Atsak, Piray

Stress and Cognition
Mechanisms regulating memory and empaty

Thesis University of Groningen with summary in English and Dutch

Cover Design and lay-out by Claudia González Arévalo (argo1983@gmail.com)
Cover illustration design by artist: Tomoki Hayasaka

Printed by :  Lovebird design & printing solutions
www.lovebird-design.com

ISBN: 978-90-367-5754-6

RIJKSUNIVERSITEIT GRONINGEN

Stress and Cognition

Mechanisms regulating memory and empathy

Proefschrift

ter verkrijging van het doctoraat in de
The Faculty of Medical Sciences
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
Maandag 1 October 2012
om 16:15 uur

door

Piray Atsak
geboren op 18 July 1981
te Antalya, Turkije

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CONTENTS

Scope of this Thesis

Stress and Cognition

Cognition refers to the acquisition, transformation, storage and use of knowledge and includes many different mental processes such as attention, memory, language, perception, reasoning and decision-making. Cognition allows us to interpret the world in which we are living, learn from the past and communicate with others and thereby make adaptive changes when our environment is changing or suddenly becomes a threat. It is well established that stress induced by environmental challenges (i.e., a stressor) can have pronounced effects on cognition at many different levels (Lupien et al., 2009; Roozendaal et al., 2009; Campeau et al., 2011). Stress invokes behavioral and physiological changes that help the organism to cope with the situation and thereby promotes survival. This thesis will investigate the effects of stress on two different aspects of cognition in rats, namely social cognition and memory. Part I will investigate the role of social information as a valuable trigger of stress, studied in a rodent model of empathy and Part II will focus on the involvement of the endocannabinoid system in regulating the effects of stress hormones on memory.

Empathy: A focus on rodent models

While the study of empathy has had a difficult history, marked by many different definitions of the concept (Preston and de Waal 2003; de Vignemont and Singer 2006; Leiberg and Anders 2006; Decety and Meyer 2008), empathy-related responding is thought to play a key role in prosocial behavior, altruism and moral reasoning (Eisenberg 2000; de Waal 2008; Decety 2010). Several theoretical models have described distinct emotional and cognitive facets of empathy (Preston and de Waal 2003; Decety and Jackson 2004; Leiberg and Anders 2006; de Waal 2008). Although empathy is not unique to humans, humans are particular for high-level cognitive abilities such as executive function, language, theory of mind that are layered on top of the phylogenetically older social and emotional capacities (Preston and de Waal 2003; de Waal 2008; Decety and Meyer 2008; Decety 2010). According to Panksepp and Lahvis (2011), emotional empathy requires that two conspecifics share an affective state, however the degree of empathy can be modulated by cognitive processes, such as the integration of information related to past experience of the subject, familiarity or similarity with the object. Some views of empathy consider it as an evolutionarily continuous phenomenon across species that exists in all social animals in varying degrees (Preston and de Waal 2003; Panksepp and Lahvis 2011). For example, Preston and

de Waal (2003) suggested that both emotional and cognitive processes involved in empathy could be unified into an ancient perception-action mechanism. These authors view empathy as any process where the attended perception of the object's state automatically generates a similar state in the subject. A perception-action model makes empathy a superior category that includes all subclasses of phenomena that share the same mechanism such as emotional contagion, sympathy, cognitive empathy, helping behavior, et cetera. These phenomena all share aspects of their underlying process and cannot be totally disentangled. For instance, emotional contagion, a change in an individual's emotional state as a result of the perception of the other individual's similar emotional state, might be the lowest common dominator of all empathic processes (Preston and de Waal 2003; de Waal 2008).

The recent discovery of mirror neurons in the ventral premotor cortex of monkey that are active both during execution and observation of action (Gallese et al., 1996; Rizzolatti et al., 1996; Rizzolatti and Craighero 2004) prompted a series of studies in humans. These studies have shown that brain regions responsible for our own actions, sensations and emotions are recruited when we observe the actions, sensations and emotions of others (Iacoboni et al., 1999; Buccino et al., 2001; Wicker et al., 2003; Keysers et al., 2004; Singer 2004; Keysers and Gazzola 2006; Keysers and Gazzola 2009; Keysers et al., 2010). These findings led to an understanding of the neural basis of empathy: seeing states of others triggers the representation of corresponding states in our brain. Although empathy has been exhaustively studied in higher primates and humans, studies focusing on the existence of this phenomenon in low-order social animals, e.g. rats and mice, have largely remained missing until recent years. However, further understanding of the neural circuits underlying empathy would require the usage of invasive techniques such that manipulations of neural activity in targeted brain regions by intracranial drug infusions or by using combination of genetic and optical tools as referred together as optogenetics in behaving animals. These methods have been established and commonly used in rodents but currently not applicable in humans and have limited practice in non-human primates (but see (Diester et al., 2011)). Thus, establishing rodent models of empathy is timely and would present a valuable tool in this field. A small number of studies performed more than four decades ago evidenced that rodents are attentive to the affective state of conspecifics; however, a further interest in this field was not pursued for a long time (Church 1959; Rice and Gainer 1962). Recently, Langford et al. (2006) demonstrated that pain sensitivity of mice is modulated by the observation of a

cagemate's similar pain response in a way that a mouse that observes the other in pain also displays more pain response, suggesting the existence of true adult-adult empathy in mice. Further evidence stemmed from studies that tested the social transfer of fear in rodents. Some of these studies indicated that animals that observe the conditioned fear responses of demonstrators to cue, either express freezing behavior (fear response in rodent) during the observation of fear responses of demonstrators to the cue or display freezing when they are only tested with the cue or context that predicted the distress of the demonstrator (Chen et al., 2009; Bruchey et al., 2010; Jeon et al., 2010; Kim et al., 2010). Response to the observation of fear behavior of the demonstrator required observers to have a prior experience with the aversive stimuli that was associated with the cue (Bruchey et al., 2010; Kim et al., 2010) whereas in other studies, observers were naïve and did not require the presence of demonstrator expressing freezing to the cue (Bruchey et al., 2010; Jeon et al., 2010). Jeon et al. (2010) also found that observers express freezing when they are adjacent to the demonstrator mouse receiving repeated footshocks. This latter effect is consistent with the emotional contagion phenomenon since the freezing behavior of the demonstrator and observer mice occurred at the same time.

Other evidence for rodent empathy originated from studies that focused on the modulation of learning by social cues in rodents, findings of these studies showed that a brief social interaction with a distressed conspecific affects the subsequent fear learning, retention and extinction (Bredy and Barad 2009; Bruchey et al., 2010; Knapska et al., 2010; Panksepp and Lahvis 2011). For instance, Knapska et al. (2010) showed that an interaction with a distressed conspecific, shortly after it underwent a fear-conditioning paradigm, facilitated the acquisition and retrieval of contextual fear conditioning in observers. Interestingly, in another study, Knapska et al. (2006) reported that the interaction with a distressed conspecific induces the expression of the neuronal activity-dependent transcription factor c-fos in many regions of the amygdala in observers similar to that in the distressed conspecific. These findings suggested that a specific pattern of amygdala activation occurs following the experience of self-distress and the experience of distress in others. The amygdala contributes to cognitive processes such as emotion, reward, learning, memory and attention (Davis and Whalen 2001; Adolphs 2003; Murray 2007). Its role in aversive learning and fear expression is well established (LeDoux 1996; LeDoux 2000; Davis and Whalen 2001; Maren 2001; Phelps and LeDoux 2005). Lesion and functional neuroimaging studies in humans that used pictures of emotional facial expressions as social signals also indicated that the amygdala

participates in processing of information about basic emotions of others (Adolphs 2003; Phelps and LeDoux 2005).

Empathy is modulated by some factors such as familiarity or similarity with the conspecific (species, age, gender), past experience (with the situation of observed distress) and salience of the stimulus from the conspecific (Preston and de Waal 2003). Few studies of rodent models of empathy focused on the modulation of empathy by familiarity with the conspecific or past experience with the observed distressing situation. Langford et al. (2006) found that familiarity is strictly necessary for empathic response in mice, whereas Jeon et al. (2010) showed that the empathic response was only enhanced by familiarity but was not necessary for the response, per se. Most rodent empathy studies reported that exposing observers to a prior similar distressing experience is required for empathic response (Langford et al., 2006; Bredy and Barad 2009; Kiyokawa et al., 2009; Knapska et al., 2010) but some found effects also in naïve animals (Bruchey et al., 2010; Jeon et al., 2010). The channel or sensory modality that is vital to convey the information between conspecific have been examined by a number of studies, these demonstrated a role for visual (Langford et al., 2006; Jeon et al., 2010), olfactory (Bredy and Barad 2009; Kiyokawa et al., 2009) and auditory channels (Chen et al., 2009; Kim et al., 2010), suggesting that sensory modality seems to depend on the nature of the employed paradigm and the expression of the distress response.

Outline of this thesis

The overall aim of this thesis is to examine the multifaceted effects of stress on cognition. The thesis consists of two parts, Part I comprises of a single chapter with its own introduction and discussion. This part explores whether a rat's stress response can affect the behavior of other rats, which can represent a simple form of empathy. Part II, which consists of a separate introduction, four empirical and discussion chapters, deals with the neurobiological mechanism of stress hormone effects on different memory functions. In particular, this part examines the role of the endocannabinoid system in regulating the effects of glucocorticoids on different memory processes.

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Experience Modulates Vicarious Freezing in Rats: A Model for Empathy

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PLoS One (2011) 6 :e21855.

CHAPTER 1

ABSTRACT

The study of the neural basis of emotional empathy has received a surge of interest in recent years but mostly employing human neuroimaging. A simpler animal model would pave the way for systematic single cell recordings and invasive manipulations of the brain regions implicated in empathy. Recent evidence has been put forward for the existence of empathy in rodents. In this study, we describe a potential model of empathy in female rats, in which we studied interactions between two rats: a witness observes a demonstrator experiencing a series of footshocks. By comparing the reaction of witnesses with or without previous footshock experience, we examine the role of prior experience as a modulator of empathy. We show that witnesses having previously experienced footshocks, but not naïve ones, display vicarious freezing behavior upon witnessing a cage-mate experiencing footshocks. Strikingly, the demonstrator's behavior was in turn modulated by the behavior of the witness: demonstrators froze more following footshocks if their witness froze more. Previous experiments have shown that rats emit ultrasonic vocalizations (USVs) when receiving footshocks. Thus, the role of USV in triggering vicarious freezing in our paradigm, is examined. We found that experienced witness-demonstrator pairs emitted more USVs than naïve witness-demonstrator pairs, but the number of USVs was correlated with freezing in demonstrators, not in witnesses. Furthermore, playing back the USVs, recorded from witness-demonstrator pairs during the empathy test, did not induce vicarious freezing behavior in experienced witnesses. Thus, our findings confirm that vicarious freezing can be triggered in rats, and moreover it can be modulated by prior experience. Additionally, our result suggest that vicarious freezing is not triggered by USVs per se and it influences back onto the behavior of the demonstrator that had elicited the vicarious freezing in witnesses, introducing a paradigm to study empathy as a social loop.

INTRODUCTION

The study of the neural basis of empathy has received a surge of interest in the last years following the description of brain activity in humans that suggests that the representations of a subjects' own emotional states and sensations are partially activated when witnessing the disgust, pain or pleasure of others (Hutchison et al., 1999; Carr et al., 2003; Wicker et al., 2003; Keysers et al., 2004; Morrison et al., 2004; Singer, 2004; Avenanti et al., 2006; Decety and Jackson, 2006; Decety and Lamm, 2006; Keysers and Gazzola, 2006; Jabbi et al., 2007; Jabbi et al., 2008). In particular, this evidence has been taken to suggest that a neural mechanism, similar to the mirror neurons found in the ventral premotor and inferior parietal lobe of the monkey, which respond both during the execution of goal directed actions and the observation of the same actions executed by others (Gallese et al., 1996; Umiltà et al., 2001; Kohler et al., 2002; Keysers et al., 2003; Fogassi et al., 2005; Fujii et al., 2008; Rozzi et al., 2008; Caggiano et al., 2009), could be at work in emotional and somatosensory brain circuits as well (Bastiaansen et al., 2009; Keysers et al., 2010). Testing this idea would require single cell recordings and experimental manipulations of the brain regions involved in empathy. Such invasive techniques are not readily applicable in humans (but see (Hutchison et al., 1999; Mukamel et al., 2010)); therefore an animal model of emotional empathy would be essential to further our understanding of empathy.

It has been proposed that empathy exists in social animals because the detection of discomfort, distress or fear in conspecifics carries information of high survival value (Preston and de Waal, 2003). In the context of developing an animal model of empathy, here we will focus on whether rats and mice, the two most readily available laboratory mammals, show such social transmission of distress cues. Social transmission of information in rats does occur in a wide range of behaviors such as food preference (Galef, 1985; Galef and Beck, 1985), motor (Kohn and Dennis, 1972; Zentall and Levine, 1972) and avoidance behaviors (Masuda et al., 2009). Moreover, rats, can respond with fear and learn from fear reactions of others; for instance, a neutral stimulus can acquire aversive value after an observation of conditioned responses of another rat (Church, 1959; Bruchey et al., 2010; Kim et al., 2010). Additionally, interactions with a distressed conspecific seem to recruit the amygdala that is also active when experiencing first hand distress (Knapska et

al., 2006). Also mice show evidence of similar social transmission: the observation of a conspecific being shocked has been shown to induce vicarious freezing in mice (Jeon et al., 2010) and to enhance subsequent fear learning in this species (Chen et al., 2009a). Vicarious behavior in mice seems to be regulated by the degree of relatedness between the interacting individuals (Langford et al., 2006; Jeon et al., 2010). Together, these evidences suggest that rodents are sensitive to what happens to other rodents. Rodents might therefore provide a powerful animal model for studying and manipulating the neural mechanisms of empathy.

In the effort to develop animal models of empathy, it is important to determine what aspect of empathy can actually be modeled. Current conceptualizations of empathy define it as being composed of two components/processes. First, if an individual has an affective reaction that resembles that of another and is triggered by perceiving or imagining the state of that other individual, the individual is said to experience 'emotional contagion' (de Vignemont and Singer, 2006; Singer and Lamm, 2009). Emotional contagion occurs early in human development: babies are more likely to cry if they hear other babies cry. Second, if that individual is also aware of the fact that its emotional reaction is triggered by that of another, it experiences true empathy. This distinction is important, because empathy proper is more likely to trigger prosocial behavior than emotional contagion. In animals, it is however often impossible to assess whether they are aware of the source of their emotions, and accordingly to disentangle models of emotional contagion from models of empathy.

Since empathy in humans has been shown to be modulated by experience (see Refs. (Preston and de Waal, 2003; de Vignemont and Singer, 2006; de Waal, 2008; Bastiaansen et al., 2009) for reviews) in this study we aimed at establishing a paradigm to study both empathy/emotional contagion itself and its modulation by prior experience. A vast number of studies in the literature reported gender differences in empathy and social perception (Hoffman, 1977; Eisenberg and Lennon, 1983; Connellan et al., 2000; Alexander and Hines, 2002; Baron-Cohen and Wheelwright, 2004; Baron-Cohen et al., 2005), moreover, gender differences in social modulation of behaviors have been reported in rats (Westenbroek et al., 2003; Westenbroek et al., 2005; Yee and Prendergast, 2010) with stronger effects in females. We therefore use female rats in this study.

In Experiment 1, we examined the behavior of two interacting female rats while one of them, the demonstrator, experiences a series of unconditioned aversive stimulus (5 footshocks) while the other, the witness, can hear, see and smell the reaction of the demonstrator. To investigate whether prior experience with a similar aversive stimulus would modulate the reaction of the witness, we compared the behavior of witnesses that had previously experienced footshocks with that of witnesses that had not. Finally, we also quantified the relationship between the witnesses' behavior and that of the demonstrators to examine if the way that the witness responds to the behavior of the demonstrator might in turn influence the behavior of the demonstrator.

We predicted that witnessing the distress reactions of the demonstrator would alter the behavioral pattern of the witness and make the witness' behavior resemble that of the demonstrator, for instance by showing an increased freezing or by expressing other distress-related behaviors. Moreover, we expected such vicarious fear responses to be more pronounced in witnesses that had previously experienced footshocks. Finally, it is reported that rats, when paired with a conspecific, express less conditioned fear responses, suggesting the existence of social buffering effects (Kiyokawa et al., 2004). Furthermore, the stress status of the partner plays an important role in social buffering effects, e.g. a non-shocked partner (not pre-exposed to footshocks) is more effective in attenuating fear responses than a shocked partner (pre-exposed to footshocks) (Kiyokawa et al., 2004). These findings led us to expect that demonstrators paired with naïve witnesses show less distress than those paired with experienced witnesses, because of the differential social buffering by their paired witness group.

Next, we set out to explore the contribution of various components of the auditory channel in triggering the vicarious freezing in the experienced witness rats. It is well documented that rats emit ultrasonic vocalizations (USVs) and that the frequency and temporal pattern of such vocalizations are determined by specific environmental factors (Brudzynski, 2005; Burgdorf et al., 2005; Ehret, 2005; Brudzynski, 2007). USVs have been thought to play an important role in the communication between conspecifics but their exact function remains unclear. It has been proposed that they can serve to: localize conspecifics, transfer emotionally valenced information across conspecifics and warn other individuals

of external dangers to promote escape or dispersion. (see Refs. (Burgdorf et al., 2005; Ehret, 2005; Brudzynski, 2007) for review). Furthermore it has been previously shown that rats emit USVs at a certain frequency (~22 kHz) in aversive conditions (e.g. during fear conditioning) and in the presence of cues that predict danger (Blanchard et al., 1991; Wohr et al., 2005). Additionally, a recent study showed that USVs can modulate social transmission of fear in rats (Kim et al., 2010), however not many studies in the literature examined the role of USVs in potential empathy paradigms. Thus, we set out to test the role of USVs in our potential model of empathy. First, we recorded the USVs produced during the social interactions in Experiment 1 in order to establish the degree of communication between witness and demonstrator pairs. Second, in Experiment 2, we used these recorded vocalizations and played them back to separate groups of naïve and experienced animals while monitoring their behavior, freezing in particular. We produced two kinds of auditory stimuli from the recordings of Experiment 1: i) 22 kHz ultrasonic vocalizations (all other recorded sound were filtered out) ii) 2-4 kHZ control sound that share same temporal characteristics with USVs.

METHODS

Subjects. Female Long-Evans rats (250-300 g) from Harlan US Davis were kept in a temperature controlled (22 ºC) room and maintained on a reversed 12-h light: 12-h dark cycle (07:00 lights off - 19:00 lights on). Rats were socially housed as 2-4 rats per cage and had *ad libitum* access to food and water. All experiments are conducted during the dark cycle between 09:00 and 13:00 h. All experiments were conducted in strict accordance with the European Community's Council Directive (86/609/EEC) and all experimental procedures were approved by The Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RuG, approval number: 4669).

Experiment 1

Groups. Adult female rats were randomly assigned to one of the witness or one of the demonstrator groups, each witness and demonstrator pair is composed of cage-mates and therefore housed together from arrival till the end of the experiment. Witnesses and demonstrators were divided into the following subgroups: Witness groups - Naïve Control Witness, Naïve Shock Witness, Experienced Control Witness

Table 1: Conditions and Groups in Experiment 1

Condition	Witness	Demonstrator
Control	NcW Naive control Witness	D(NcW)
	EcW Experienced control	D(EcW)
Shock	NsW Naive shock Witness	D(NsW)
	EsW Experienced shock	D(EsW)

Each row indicates the Witness group and its paired Demonstrator group.

and Experienced Shock Witness; Demonstrator groups - Demonstrator paired with Naïve Control Witness, Demonstrator Paired with Naive Shock Witness, Demonstrator paired with Experienced Control Witness, Demonstrator paired with Experienced Shock Witness (see table 1 for the explanation and abbreviations of the experimental groups and pairs). Rats were handled and habituated 3 minutes to the experimenter everyday for 10 days preceding the experiment. All rats were habituated to the transportation and experimental room for 20 minutes/day for 3 days prior to the experiment.

Apparatus. To ensure that experienced witnesses could be familiarized with footshocks prior to the Empathy Test without generating conditioned fear for the context of the Empathy Test, two different chambers (context A and B) were used for the Pre-Exposure and Empathy Test in a counterbalanced fashion. Each chamber consisted of two adjacent animal compartments - witness compartment and demonstrator compartment (each D24cm x W25cm x H34cm) divided by a perforated transparent Plexiglas divider. The dimensions of the two chambers were identical but the two contexts (A and B) were modified to maximize their discriminability by the animals. Context A had metal-coated sides, a transparent front door and lid, and was illuminated using a dim red light. Context B had side panels coated with a striped pattern using latex-based colors, a patterned solid front door and lid, and was illuminated using a bright white light. In both contexts (A and B), the demonstrator area had a stainless steel rod floor to deliver shocks while a solid Plexiglas sheet covered the witness area's floor. The demonstrator

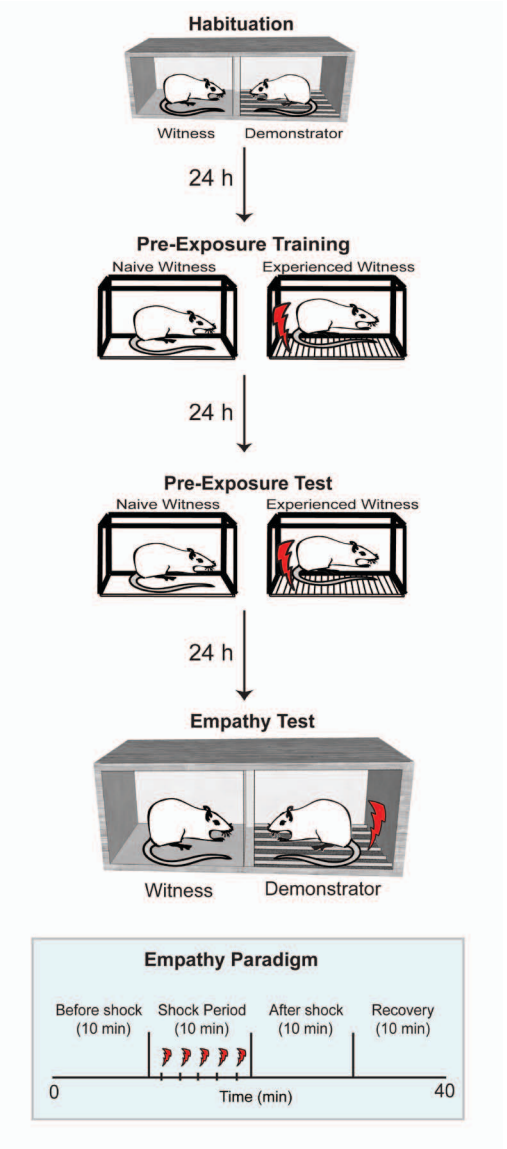

Figure 1. Illustration of experimental design of Experiment 1. Pairs of rats were exposed to the Empathy Test context for 15 minutes (Habituation). Twenty-four hours later, witnesses were placed in the other context, and either received or not a number of footshocks (Pre-Exposure Training). Twenty four hours later, the witnesses were tested for long-term retention of this experience by replacing them in the pre-exposure context and measuring freezing (Pre-Exposure Test). Twenty-four hours later, demonstrator - witness pairs were placed again in the 2 compartments of the Empathy context. This time, the demonstrator (right) receives shocks through the floor grid while the witness (left) can hear, see and smell the demonstrator through a perforated Plexiglas dividing screen. The lowest panel schematizes the time course of the Empathy Test session.

area of each chamber (context A or B) was used for the Pre-Exposure training of the experienced witnesses. Experienced witnesses that received the footshock in context A, were then tested in context B in the Empathy Test or vice-versa. Between animals, chambers were wiped twice with different substances to ensure the contexts differed in odor: context A- 70% alcohol and then 3% mint soap solution and context B- 3% vinegar and then antibacterial soap solution.

Pre-Exposure. All witnesses were placed in the Pre-Exposure environment individually and after 15 minutes of exploration, only experienced witness groups received 4 footshocks (1 second each, 0.8 mA) separated by random intervals ranging between 240 and 360 seconds (Fig. 1). The pre-exposed rats were housed individually for 1h after pre-exposure before being returned to their home cages. Twenty-four hours later, both naïve and experienced witness rats were individually tested in the same Pre-Exposure context for 5 minutes (this session will be referred as Pre-Exposure test) and freezing behavior was scored during the last 3 minutes. As this test session could lead to extinction of the acquired fear, at the end of the 5 minutes of Pre-Exposure test, experienced witnesses received one reminder footshock (1 second, 0.8 mA) before they were taken out of the chamber. Again, the rats were then housed individually for 1h before returning to their home cage.
Empathy Test. All witness-demonstrator pairs were habituated to the Empathy Test environment a day prior to the Pre-Exposure training of witnesses. In the Empathy Test, the witness and demonstrator constituting a pair were placed in the two adjacent areas of the Empathy Test chamber for a total time of 40 minutes (Fig. 1). After 10 minutes of baseline, in the shock condition, five footshocks (each footshock 5 seconds, 0.8 mA) separated by random intervals of either 2 or 3 m, were delivered to the demonstrator rat only (Fig. 1). In the control condition, the exact same procedure was used, except that a Plexiglas floor separated the demonstrator rat from the metal grid through which the shocks were delivered. This ensured that any sounds or vibrations generated by the shock device would be identical between the shock and control conditions, but the actual footshocks would only reach the experimental but not the control demonstrators. Any differences in freezing rate between the two conditions therefore cannot be due to classical conditioning to the sound of the shock device. After the last shock delivery, rats were left in the box for an additional 20 minutes.

Behavioral Scoring and Analysis. The entire test sessions were videotaped with a CCD black and white camera (Model SSC-M370 CE, Sony, Japan) mounted on the chamber and connected to an MPEG-encoder PC. Movies were stored in MPEG-2 digital format for later behavioral scoring. Live image from the same camera was transferred to a PC running a video-tracking system (Ethovision 3.1; Noldus information technology, Wageningen, Netherlands) for quantification of general movement and locomotor activity of the witness groups. Locomotor activity of witnesses is sampled as 5 minute time-bins and the percentage change in locomotion was calculated by subtracting the locomotor activity measured in the first 5 minutes (taken as a baseline) from the locomotor activity sampled in the subsequent 5 minute time-bins (in total 8 time-bins were used: 1st and 2nd -before shock, 3rd and 4th -shock period, 5th and 6th -after shock and 7th and 8 th recovery period, Fig. 2b illustrates only the first 6 time-bins). Additional video-tracking analysis was run to quantify the amount of time spent by witnesses in close distance to the demonstrator. For this analysis, the observer's compartment was divided in a far and a close half, relative to the screen dividing the two rats (each zone is 12.5 cm wide) and time spent in the zone close to the demonstrator's compartment (window zone) is calculated in three 10 minute time periods, each corresponding to before shock, shock and after shock periods, respectively.

Freezing behavior was scored in the Pre-Exposure training, Pre-Exposure test and in the Empathy Test sessions. A trained researcher that was blind to the experimental condition, performed the behavioral scoring from the digital movies using Observer XT (Noldus information technology, Wageningen, Netherlands) and Jwatcher (http://www.jwatcher.ucla.edu/). A rat was considered to be freezing if it was (i) in the stereotypical crouching posture and (ii) not moving except for respiration related movements. In the Empathy Test, total time of freezing scoring consisted of 14 minutes divided in 6 time-bins per rat. The first time bin lasted from -2 minute to 0 relative to the onset of the first shock. The other five time-bins corresponded to the time following each of the 5 shock trials (since the inter-shock interval was either 3 or 2 minutes, the time-bins used corresponded to 3 time-bins of 2 minutes and 2 time-bins 3 minutes). For control groups, the same scoring schedule was used. Freezing scores were calculated as the percentage of time during each bin that the rats spent freezing. Average percentage freezing in shock period was calculated by averaging the freezing scores in 5 time bins

following the footshock trials.

Ultrasonic Vocalization Recordings and Analysis. Sounds were recorded with a high-frequency omnidirectional microphone (Earthworks M30, frequency range 5-30kHz, Earthworks Inc., Milford NH) mounted on the chamber, and amplified (Edirol FA-66, Roland Corporation, Los Angeles, CA). Sounds were digitized at 96 kHz, 16 bits and stored in wav format using Adobe Soundbooth CS3 (Adobe Inc.) on a Macintosh computer. In order to count the number of USVs emitted by witness-demonstrator pairs, wav files were processed in Matlab (Mathworks Inc.) to create sound spectrograms using short-time fast Fourier transform (sFFT) with a window of 256 time points and an overlap of 75%, resulting in a final frequency resolution of 1.5 kHz and time resolution of 0.6 ms. Frequencies outside 15-30 kHz were truncated. Time points containing USVs were separated from those containing only environmental noise by considering the standard deviation of the (filtered) power spectrum of each time point.

Time points containing USVs were clearly identifiable as having a higher standard deviation in the power spectrum with respect to time points containing only environmental noise. We therefore set the time points containing only noise to 0 dB, and summed the power of each time point across frequencies. The resulting vector was smoothed with a moving average of 100 time points (corresponding to approximately 66.67 ms) to increase the signal-to-noise ratio. The nonzero time points of this vector were used to calculate the number of calls, and to compute the distribution of the estimated number of calls for different durations. Only the number of emitted USV in the time frame of freezing scores (6 time bins, see behavioral scoring and analysis for details) was taken into consideration. In order to check the accuracy of the algorithm, the number of USVs in the recording of 5 different demonstrator-witness pairs was quantified both by the algorithm and manually. The number of calls detected by algorithm matched the number of manually counted calls during the same interval.

Experiment 2

Groups, Chambers and Experimental Design. Rats were handled and habituated 3 minutes to the experimenter everyday for 10 days preceding the experiment. All rats were accustomed to the transportation and experimental room for 20

minutes/day for 3 days prior to the experiment. On the first day of the experiment, all rats were habituated to the Sound Test chamber (D25xW40xH40) for 15 minutes. Then, rats were divided into two groups: Experienced and Naïve. Experienced animals were trained with footshocks according to the Pre-Exposure training schedule described in Experiment 1, whereas the other animals were kept naïve to footshock. On the following day, animals were placed in the Sound Test chamber and Control sounds or USV sounds were played back from a high frequency loudspeaker (Precision 8D Studio Monitor, Tannoy Ltd., Scotland, UK) through the holes in the Plexiglas divider also used in Experiment 1. In pilot experiments, playback loudness was adjusted to lead to the same sound intensities in the chamber of the witness rats as in Experiment 1. Since the distance between the speaker and the animal depends on the place preference of the animal, we set the distance of the speaker such that the maximal distance (45 cm) or minimal distance (5 cm) between rat and speaker corresponded to the maximum or minimum distance between the witness and demonstrator pairs in the Empathy Test. The total duration of the Sound test was 40 minutes, however only the time window of interest is analyzed (see below for detailed explanation).

Auditory Stimulus and Playback. In this experiment two different sounds (USV and Control sounds) were played back to naïve and experienced rats. In order to prepare the USV stimuli for playback, the sound tracks recorded from the EsW-D(EsW) pairs during Experiment 1 were band-pass filtered in the range between 17 and 25 kHz in Adobe Soundbooth CS3 (Adobe, San Jose, CA). No USVs outside this frequency range were observed. Control sounds were generated from the same sound track recorded in Experiment 1 by using the SOX software (http://sox.sourceforge.net/). USVs in each recorded file were pitched down 35 semitones to a range of 2.6 - 4 kHz, while intensity and temporal characteristics were preserved. This range for the control sound was selected on the basis of the previous findings in the literature that rat effectively discriminates 4 kHz sounds from USVs (Bang et al., 2008). Sound presentation started after 10 minutes of baseline at the point in which the first electroshock was given in the recording session (Experiment 1), so as to lead to a similar timing as in the Empathy Test in Experiment 1 (footshock exposure of demonstrator started after 10 minutes of baseline). In addition to this main auditory experiment (Experiment 2) we also conducted a pilot experiment to explore the contribution of other auditory signals contained in the sound track

recorded in Experiment 1. In this pilot experiment, the same rats that had only been exposed to the control sound in Experiment 2 were place in the test chamber once more and exposed to a playback of the unfiltered recording (USV and audible sounds) of the Empathy Test. In Experiment 2 and in the pilot experiment, freezing behavior was scored and analyzed using the same time window as in Experiment 1 but only in 2 time-bins corresponding to 2 minutes before the onset of the playback and 12 minutes during sound playback, respectively.

Behavioral Scoring and Analysis. Behavioral scoring was performed live with Ethovision 3.1. (Noldus information technology, Wageningen, Netherlands). 20% of the animals were also scored blindly and the correlation coefficient between blind and live scoring was found to be nearly perfect (pearson correlation, r_p=0.96, $p<0.05$).

Statistical Analysis. A separate analysis was performed on witnesses' and demonstrators' freezing levels. In both cases, we analyzed between and within group changes in freezing behavior using a two-way mixed effect analysis of variance (ANOVA) with time (before and after shock) as a within factor and group (either witness or demonstrator groups) as a between factor. In the analysis of the dynamics of interaction between demonstrator and witness, we analyzed the freezing behavior of demonstrators and witnesses separately. In both cases, we analyzed changes in freezing behavior using a two-way ANOVA with time (6 time bins) as within factor and group (2 shock groups) as between factor. Planned comparisons were conducted using unpaired t-tests to compare the differences between groups, while planned comparisons using paired t-tests were performed to compare the differences between time bins. A two-way mixed effect ANOVA model was used, with factors for time bins (within) and group (between) for the analysis of locomotor activity of witnesses. Further *post hoc* tests were performed for more detailed comparisons between witness groups and time bins. Similarly, differences in the time spent in the window zone were tested with a two-way mixed effect ANOVA with time bins as within and group as between factors, followed by *post hoc* tests. The p values resulting from the latter two analyses were corrected for multiple comparisons with the Bonferroni method. Pearson's correlation was used to calculate the relationship between freezing of EsW and freezing of D(EsW), and between USV and average freezing of demonstrators and

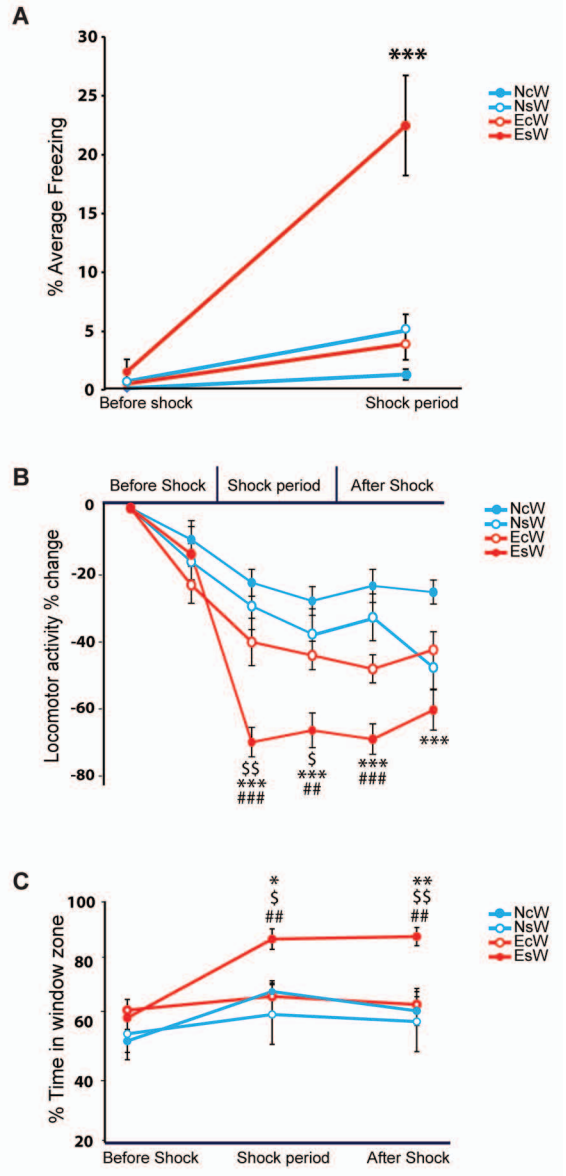

Figure 2. Behavior of 4 witness groups in Empathy Test. Naïve control witness (NcW), experienced control witness (EcW), naïve shock witness (NsW), experienced shock witness (EsW). (A) % Average freezing before shock and during shock period by witnesses. ***p<0.001 EsW compared to all the other witness groups. (B) % Change in locomotor activity before shock, shock and after shock periods. % Change in locomotion is relative to the first time bin that served as baseline and thus has a value of zero by definition. $p<0.05, $$p<0.01 EsW compared to EcW; ##p<0.01, ###p<0.01 EsW compared to NcW ; ***p<0.001 EsW compared to NsW. (C) % Time spent in window zone by witnesses. $p<0.05, $$p<0.01 EsW compared to EcW ; ##p<0.01 EsW compared to NcW; *p<0.05, **p<0.01 EsW compared to NsW. All data is presented as mean ± S.E.M (n = 11-15 per group).

as well as between USV and average freezing of witnesses. In the analysis of USV, the percentage of pairs that emitted USV was calculated and compared between NsW-D(NsW) and EsW-D(EsW) pairs with t-test.

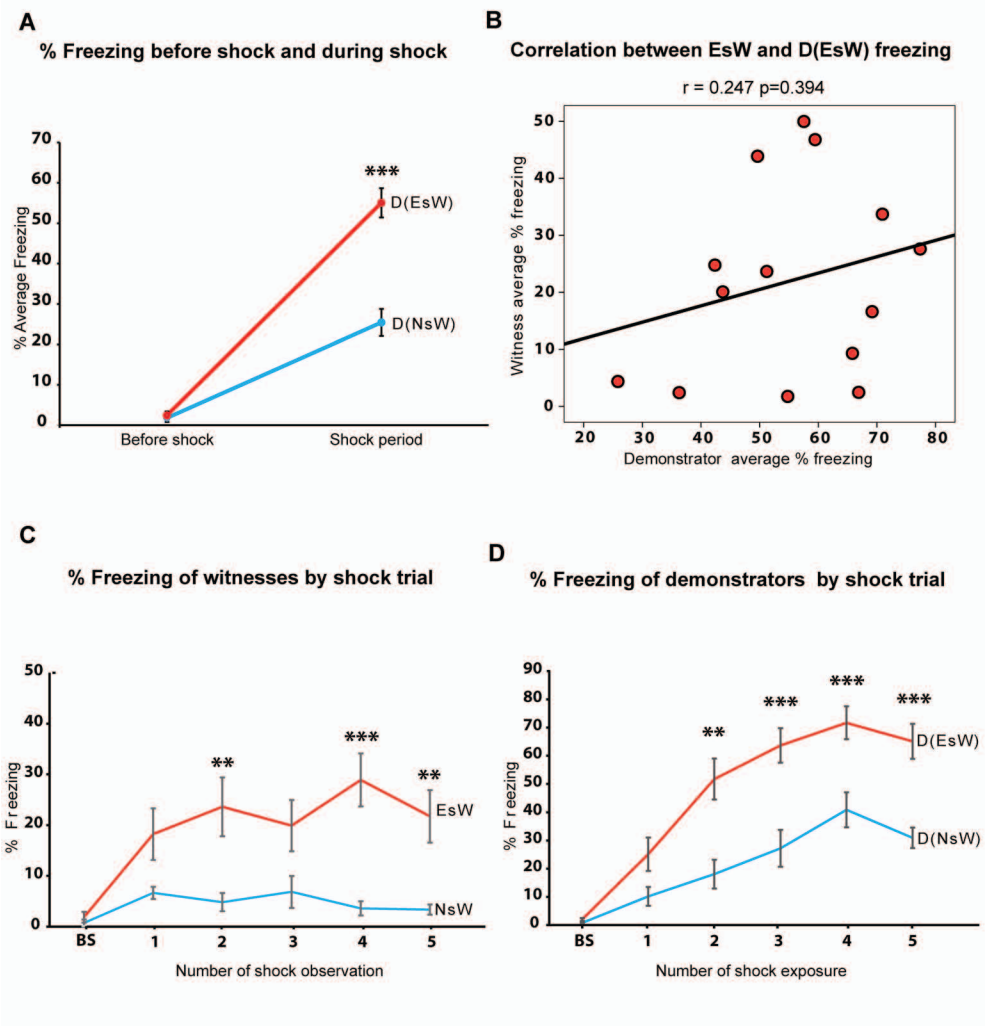

Figure 3. Social modulation of freezing in witnesses and demonstrators. (A) % Average freezing before shock and during shock period by demonstrators paired with naive (D(NsW) and experienced (D(EsW) witnesses. (B) Correlation between freezing levels of experienced shock witness (EsW) and their paired demonstrator (D(EsW)). (C) % Freezing levels of naïve (NsW) and experienced shock witness (EsW) before shock (BS) and during footshock trials (1st to 5th). (D) % Freezing of demonstrator group paired with naïve (D(NsW) and experienced (D(EsW)) witnesses before shock (BS) and during footshock trials (1st to 5th). **p<0.01, ***p<0.001 compared to respective groups . All data is presented as mean ± S.E.M (n = 11-15 per group).

RESULTS

As we were interested in the effect of prior experience with footshock on vicarious fear, we first verified whether Pre-Exposure training with footshock led to the formation of a long-term memory for the aversive event in experienced witnesses. To this end, we compared the freezing behavior of experienced and naïve witnesses in the Pre-Exposure test. We found that experienced witness rats, that received footshocks on the Pre-Exposure training, froze significantly more than naïve witnesses (36.6 5.2 %, vs. 1.2 ± 0.7 % (mean±SEM)) in the Pre-Exposure test ($t(20) = -3.276$, $p<0.001$). This finding confirmed that a long-term memory of the Pre-Exposure event was formed in the experienced witnesses.

Vicarious fear

To investigate whether rats display vicarious fear when observing a conspecific receiving footshocks, freezing behavior was compared across witness groups (Fig. 2a). A 4 Groups (NsW, EsW, NcW, EcW) x 2 time period (before shock vs. shock period) mixed effect ANOVA for freezing levels revealed a significant main effect of group ($F_{3,96}=12.519$, $p<0.0001$), time period ($F_{1,96}=45.201$, $p<0.0001$) and interaction of group by time period ($F_{3,96}=14.939$, $p<0.0001$). Following planned comparisons showed that EsW displayed higher freezing levels in the shock period compared to all other witness groups ($p<0.0001$ compared to NsW, NcW, EcW). These results indicate that in our experiment, rats express vicarious freezing behavior when observing a conspecific being shocked but only when they have had prior experience with footshock.

We also analyzed the locomotor activity of the four witness groups using video-tracking. This data provides an overall measure of the witnesses' locomotor activity throughout the whole Empathy test period (Fig. 2b). A 4 Groups (NsW, EsW, NcW, EcW) x 7 time bins (each consists of 5 minutes) mixed effect ANOVA for locomotor activity, indicated a significant main effect of group ($F_{3,48}=7.84$, $p<0.0001$) and effect of time bins ($F_{6,288}=19.748$, $p<0.0001$), and a significant effect of interaction between group and time bins ($F_{18,288}=2.983$, $p<0.0001$). Further post-hoc analyses pointed out that EsW exhibited a significantly larger reduction of locomotor activity in time bins corresponding to shock period and

to after shock period (see Fig. 2b for the significant differences relative to other groups). This confirmed the results derived from the analysis of freezing behavior. Locomotion of the four witness groups reconverged during the last 10 minutes of the Empathy Test when all groups showed a similar level of activity (Data not shown). Additionally, by using the video-tracking system, we could also assess whether the witness rats preferred to be close to the demonstrator during the Empathy test session (Fig. 2c). To this end, we divided the witnesses' compartment

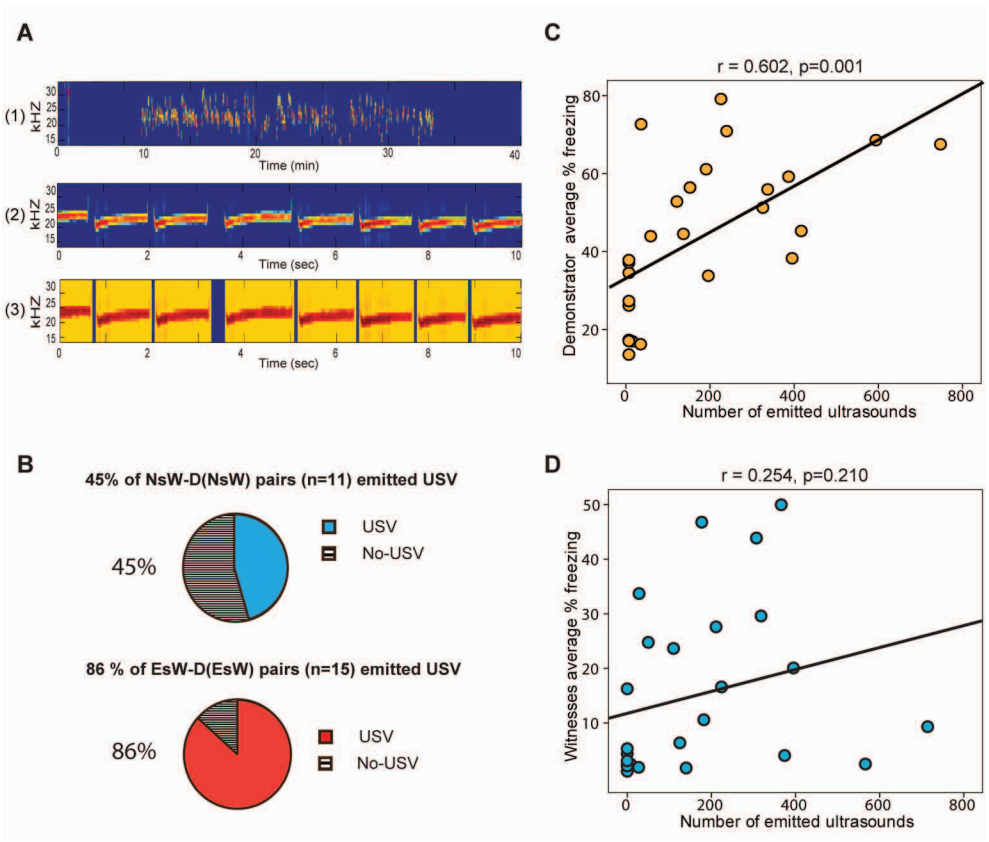

Figure 4. (A) Example sound spectrograms illustrating (1) a 40 min sound track containing USVs recorded in Experiment 1, (2) USVs in a 10 second time window detail, (3) the result of the automated detection of USVs in Matlab, with epochs containing a single 22 kHz-USV shown in yellow. (B) % of naïve shock witness (NsW)-demonstrator (D(NsW) pairs and % experienced shock witness (EsW) and Demonstrator (D(EsW) pairs that emitted USVs. (C) Correlation between the number of emitted USVs and % average freezing response in shock period by both demonstrator groups (paired with naïve shock witness D(NsW) and paired with experienced shock witness D(EsW) together). (D) Correlation between the number of USVs and % average freezing behavior in shock period by naïve shock witness (NsW) and experienced shock witness (EsW) groups.

in 2 equal zones: a window zone close to demonstrator and a wall zone far from the demonstrator. A 4 Groups (NsW, EsW, NcW, EcW) x 3 time periods (before shock, shock, after shock) mixed effect ANOVA comparing the proportion of time spent in the window zone revealed a significant effect of group ($F_{3, 48}$=3.063, p<0.05) and effect of time period ($F_{2, 96}$=26.394, p<0.0001) and as well as significant effect of interaction between group and time period ($F_{6, 96}$=5.846, p<0.0001). Following post hoc comparisons showed that the EsW group spent significantly more time in the window zone close to their demonstrator than all the other witness groups during shock period and after shock period (see Fig. 2c for significant differences relative to other groups).

Effect of social interaction on freezing behavior of demonstrators

A two by two mixed effects ANOVA, demonstrator groups ((D(NsW) vs D(EsW)) and two time periods (before shock and shock period), for freezing levels showed a significant main effect of group ($F_{1,24}$=35.619, p<0.0001), of time period ($F_{1,24}$=227.615, p<0.0001) and a significant interaction between group and time period ($F_{1,24}$=29.890, p<0.0001). Planned comparisons show that before shock trials both groups displayed low levels of freezing that did not significantly differ from each other (p = 0.658, Fig. 3a), and that footshock delivery led to significantly higher levels of freezing in all demonstrators exposed to footshock (comparison of freezing before shock period vs during shock trials, p<0.0001 for D(NsW), p<0.0001 for D(EsW)). However, D(EsW) expressed significantly more freezing behavior than D(NsW) (p<0.0001, Fig. 3a) during the shock period. To further explore the relationship between freezing displayed by the demonstrator and the witness rats, we examined the correlation between freezing levels displayed by D(EsW) and EsW rats (the group of witness rats which displayed vicarious freezing). We found no significant correlation (Pearson r=0.247 p=0.394, Fig. 3b), suggesting that prior experience, rather than differences in freezing displayed by demonstrators (D(EsW) vs. D(NsW)), underlies the differences observed in the behavior of the two shock witness groups.

To further investigate the dynamics of the demonstrator – witness interaction, we conducted analyses to look at the effect of time on the difference of freezing between the demonstrator and witness groups separately (Fig 3c, 3d). A 2 shock witness groups (NsW, EsW) x 6 time bins (before shock, 1st to 5th shock

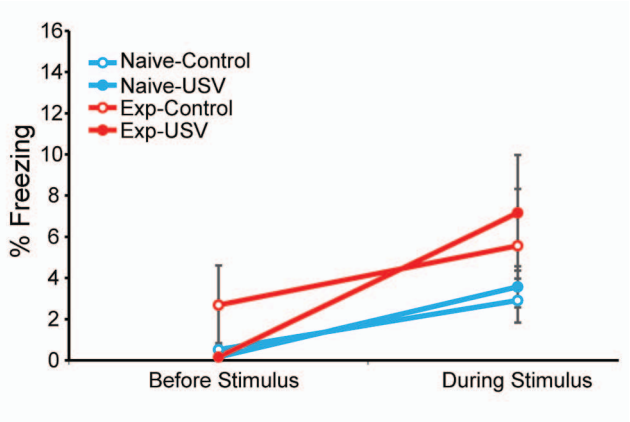

Figure 5. % Freezing behavior of Naïve (Naïve-Control, Naïve-USV) and Experienced groups (Experienced-Control and Experienced-USV) before and during control and USV sound stimulus in Experiment 2. All data is presented as mean ± S.E.M (n = 10-11 per group).

trials) mixed effect ANOVA for freezing behavior indicated a significant effect of group ($F_{1,24}$=11.259, p<0.01) and effect of time ($F_{5,120}$=3.594, p<0.01). Planned comparisons further unveiled that a significant increase in freezing behavior of EsW relative to the baseline emerged after the 1st footshock trial (p=0.041 compared to baseline) and that after this initial increase, freezing levels remained stable in the following footshock trials (no difference between 1st shock trial compared to 2nd – 5th, p>0.05). Freezing levels of EsW significantly differed from NsW in some of the footshock trials, but the difference was not significant in all cases (Fig. 3c).

A 2 shock demonstrator groups (D(NsW), D(EsW)) x 6 time bins (before shock, 1st to 5th shock trials) mixed effect ANOVA for freezing behavior revealed a significant effect of group ($F_{1,24}$=34.585, p<0.00001), effect of time ($F_{5,120}$=36.406, p<0.00001) and as well as significant interaction of group and time ($F_{5,120}$=4.052, p<0.01). Planned comparisons showed that freezing displayed by both groups of demonstrators (D(NsW) and D(EsW)) increased gradually over footshock trials: Freezing levels of the D(EsW) showed a significant increase on the 1st shock trial relative to baseline (p=0.004), and increased again after the 2nd shock trial (p=0.001 relative to the 1st). Importantly, the significant difference in freezing levels between D(EsW) and D(NsW) only emerged after the 2nd shock trial and remained significant in the all subsequent shock trials (Fig. 3d). Collectively, these findings show that the differences in freezing between NsW-D(NsW) and EsW-D(EsW) have a different time course for the demonstrators and witnesses. This

difference peaked around the 1st shock trial for EsW, but after the 2nd in both demonstrator groups (D(NsW) and D(EsW)).

Alarm calls during the Empathy Test

Analyses of the USVs revealed that not all pairs of rats submitted to shocks emitted USV, and that a larger proportion of EsW-D(EsW) than NsW-D(NsW) pairs emitted USVs (86% versus 45%, $p<0.05$, Fig. 4b). Separate correlation analysis between the number of USVs emitted and proportion of freezing displayed by witness groups (EsW and NsW) and demonstrator groups (D(EsW) and D(NsW)) show a significant correlation between emitted USVs and mean percentage freezing for the demonstrator groups ($r=0.602$, $p=0.001$, Fig. 4c), but not for the witness groups ($r=0.254$, $p=0.210$, Fig. 4d). This shows that differences in the number of USVs emitted by each pair is mainly explained by differences in the freezing behavior of the demonstrators, suggesting that they might be the prime source of USVs.

Next, we examined whether these alarm calls induced freezing in naïve or experienced rats, to which end we performed Experiment 2, a sound playback experiment. Analysing Experiment 2 using a mixed effects ANOVA with freezing as the dependent variable and a 4 groups (Naïve-Control, Naïve-USV, Experienced-Control, Experienced-USV) x 2 time periods (before sound stimulus and during sound stimulus) design revealed a significant effect of time period ($F_{1,37}=18.480$, $p<0.0001$), but no significant effect of group ($F_{3,37}=1.006$, $p=0.401$) and no significant interaction of group and time period ($F_{3,37}=1.361$, $p=0.270$). Although there was a significant increase in freezing levels in both experienced and naïve rats during the presentation of any sounds (USV and control sound stimuli), the playback of USVs did not increase the freezing levels above and beyond that of the control sounds in experienced or naïve listeners (Fig. 5).

Finally, to examine if auditory information other than USVs could have triggered freezing in our experiment, we performed a pilot experiment in which we played back the unfiltered recording of Empathy test (USV together with other audible sounds) and we found that the listening rats did displayed freezing behaviour when faced with the combination of USVs and environmental sounds, and that this freezing was stronger in experienced than naïve listeners ($t(17) = 2.177$,

$p<0.05$, Fig. S1). Importantly, although these rats were not experimentally naïve, we did not observe any difference in freezing behavior before the onset of the sound stimulus (Fig. S1).

DISCUSSION

In this study, we describe a paradigm to potentially study empathy in rats and, in particular, the role of prior experience in modulating the empathic response: a demonstrator rat was exposed to footshocks while a cage mate witnesses its distress. We found that demonstrator rats receiving footshocks displayed typical fear responses to this distressing experience, including freezing and emission of USVs and that witness rats that had previously experienced shocks themselves (EsW) displayed similar, albeit less intense, fear responses, including augmented freezing and reduced locomotion. Thus, our experiments confirm that rats can express vicarious fear responses even though not experiencing firsthand pain or distress. This vicarious response was significantly reduced (and no longer significant) in witness rats that had not experienced electroshocks in the past. We further found that the difference in vicarious behavior of the witnesses fed back onto the behavior of the demonstrators that had triggered it in the first place: D(EsW) that were shocked in the company of experienced witnesses progressively froze more than D(NsW) that were exposed to footshocks of the same intensity in the company of naïve witnesses. Finally, the playback of USVs alone did not trigger such vicarious freezing more than control sounds.

Prior Experience modulates vicarious freezing in rats

Recent studies put forward that mice can display empathic behaviors. In one study, the writhing behavior of a mouse in response to abdominal pain was enhanced if witnessing another mice writhe (Langford et al., 2006). The second study showed that mice express freezing when observing a conspecific being shocked (Jeon et al., 2010). The fact that we found a significant elevation of freezing in EsW rats while observing demonstrator rat receive shocks confirms that a similar form of vicarious distress behavior can be observed in another species of social rodents, the rat. Moreover, that vicarious freezing was lower in NsW compared to EsW adds to our understanding of this phenomenon by showing that having prior experience with footshock can modulate this vicarious reaction. Our findings are

in line with the study indicating that conditioned fear responses elicit significant freezing in rats that previously experienced an aversive event but not in naïve rats (Kim et al., 2010).

The fact that vicarious freezing in NsW was not only lower but also failed to differ significantly from baseline apparently contrasts with the study reporting strong vicarious freezing behavior displayed by naïve mice (Jeon et al., 2010). Many differences between the two experiments could account for this discrepancy. For example, the intensity and the frequency of the aversive stimulus (footshock) that the witnesses observe seem to play a very important role in modulating empathic responses ((Jeon et al., 2010)supplementary material). Therefore it is reasonable to think that NsW in our experiment might have shown more vicarious freezing if demonstrators had been exposed to more intense or frequent footshocks. Future experiments will be required to determine the adequate intensity and frequency of the footshock to elicit empathic response in naïve witnesses and examine how much prior experience can further augment this response. Moreover, because most other developed empathy models in rodents used male mice (Langford, 2006; Chen et al., 2009a; Jeon et al., 2010) whereas in our study we used female rats, it is plausible that there might be species and/or gender differences in vicarious fear behaviors. Species differences have been suggested by studies reported conflicting findings in social modulation of learning between mice and rats. For example, one study indicated that brief social interaction with a recently fear-conditioned conspecific improves the subsequent fear learning in rats (Knapska et al., 2010), whereas similar social interaction impairs fear learning in mice (Bredy and Barad, 2009). Gender differences, on the other hand, would dove-tail with gender differences in social support (Westenbroek et al., 2003) in rats and in social interest in human infant (Connellan et al., 2000) and chimpanzees (Alexander and Hines, 2002). Additionally, gender differences in self reported human empathy and in functional activity associated with the human mirror neuron system have been also reported (Cheng et al., 2008; Cheng et al., 2009). Nonetheless, it was recently found, with a paradigm somewhat different form the one used in our study, that prior experience plays a crucial role in social transmission of fear between male rats as well (Kim et al., 2010). Future experiments testing rats and mice of both sexes in the same paradigms will be necessary to examine the presence of gender and species differences in vicarious freezing.

In our study, we did not examine the effects of the estrous cycle on the vicarious freezing behavior, therefore variance in our data could in part be due to differences in estrous cycle. There is evidence that estrous cycle could affect anxiety and fear responses and therefore affect freezing behavior in female rats (Frye et al., 2000; Marcondes et al., 2001; Chen et al., 2009b), however other studies reported no influence of estrous cycle on anxiety levels, fear responses or social interaction in female rats (Hiroi and Neumaier, 2006; Stack et al., 2010).

Other animal studies reported that past experience play a role in reinforcing social transmission of fear and avoidance behavior in rats and empathy in pigeons (Church, 1959; Watanabe and Ono, 1986; Masuda et al., 2009; Kim et al., 2010). Moreover, there is evidence of prior experience dependent modulation of empathic behavior in humans (see Ref. (Preston and de Waal, 2003) for a review). In particular, functional magnetic resonance imaging studies reported that in humans, hearing piano does not activate the premotor cortex, if one has never played the piano. Five lessons of piano playing, however, are sufficient for the sound of piano to activate areas of the premotor cortex involved in playing the piano (Lahav et al., 2007). These results have been interpreted as evidence for Hebbian learning: a particular set of sounds (piano notes) becomes associated with a particular inner state (premotor activity required to play the piano) because each time the premotor neurons fire, the participant can hear the consequences of this action, namely the piano notes (Keysers and Perrett, 2004; Keysers and Gazzola, 2009).

Our results are compatible with a Hebbian learning account for the modulation of empathic behaviors by prior experience. When experiencing footshocks, rats will experience their own pain together with the sound and smell of their own reactions (emission of vocalizations, release of pheromones, and sound of running during the shocks alternated with the silence associated with freezing). The sensory consequences of these pain responses could become associated with the experience of pain during footshocks. Once this association is established, perceiving similar sounds and smells while a demonstrator is shocked and reacts accordingly, would trigger, by association, a vicarious form of the first-hand experience of being shocked, including vicarious freezing. Rats that have not experienced this particular type of distress would be expected to have some, albeit

weaker associations between the sensory consequences of the demonstrators distress and their own distress. Such weaker associations would originate from the naïve rats experience with other forms of stressors (flying in from the US, grabbing from their home cage, handling by unknown humans etc). These other stressors have probably led to somewhat similar/overlapping behaviors (e.g. squeaking, trying to run away, USVs), that could have been Hebbianly associated with the similar states of distress in these rats. Indeed, in our experiment, there is a trend for NsW to demonstrate more freezing than the NcW. In addition, because sensing the distress of others is such a valuable source of information about dangers, one might suspect that certain expressions of distress may be inborn triggers of vicarious emotions and behavior, and thereby cause some vicarious freezing without any need for Hebbian learning.

There might however be other, less specific routes for prior experience to influence vicarious freezing. The prior experience of stress in experienced witness groups might have altered their emotional and cognitive state. For instance, the distress during Pre-Exposure could have generated a state of heightened anxiety that would prime these animals to be more sensitive to distress signals in the empathy test or to express their own distress-behavior more readily upon sensing the distress of others (Li et al., 2008; Masuda et al., 2009). Or, the prior experience might modulate the attentional and motivational states of the witnesses towards the behavior of their conspecifics, including their demonstrators. More attention to the demonstrators would then increase vicarious freezing. In support of that possibility, EsW spent more time close to the demonstrator during and after the shock trials.

One of the core benefits of developing a potential rodent model of experience-dependent empathy is that it will afford us the possibility to disentangle these alternative accounts. For instance, repeating Experiment 1 with the addition of a group that would have experienced a different, but similarly intense, stressor during Pre-Exposure (e.g. immersion in ice water) would be highly instructive: a Hebbian account would predict this new group to freeze less, anxiety or attention accounts, as much, as the electroshock-pre-exposed group.

Prior experience of witnesses influence the demonstrator's response

We also found that during the shock exposure, D(EsW) expressed more freezing

than D(NsW). Given that demonstrator rats were randomly assigned to these two groups and received the exact same treatment throughout the experiment, the only systematic difference between these groups has to originate from systematic differences in the treatment received by their witnesses. The possible explanation for the difference in freezing behaviors of two demonstrator groups might be due to differential social buffering effects by their paired witness groups. Kiyokawa et al showed that the stress status of a partner could influence the social buffering effect in rats. In particular, rats paired with a naïve partner expressed less fear responses in a conditioning context than animals paired with previously shock-exposed partners (Kiyokawa et al., 2004). Our finding is in line with this observation: demonstrators paired with naïve witnesses showed significantly less freezing responses compared with demonstrators paired with shock pre-exposed witnesses. Issues requiring further study include the channel that is responsible for the influence exerted by the witnesses on the demonstrators and whether the difference in freezing between the demonstrators represents (i) a differences in their distress (Keysers and Perrett, 2004; Wohr et al., 2005; Brudzynski, 2007) or (ii) a difference in the propensity to display signs of distress. An analogy to human behavior might clarify these latter alternatives. Would we be genuinely more distressed by a shock if the people around us showed more signs of concern or would we simply be more encouraged to show our distress? Disentangling these possibilities will be an interesting challenge for future research. Importantly, this finding begs us to remember that social interactions are not one-way streets: the demonstrators influenced the witnesses, but the witnesses also influenced the demonstrators. While this conclusion may seem trivial, it actually brakes new grounds in the context of empathy research: most current models of empathy for pain or distress in human neuroscience used prerecorded stimuli (Wicker et al., 2003; Morrison et al., 2004; Jackson et al., 2005; Avenanti et al., 2006; Decety and Lamm, 2006; Gazzola et al., 2006; Jabbi et al., 2007; Jabbi and Keysers, 2008) or used live interactions but prevented participants from viewing the reactions of their partner (Singer, 2004; Singer et al., 2006). Accordingly, these experiments were unable, by design, to study how the observer's response influences the experience of the demonstrator. Our finding begs us to design experiments in which this feedback-loop and its neural mechanisms can be studied more explicitly in humans as well as in rodents.

The social nature of our experiment is also evidenced by video-tracking data that shows the EsW opted to spend more time in the vicinity of their demontrators than any other witness groups, and by audio recordings that show, the EsW-D(EsW) pairs communicated through more USVs than the NsW-D(NsW) pairs. The fact that the difference in freezing between the demonstrators peaked later than that in the witnesses further suggests that the behaviour of the witnesses could have contributed to that of the demonstrators.

USV playback alone does not trigger significant vicarious freezing

In the second part of our study (Experiment 2), we examined the contribution of various components of the auditory channel in triggering the vicarious freezing. In both naïve and experienced rats, USVs only produced modest freezing rates (~5%) that did not exceed the freezing response to control sounds. Therefore, USVs alone cannot account for the bulk of the vicarious stress response in our experiment, where freezing rates reached over 20% in EsW in Experiment 1. Although, the primary function of the rodent USVs remains poorly understood, 22 kHz USV have often been associated with negative and 50 kHz USVs, with positive states (Blanchard et al., 1991; Brudzynski and Chiu, 1995; Panksepp and Burgdorf, 2000; Burgdorf et al., 2005). However, it remains unclear whether and when 22kHz USV can trigger defensive behavior (fleeing or freezing) (Blanchard et al., 1991; Brudzynski and Chiu, 1995; Mongeau et al., 2003; Allen et al., 2007). At least in our experiment, and with the quality of playback achieved by our equipment, we concluded that USVs playback alone did not produce very robust freezing in naïve or experienced animals. In other situations, USVs might play a more important role (Kim et al., 2010).

Additionally, in a pilot experiment, by playing back the recorded ultrasounds together with additional audible sounds associated with the behavior of the demonstrator's distress, we observed an experience dependent increase in freezing (Fig. S1). This preliminary finding suggests that audible sounds derived from the fear response of the demonstrator rat might convey distress signals to the witness. In particular the sound of the actions of the demonstrator rat (loud metallic sounds of running intermixed with conspicuous silence) might play an important role in this communication. In monkeys and humans, the sound of the actions of one individual triggers activity in premotor and somatosensory cortices

of the listener that mirrors the activity in those of the first individual (Kohler et al., 2002; Keysers et al., 2003; Gazzola et al., 2006; Etzel et al., 2008). Whether similar mirror mechanisms are at work in the rat remains to be explored.

Given that previous studies have shown that visual (Kohn and Dennis, 1972; Langford et al., 2006) and olfactory cues (Brechbühl et al., 2008) can also play a role in social communication in rodents, our pilot data suggests that social modulation and empathy seem to be a multimodal phenomenon, with the dominant modality likely to vary from paradigm to paradigm.

CONCLUSIONS

In conclusion, placing two rats in adjacent compartments and exposing one of the two to footshocks is a simple and viable paradigm to study the way in which distress reactions of a rat influences the behavior of the other rat. Additionally, prior experience of footshocks increases the propensity of a rat to freeze in response to the distress of another. Our paradigm also evidences that the vicarious freezing of the witnessing rat can in turn influence the behavior of the demonstrating rat, closing the social loop.

As mentioned in the introduction, emotional contagion refers to cases in which an emotion in one individual triggers a similar emotion in another, while empathy proper requires that the other is aware of the fact that the triggered emotion is not his/her, but that of another person. Because it is impossible to assess whether rats have any form of awareness of their own emotions (i.e. have feelings), and of the source that triggered the emotion, it is difficult to equate our results with emotional contagion or empathy (Singer et al., 2006). Even the degree to which the witnesses in our experiment only showed similar behaviour to that of the demonstrator or felt the same emotion remains veiled. All we can state is that the witnesses reacted with a typical distress behavior to the distress of another rat, and that this represents a potential model for human empathy.

SUPPORTING INFORMATION

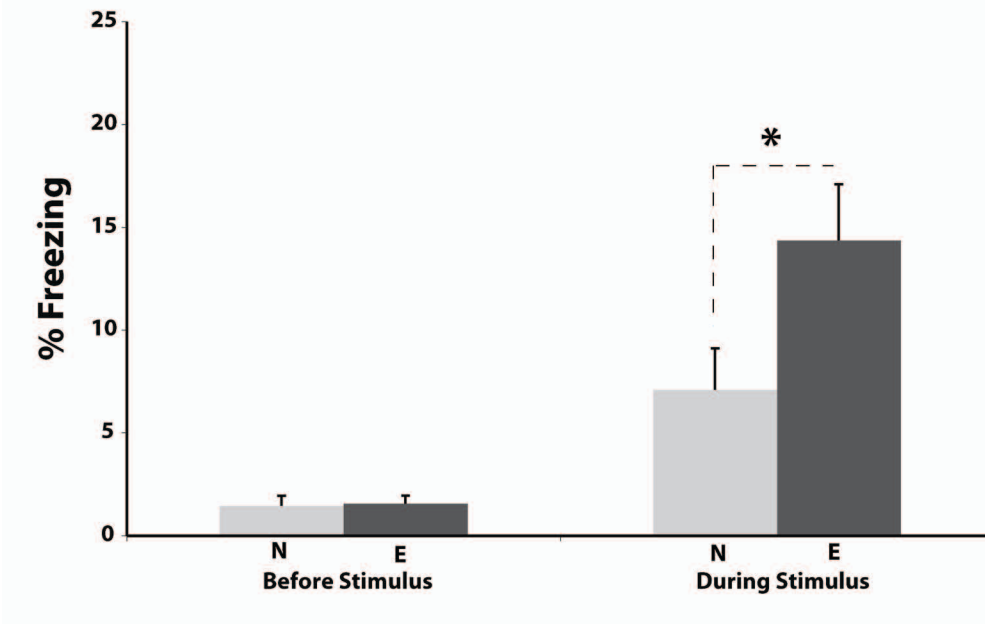

Figure S1. % Freezing behavior of Naïve (N) and Experienced (E) groups before and during the playback of the unfiltered recording (22 kHz USVs and the audible sounds < 20 kHz) from the EsW-D(EsW) pairs in Empathy Test. All data is presented as mean ± S.E.M (n = 9-10 per group). *p<0.05, Experienced group compared to Naive. Figure S1. % Freezing behavior of Naïve (N) and Experienced (E) groups before and during the playback of the unfiltered recording (22 kHz USVs and the audible sounds < 20 kHz) from the EsW-D(EsW) pairs in Empathy Test. All data is presented as mean ± S.E.M (n = 9-10 per group). *p<0.05, Experienced group compared to Naive. Figure S1. % Freezing behavior of Naïve (N) and Experienced (E) groups before and during the playback of the unfiltered recording (22 kHz USVs and the audible sounds < 20 kHz) from the EsW-D(EsW) pairs in Empathy Test. All data is presented as mean ± S.E.M (n = 9-10 per group). *p<0.05, Experienced group compared to Naive.

ACKNOWLEDGEMENTS

Authors would like to thank Angelika Jurdzinski for her technical help in the first experiment, Gert ter Horst for providing experimental space and equipment, Christel Westenbroek for useful discussions over the early version of the experimental design.

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General Introduction

CHAPTER 2

CONTENTS

1. Stress and Stress Mediators

1.1. Stress

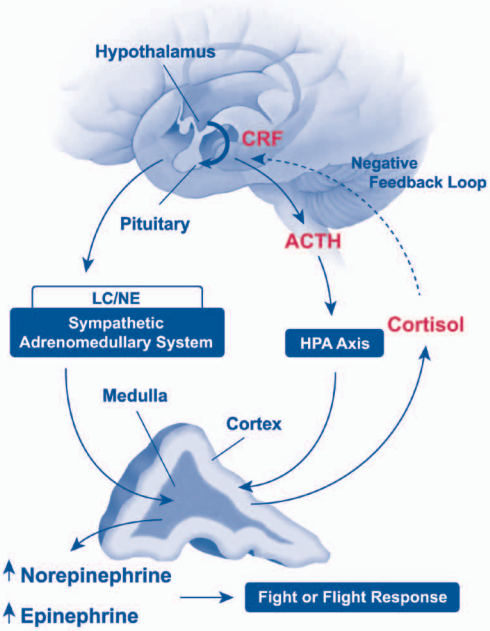
Stress is typically defined as any stimulus that represents a perceived or actual threat to the psychological and physiological equilibrium or homeostatic functioning of an organism (Selye, 1976). As a response to stress, the organism strives to reinstate the disturbed homeostasis by activating neuromodulatory systems. Released hormones promote the organism's ability to cope with stress by acting on target systems in the periphery but also inducing a myriad of effects on the brain. In addition to preparing an individual for the acute consequences of dangerous or threatening situations and the return to homeostasis, an important function of the stress response is to induce long-term adaptive changes (McEwen, 1998; McEwen, 2001). An inability to appropriately adapt to repeated stress can produce a vulnerable phenotype that is associated with a high risk for a wide array of mental disease states, including depression and post-traumatic stress disorder (Sapolsky, 2000; McEwen, 2001; Kim and Diamond, 2002).

1.2. The neuroendocrine stress response

Stress leads to an activation of the autonomic nervous system and hypothalamus–pituitary–adrenal (HPA) axis (Box 1). Activation of the autonomic nervous system results in the release of the catecholamines epinephrine and norepinephrine from the adrenal medulla and presynaptic nerve terminals (Miller and O'Callaghan, 2002; Smith and Vale, 2006; Ulrich-Lai and Herman, 2009). These catecholamines trigger an elevation in heart rate and respiration, increase blood pressure and promote energy mobilization to contribute directly to the fight-flight response with an acute preservative impact on survival systems. In the central nervous system, exposure to a stressful event rapidly activates the locus coeruleus (LC), which is the main source of norepinephrine in the brain. These LC neurons project to other brain areas, such as the prefrontal cortex, cerebellum, amygdala, and hippocampus and innervate these areas with noradrenergic signals via α- and β-adrenoceptors. Peripherally released adrenaline can also stimulate ascending vagal afferents that innervate the nucleus of solitary tract, from which noradrenergic neurons project to the basolateral complex of the amygdala (BLA) and LC. Therefore the brain is a major target for catecholamines that are released upon exposure to stressful events and arousing experiences. On the other hand,

activation of the HPA-axis triggers a cascade of events that eventually culminates in the release of glucocorticoids (cortisol in human, corticosterone in rats) from the adrenal glands (Miller and O'Callaghan, 2002). First, corticotropin-releasing factor (CRF) is secreted in response to a stressor by the medial parvocellular region of the paraventricular nucleus of the hypothalamus (PVN). This leads to the release of adrenocorticotropin hormone (ACTH) from the adrenal pituitary gland into circulation, which subsequently stimulates the release of glucocorticoids from the adrenal cortex. Blood concentrations of glucocorticoids then rise to peak 15-30 minutes after stress, and decline slowly to pre-stress levels. In the periphery, glucocorticoids exert immunosuppressive actions and increase blood glucose levels by affecting diverse metabolic processes. Glucocorticoids are highly lipophylic and, thus, directly enter the brain and bind to mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (McEwen et al., 1968). MRs have a high affinity for the natural steroids corticosterone and aldosterone and are almost saturated during basal corticosterone levels whereas GRs become occupied by higher levels of corticosterone (de Kloet et al., 2005). The catecholamine component of the stress response can be thought as representing

Box 1: The HPA Axis and Stress Response System



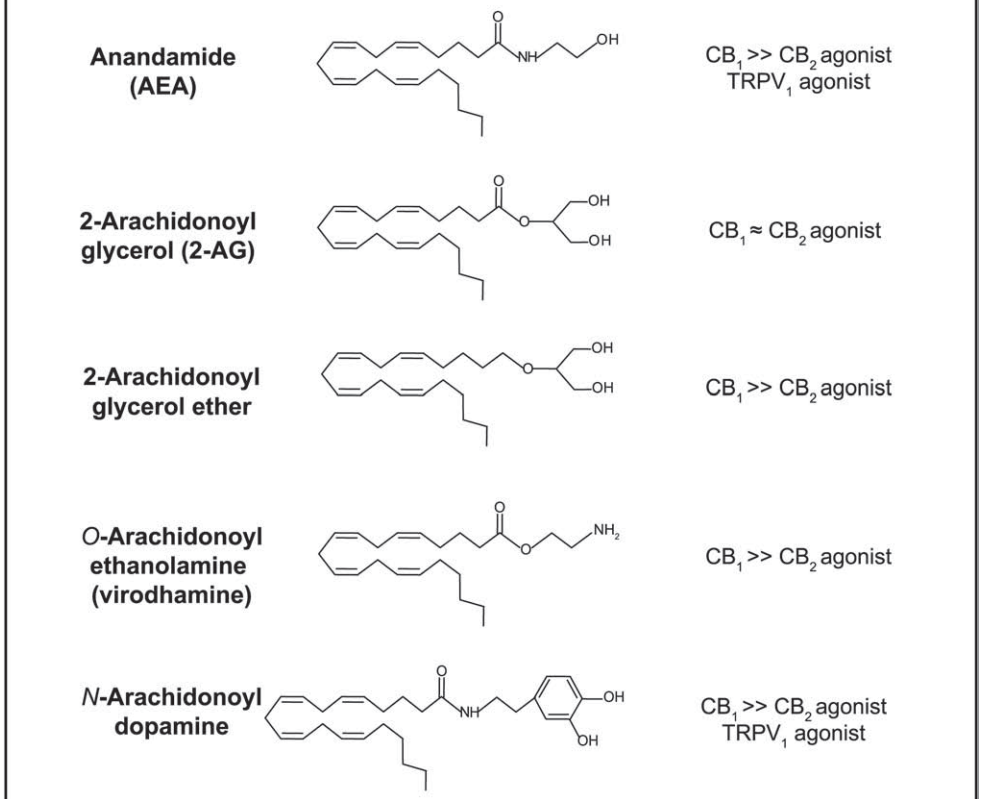
A vast amount of research has been conducted to understand the intricate cascade of events that occur once the brain detects a disruption in homeostasis (a stressor) and the hormonal responses driven by these systems. The stress system has two modes of operation and the key components of the system are the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system. The fast mode involves the corticotropin-releasing factor (CRF)-driven activation of the noradrenergic neurons of the locus coeruleus/norepinepherine (LC/NE) system in the brain. The LC/NE system is primarily responsible for the immediate "fight or flight" response driven by epinephrine and norepinephrine. The second mode initiates also with the secretion of CRF from hypothalamic paraventricular neurons, this induces the release of adrenocorticotropin hormone (ACTH) from the posterior pituitary. ACTH, then, stimulates the adrenal cortex to secrete cortisol (in humans) and corticosterone (in rats and mice). Under normal conditions, the production of CRH and ACTH fluctuate in a predictable circadian cycle and are inhibited by high levels of blood cortisol via a well-described negative feedback loop.
Corticosteroids operate in both stress-system modes through mineralocorticoid and glucocorticoid receptors (MRs and GRs), which are expressed abundantly in the neurons of limbic structures. MR is implicated in the appraisal process and the onset of the stress response. GR, which is only activated by large amounts of corticosteroid, terminates the stress reactions, mobilizes the energy resources required for this purpose and facilitates recovery. GR promotes memory storage in preparation for future events, however impairs some cognitive functionssuch as memory recall and working memory when the circulating levels are still high.

the first wave, and the glucocorticoid component as being the second wave. Catecholamines are secreted and trigger second messenger cascades in postsynaptic target tissues within seconds, whereas glucocorticoids are secreted following a latency of minutes to hours. The hormone can exert nongenomic actions (actions not involving transcriptional events) in a rapid fashion or slow genomic actions resulting from transcriptional events that take hours to emerge. Despite the differential time course of actions of these hormones, there appears to be an overlapping presence of noradrenaline and corticosteroids in time and space that allows the stage for interactions, provided that signals transduced by receptors of these ligands act in the same time frame (Joels et al., 2011).

2. Endocannabinoid System

The endocannabinoid system is a relatively unique system, exerting modulatory actions in both central tissue and in the periphery. Cannabinoid receptors type 1 (CB1) and type 2 (CB2) are activated by three major classes of ligands: Endocannabinoids (endogenously produced), plant cannabinoids (such as tetrahydrocannabinol (THC), produced by the cannabis plant) and synthetic cannabinoids (such as WIN55,212-2). The most well-defined endogenous cannabinoid ligands *N*-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) are neuroactive lipids that are produced within the brain by neurons and glia cells and function primarily as interneuronal signaling molecules (Freund et al., 2003; Kano et al., 2009) (Box 2). Endocannabinoids are released on demand in response to altered neuronal activity. The synthesis of AEA and 2-AG occurs via separate enzymatic cascades and are evoked by neuronal depolarization, elevations in intracellular calcium, and activation of several metabotropic and excitatory ionotropic neurotransmitter receptors (Freund et al., 2003; Di Marzo et al., 2005; Kano et al., 2009). Both CB1 and CB2 receptors are G protein-coupled and the CB1 receptor is expressed almost ubiquitously throughout the brain, including the olfactory bulb, neocortex, pyriform cortex, hippocampus, amygdala, basal ganglia, thalamic and hypothalamic nuclei, cerebellar cortex, and brainstem nuclei (Katona et al., 1999; Katona et al., 2001; Freund et al., 2003) whereas CB2 receptors are generally present peripherally in immunological tissues, but they have also been found within the central nervous system on neurons and glia cells with their expression mainly related to conditions of inflammation, in brain regions like the hippocampus and cerebellum (Begg

Box 2: The chemical structure of endogenous cannabinoids.



et al., 2005). The termination of endocannabinoid signaling is determined by metabolic enzymes: Fatty acid amide hydrolase (FAAH) is the primary catabolic enzyme for AEA, and hydrolyzes it into ethanolamine and arachidonic acid and 2-AG is primarily metabolized by monoacylglyceride lipase (MAG lipase) to form glycerol and arachidonic acid (Freund et al., 2003; Di Marzo, 2008). Recent studies indicated an involvement of the endocannabinoid system in many different brain functions, including synaptic plasticity, stress response, locomotion, appetite, anxiety, reward, pain and learning and memory(Kano et al., 2009).

2.1. Role as a retrograde messenger in synaptic plasticity

It is now widely accepted that postsynaptic depolarization induces an elevation of intracellular Ca^{2+} concentrations which trigger the biosynthesis of endocannabinoids. Endocannabinoids are then released from postsynaptic

neurons into the synapse, act as a retrograde messenger at various synapses in the brain and contribute to several forms of short-term and long-term synaptic plasticity. The released endocannabinoids activate presynaptic CB1 receptors and suppress transmitter release either transiently or persistently. Endocannabinoids are known to mediate the transient suppression of inhibition or excitation by inhibiting the release of glutamate (short-term: depolarization induced suppression of excitation - DSE or long-term depression at excitatory synapses – LTDe) or GABA (short-term: depolarization induced inhibition - DSI, long-term depression at inhibitory synapses - LTDi), respectively (Hashimotodani et al., 2007; Kano et al., 2009). The molecular identity of the retrograde endocannabinoid ligand that mediates DSI/ DSE or LTD has not been proven conclusively. The available evidence suggests that 2-AG is a more likely candidate than AEA to mediate DSI/DSE whereas the contribution of AEA and 2-AG to LTD seems to depend on the brain region investigated. For instance, AEA is reported to mediate LTDi in the amygdala (Azad et al., 2004) whereas 2-AG is assumed to contribute to hippocampal LTDi (Chevaleyre and Castillo, 2003). In addition to this, there are number of studies demonstrating that CB1 receptor activation inhibits neurotransmitter release. The neurotransmitters that are reported to be affected by the CB1 receptor include glycine acetylcholine, norepinephrine, dopamine, serotonin, and cholecystokinin (Kano et al., 2009).

2.2. Role of endocannabinoids in mediating the effects of stress and glucocorticoids

Besides these functions, endocannabinoids also play important role in the stress response, in particular in the return to homeostasis after stress. Since an excessive and prolonged stress or glucocorticoid response can result in deleterious effects on cardiovascular, immune, metabolic, and neural systems (Chrousos, 2009), an appropriate inhibition of the stress response is essential once the perceived stressor has subsided. This inhibition is triggered by slow as well as rapid feedback mechanisms. Classically, slow feedback inhibition represents a mechanism by which glucocorticoids shut down the HPA-axis via transcriptional regulation of gene expression. In addition to this slow feedback, glucocorticoids also mediate a rapid negative feedback regulation of the HPA axis (Dallman, 2005). Recent findings revealed that the mechanism of rapid glucocorticoid feedback inhibition of hypothalamic hormone secretion involves endocannabinoid release in the PVN. This provided a link between the actions of glucocorticoids and cannabinoids

in the hypothalamus (Di et al., 2003). In addition to an inhibition of the stress response by negative feedback mechanisms, behavioral and hormonal responses to repeated and predictable exposure to the same type of stressor exhibit stress habituation. Stress habituation is a progressive decrease in the expression of glucocorticoid secretion after repeated applications of the same stressor and represents a protective mechanism to avoid needless hormone secretion while maintaining the ability to mount a hormonal response. Much of the findings regarding the mechanisms of stress habituation have focused on the role of glucocorticoids (Jaferi et al., 2003; Jaferi and Bhatnagar, 2006). However, recent studies have also implicated endocannabinoid signaling system in this process (Patel et al., 2005; Patel and Hillard, 2008).

2.3. Role of endocannabinoid system in learning and memory

It has long been recognized that THC intake causes memory impairment in humans. In laboratory animals, effects of exogenously applied cannabinoid agonists on learning and memory have been investigated intensively using various behavioral paradigms (Kano et al., 2009; Akirav, 2011; Zanettini et al., 2011). These studies have revealed that in cannabinoid-treated animals, certain aspects of memory are largely affected, while other aspects remain intact. Since memory is assessed through behavior in animals and endocannabinoid signaling can affect many behavioral and physiological processes, it is essential to control for side effects of the cannabinoid modulation on the behavior in order to confidently attribute the effects to learning and memory processes per se.

Although there is no consensus regarding the direction of endocannbainoid effects on memory, disruptive effects of cannabinoid agonists and antagonists have been reported in many different behavioral paradigms and on different memory processes. Systemic administration of CB1 receptor agonists such as THC or WIN55212-2 shortly before a learning experience has been shown to impair the acquisition of water maze, contextual fear memory and object recognition training in rodents, whereas the CB1 receptor antagonist/inverse agonist SR141716 (rimonabant) blocks these impairing effects and even enhances the acquisition per se (Lichtman et al., 1995; Da and Takahashi, 2002; Pamplona et al., 2006). Extensive evidence also indicates that systemic CB1 receptor agonists exert impairing effects on working memory whereas the effect on retrieval is

more controversial (Lichtman et al., 1995; Nava et al., 2001; Egashira et al., 2002; Wise et al., 2009). Pharmacological enhancement of endocannabinoid tone or the activation of CB1 receptors facilitates extinction learning whereas genetic and pharmacological blockade of CB1 receptors impairs extinction learning (Suzuki et al., 2004; Chhatwal et al., 2005; Kamprath et al., 2006; Pamplona et al., 2008). Consistently, training on a fear conditioning extinction task increases endocannabinoid levels within the BLA, suggesting that this brain region mediates the effects of endocannabinoids on fear extinction learning (Marsicano et al., 2002). Accordingly, recent findings indicate that microinjections of the CB1 receptor antagonist AM251 into the BLA or hippocampus block the extinction of inhibitory avoidance training (Ganon-Elazar and Akirav, 2009; Abush and Akirav, 2010), whereas CB1 receptor antagonism in the insular cortex impairs the extinction of conditioned taste aversion (Kobilo et al., 2007). Pharmacological manipulation of the endocannabinoid system by local infusions into the brain also affects other memory processes such as memory consolidation, recall and working memory. For instance, infusions of the CB receptor agonist WIN55,212-2 into the BLA immediately after inhibitory avoidance training enhance 48-h retention performance, suggesting an enhancing effect of endocannabinoids on memory consolidation (Campolongo et al., 2009). Moreover, a blockade of CB1 receptors in the hippocampus by AM251 is known to impair the consolidation of inhibitory avoidance training (de Oliveira Alvares et al., 2006; de Oliveira Alvares et al., 2008). Studies using intrahippocampal infusions of the agonists CP55940, THC or WIN 55,212-2 in rats also showed a disrupted performance on radial-arm maze, T-maze delayed alternation, inhibitory avoidance, spatial learning as well as place recognition tasks and were thus interpreted as suggesting that the impairing effects of endocannabinoids on learning and recall are mediated through the activation of CB1 receptors in the hippocampus (Akirav et al., 2011). Infusions of a CB1 receptor agonist into prefrontal cortex is known to impair working memory (Lichtman et al., 1995; Egashira et al., 2002; Wegener et al., 2008; Abush and Akirav, 2010; Akirav, 2011). Collectively, although most reported effects of endocannabinoids are controversial and there is no consensus in the direction of the effects on memory, endocannabinoids appear to be involved in the modulation of different memory processes by influencing multiple brain regions (Kano et al., 2009; Zanettini et al., 2011). The opposite behavioral effects of cannabinoid drugs seem to derive from differences in drug dose, route of administration and timing of

exposure; however, variations in the stressfulness of the experimental conditions employed in the different studies are implicated as well. In particular, the neural processes underlying emotional memory formation and non-emotional memories (more neutral memory) seem to be differentially sensitive to cannabinoid receptor activation (Chhatwal and Ressler, 2007). It is suggested that endocannabinoids exert effects on mood and cognition via influences on noradrenergic activity and thus the origin of the altered sensitivity to endocannabinoids might result from different level of arousal that will cause a different activation of the noradrenergic system (Carvalho and Van Bockstaele, 2011). The finding that cannabinoid drugs such as WIN55,212-2 can induce opposite effects on short- and long-term object recognition memory depending on the level of emotional arousal at encoding and thus the level of noradrenergic activity supports this view (Campolongo et al., unpublished observation). Moreover, the administration of cannabinoid-like agents has been shown to increase Fos expression in LC noradrenergic neurons (Patel and Hillard, 2003; Oropeza et al., 2005) and dose-dependently increases norepinephrine levels in limbic and cortical regions (Oropeza et al., 2005; Page et al., 2007).

3. Stress & Memory

New information needs to be encoded and stored in order to enable the organism to retrieve the accurate information and express the appropriate behavior or modify it accordingly. Memory is not a single entity but composed of several separate systems and accordingly different brain systems orchestrate various tasks to form new memories. Long–term memory can be broadly divided in at least two types: Declarative (explicit) and non-declarative (implicit) memory. Declarative memory refers to conscious knowledge of facts and events, while non-declarative memory refers to a collection of non-conscious knowledge systems that provide for the capacity of learning skills, habit formation, priming. Declarative memory is further divided in subcategories as episodic (remembering specific events from the past) and semantic memory (general knowledge). Declarative memory processes rely upon the hippocampus and related structures in the medial temporal lobe including the perirhinal, entorhinal and parahippocampal cortices.

Memories are not all created equally strong: Some experiences are well remembered, while others are remembered poorly, if at all. Why is there a difference

in the strength of our memories? Extensive evidence indicates that emotions can have lasting effects on memory. Accordingly, enhanced memory for stressful or emotionally arousing events has given the first evidence regarding an influence of stress on memory. Evolutionarily this is a highly adaptive phenomenon that helps us to remember important information. Findings from experimental studies indicate that people have good recollection of where they were and what they were doing when they experienced an earthquake or witnessed an accident (Bohannon, 1988; Neisser et al., 1996). Similarly, rats remember the place in the apparatus where they received a mild footshock or the location of an escape platform in a tank filled with water (Morris et al., 1986; Vazdarjanova and McGaugh, 1998). Extensive evidence from both animal and human studies demonstrates that stress strengthens the consolidation of memory for emotionally arousing experiences (Roozendaal and McGaugh, 2011). In contrast, acute stress can cause a temporary impairment of the recall of memory such as the blockade of memory during a job interview or an important exam. Experiments that investigated this phenomenon employed paradigms in which human subjects or rats are given a session of stress shortly before assessing their ability to recall previously learned information. The findings of these experiments indicate that in both rats and humans, stress-induced impairment was linked to high levels of circulating glucocorticoids (de Quervain et al., 1998; de Quervain et al., 2000). Similar to the effects of stress on memory consolidation, stress induces memory retrieval impairment selectively under arousing conditions via interactions with the noradrenergic system (de Quervain et al., 1998; Kuhlmann et al., 2005).

3.1. Stress hormones as mediators of stress effects on memory

Decades of research show that hormones of the adrenal medulla (epinephrine) and adrenal cortex (glucocorticoids), released during and immediately after emotionally arousing experience, mediate the multifaceted effects of stress on memory (McGaugh and Roozendaal, 2002; Joels and Baram, 2009; Roozendaal et al., 2009; Joels et al., 2011). In adrenally intact rats, systemic injections of the adrenomedullary hormone epinephrine enhance memory consolidation of inhibitory avoidance when administered shortly after training (Gold and Van Buskirk, 1975; Gold et al., 1977). Comparable effects were obtained in subsequent experiments using many different types of training tasks commonly used in experiments with rats and mice (Introini-Collison et al., 1991; Costa-Miserachs

et al., 1994). Since epinephrine does not readily cross the blood-brain barrier, its effects on memory seem to be initiated by an activation of β-adrenoceptors located on vagal afferents that project to the nucleus of the solitary tract that sends direct or indirect noradrenergic projections to the forebrain, including the BLA (Roozendaal and McGaugh, 2011). Unlike epinephrine, glucocorticoids are highly lipophylic and, thus, readily enter the brain and exert their effects by modification of gene transcription through binding to intracellular or intranuclear receptors and as a result, binding of receptor homodimers to DNA (de Kloet, 2000). Glucocorticoids may also act rapidly by interacting with membrane receptors and potentiating the efficacy of other signaling cascades (Dallman, 2005; de Kloet et al., 2005; Tasker et al., 2006).

Eventually, stress effects on cognitive functions have been attributed to extensive release of glucocorticoids that occurs as a result of chronic stress or some pathological conditions such as affective disorders (Sapolsky, 2000; McEwen, 2001; de Quervain et al., 2009; Lupien et al., 2009). Early reports found both enhancing and impairing properties of glucocorticoids on memory (Bohus and Lissak, 1968; Flood et al., 1978; Beckwith et al., 1986; Luine et al., 1993; Arbel et al., 1994). However, most of these studies only used repeated or chronic treatments that do not allow disassociating the effects of glucocorticoids on memory from the effects on other cognitive functions or directly on behavior. More recent studies investigating glucocorticoid effects on memory by focused drug manipulations targeting a time frame of a particular memory phase revealed differential effects of these hormones on different memory processes. There is now extensive evidence from animal studies that glucocorticoids are critically involved in regulating the consolidation of memory processes (Flood et al., 1978; de Kloet, 2000; Roozendaal, 2000; McGaugh and Roozendaal, 2002; Roozendaal, 2002). Acute systemic administration of corticosterone or synthetic glucocorticoid ligands typically enhances long-term memory consolidation when given either before or immediately after a training experience in rats (Flood et al., 1978; Sandi and Rose, 1994; Roozendaal and McGaugh, 1996; Roozendaal et al., 1996; Pugh et al., 1997; Roozendaal et al., 1999; Cordero et al., 2002) and human subjects (Buchanan and Lovallo, 2001; Abercrombie et al., 2003; Kuhlmann and Wolf, 2006; de Quervain et al., 2009). Glucocorticoid effects on memory consolidation follow an inverted U-shape dose–response relationship: Moderate doses enhance memory, while

higher doses are typically less effective or may even impair memory consolidation (Roozendaal et al., 1999). A series of studies in animals showed that glucocorticoids do not enhance memory consolidation for all kind of training experience but particularly for emotionally arousing events (Okuda et al., 2004; Roozendaal et al., 2006a; Roozendaal et al., 2006b). For example, Okuda et al. (2004) investigated the importance of emotional arousal in mediating glucocorticoid effects on memory consolidation by manipulating the level of training-induced arousal in rats. They reported that systemic corticosterone does not enhance memory consolidation for training experience if the rat's arousal level is decreased by extensive prior habituation to the training context. These findings indicate that glucocorticoids enhance memory consolidation for emotionally arousing training experiences but do not affect memory consolidation of emotionally neutral information. Consistent with these findings in rats, human studies reported similar results with respect to learning-associated arousal as a prerequisite for the enhancing effects of glucocorticoids and stress on memory consolidation (Buchanan and Lovallo, 2001; Abercrombie et al., 2003; Kuhlmann and Wolf, 2006; Van Stegeren et al., 2007; Wolf, 2008; de Quervain et al., 2009; Marin et al., 2010).

Besides the effects of glucocorticoids on memory consolidation, many studies demonstrated differential effects of glucocorticoids on other memory processes such as memory retrieval and working memory (Kirschbaum et al., 1996; de Quervain et al., 1998; Roozendaal et al., 2004a; Roozendaal et al., 2004b; Roozendaal et al., 2004c; Cai et al., 2006; Barsegyan et al., 2010). The elevation of glucocorticoids either naturally (i.e. by stress exposure) or induced by pharmacological manipulations shortly before retention testing induces impairing effects on the retrieval of spatial/contextual memory in rats (de Quervain et al., 1998). Interestingly, this is a temporary effect that only occurs during high circulating glucocorticoid levels and fades away once the hormone levels return to baseline (de Quervain et al., 1998). Human studies consistently reported similar findings with respect to glucocorticoid effects on memory recall impairment (Kirschbaum et al., 1996; de Quervain et al., 2000; de Quervain et al., 2003; Buss et al., 2004; Het et al., 2005; Wolf, 2008). Moreover, pharmacological activation of hippocampal GRs by a specific GR agonist resulted in comparable memory retrieval impairment, indicating that these effects involve GRs and not MRs (Roozendaal et al., 2004b).

Extensive evidence from cognitive and neurobiological research indicates that the hippocampus is an important brain region involved in memory retrieval of contextual, spatial or declarative information and is also a primary target for stress hormones. Studies investigating glucocorticoid effects on memory retrieval mostly employed hippocampus-dependent learning tasks like spatial water-maze or inhibitory avoidance training in rats and declarative memory tasks in humans. Therefore, the impairing effects of glucocorticoids on memory retrieval are largely based on the effects found in these tasks. The few studies that investigated whether glucocorticoids also impair retrieval of hippocampus-independent memory (e.g. auditory fear memory) in rats reported only small or absent effects (Schutsky et al., 2011).

4. Outline of part II

Although not many can properly define stress, everybody knows what it means to have stress. The brain is the organ that interprets and determines whether an experience is stressful or not, and accordingly produces the behavioral and physiological responses thereof. A hallmark of stress response is the activation of the autonomic nervous system, resulting in the release of catecholamines from the adrenal medulla, and of the HPA-axis that culminates in the release of glucocorticoid hormones from the adrenal cortex. These stress response systems enable the organism to cope with the situation and survive acute challenges. The stress response also induces acute changes in behavior and cognition as well as long-term adaptive responses.

Glucocorticoids, released from the adrenal cortex, have potent modulatory effects on emotional memory. Specifically, glucocorticoids have been shown to enhance memory consolidation of emotionally arousing experiences, but to impair memory retrieval and working memory during emotionally arousing test situations. Glucocorticoids are known to interact with arousal-induced activation of the noradrenergic system to selectively affect memory of emotionally arousing experiences. Glucocorticoids modulate cellular function, including learning and memory, through both genomic (slow) and nongenomic (rapid) pathways (de Kloet, 2000; Dallman, 2005). Genomic glucocorticoid actions are mediated by classical steroid mechanisms involving transcriptional regulation. Although

many glucocorticoid actions fit the time frame for a genomic mechanism, some behavioral and physiological effects of glucocorticoids have a rapid onset, occurring in seconds to minutes, that is not readily compatible with transcriptional regulation. The emerging view suggests that glucocorticoids, by influences on fast inducible signaling systems such as endocannabinoids, exert their rapid and nongenomic effects on adaptive responses to stress, behavior and memory. The endocannabinoid system is a rapidly activated retrograde messenger system in the brain that exerts diverse effects on synaptic transmission, neuronal firing, mood and memory. **Chapter 3** provides an overview of the existing literature regarding the fast actions of glucocorticoid on the noradrenergic system in influencing memory functions. This chapter finalizes with a model that suggests endocannabinoids as a well-suited candidate system to mediate at least some of the rapid actions of glucocorticoids. The other chapters of this part deal with experiments aimed at validating this model.

Prior studies indicated that glucocorticoids only enhance memory consolidation of emotionally arousing, and not neutral, experiences. It is now well established that this selectivity originates from glucocorticoid-mediated facilitation of arousal-induced noradrenergic activation within the BLA. However, the neural mechanism of how glucocorticoids can rapidly influence noradrenergic function in the context of memory consolidation remains elusive. **Chapter 4** investigates whether the endocannabinoid system is essentially involved in regulating glucocorticoid effects, via a GR on the cell surface, on the noradrenergic arousal system within the BLA in enhancing memory consolidation.

As mentioned before, extensive evidence indicates that glucocorticoid hormones not only enhance the consolidation of memory but also impair the retrieval of memory of emotionally arousing experiences. Such glucocorticoid effects on memory retrieval impairment are known to depend also on rapid interactions with arousal-induced noradrenergic activity. **Chapter 5** investigates whether the endocannabinoid system is involved in mediating glucocorticoid effects on the noradrenergic system in impairing the retrieval of contextual fear memory.

Considerable evidence indicates that glucocorticoids do not impair the retrieval of all kinds of memory. Most studies investigating glucocorticoid effects on memory

retrieval examined their effects on retrieval of spatial/contextual memory in rats or of declarative memory in humans. The few studies that also investigated the effects of glucocorticoids on retrieval of recognition memory reported small and mostly nonsignificant effects. As animal studies have not explicitly examined glucocorticoid effects on retrieval of recognition memory, **Chapter 6** investigates whether glucocorticoids impair the retrieval of two components of information acquired during a single object recognition training session, i.e., memory of the training object per se and memory of the location of the object during the training session. Further, the role of the endocannabinoid system in mediating the effects of glucocorticoids on retrieval of these different aspects of recognition memory is determined.

Chapter 7 summarizes and discusses the findings of the second part of this thesis and provides conclusions and future perspectives.

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Role of the Endocannabinoid System in Regulating Glucocorticoid Effects on Memory for Emotional Experiences

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Neuroscience (2012) 204: 104-116.

CHAPTER 3

ABSTRACT

Glucocorticoids - stress hormones released from the adrenal cortex - have potent modulatory effects on emotional memory. Whereas early studies focused mostly on the detrimental effects of chronic stress and glucocorticoid exposure on cognitive performance and the classical genomic pathways that mediate these effects, recent findings indicate that glucocorticoids exert complex and often rapid influences on distinct memory phases. Specifically, glucocorticoids have been shown to enhance memory consolidation of emotionally arousing experiences, but to impair memory retrieval and working memory during emotionally arousing test situations. Furthermore, growing evidence indicates that these different glucocorticoid effects all depend on a nongenomically mediated interaction with emotional arousal-induced noradrenergic activation within the basolateral complex of the amygdala. In this paper, we present a model suggesting that the endocannabinoid system, a lipid-based retrograde signaling system, might play an important role in mediating such rapid glucocorticoid influences on the noradrenergic system in modulating memory of emotionally arousing experiences.

INTRODUCTION

Stress activates the hypothalamus–pituitary–adrenal (HPA)-axis, which results in the release of glucocorticoid hormones (cortisol in human, corticosterone in rodents) from the adrenal cortex. These hormones are known to influence the organism's ability to cope with stress, influencing target systems in the periphery, but also inducing a myriad of effects on the brain. In addition to preparing an individual for the acute consequences of threatening situations and the return to homeostasis, an important function of stress is to induce long-term adaptive responses (McEwen 1998; McEwen 2001). It has long been recognized that glucocorticoids readily enter the brain and affect cognition. Early reports on both enhancing and impairing properties of glucocorticoids on memory (Bohus and Lissak 1968; Flood et al., 1978; Beckwith et al., 1986; Luine et al., 1993; Arbel et al., 1994) have indicated that these hormones have complex effects on cognition. More recent studies investigating glucocorticoid effects on distinct memory phases and studies examining their interaction with emotional arousal helped to disentangle the multifaceted actions of these stress hormones. For example, there is now extensive evidence that glucocorticoids enhance the consolidation of long-term memory of emotionally arousing experiences (Roozendaal et al., 2006a). In contrast, elevated glucocorticoid levels are known to impair memory retrieval and working memory during emotionally arousing situations (de Quervain et al., 2009). Yet, these hormones have little effect on memory of more mundane experiences. Findings of experiments in both animals and humans investigating the neurobiological basis underlying this selectivity indicate that glucocorticoid modulation of these different memory phases all require arousal-induced noradrenergic activation within the basolateral complex of the amygdala (BLA) (Roozendaal et al., 2006a). Importantly, as many of these glucocorticoid influences on the noradrenergic system have an onset that appears too rapid to be mediated by their classic genomic actions, such findings, as well as other recent reports of rapid glucocorticoid effects, have rekindled the interest in this fascinating field and led to the discovery of novel nongenomically mediated mechanisms of glucocorticoid action on cellular function, neural plasticity and memory (de Kloet et al., 2008).

The endocannabinoid system is one emerging candidate system thought to

mediate nongenomic glucocorticoid actions in the brain (Hill and McEwen 2009). Endocannabinoids, i.e., anandamide and 2-arachidonoylglycerol (2-AG), are synthesized on demand through cleavage of membrane precursors and serve as retrograde messengers at central synapses (Hashimotodani et al., 2007). They regulate ion channel activity and neurotransmitter release (Freund et al., 2003). CB1 and CB2 cannabinoid receptors are G-protein-coupled receptors (Matsuda et al., 1990; Gerard et al., 1991). Whereas the CB1 receptor is mainly expressed in the brain, but also in the lungs, liver and kidneys, the CB2 receptor is predominantly found in glia and peripheral tissues (Morgan et al., 2009; Pacher and Mechoulam 2011). Cannabinoid receptors are activated by three major classes of ligands: Endocannabinoids (produced by the mammalian body), plant cannabinoids (such as tetrahydrocannabinol (THC), produced by the cannabis plant) and synthetic cannabinoids (such as WIN55,212-2). In the last decade, it became clear that the endocannabinoid system modulates a wide range of physiological processes and is also essential for an adaptive regulation of the stress response (Patel and Hillard 2008; Kano et al., 2009; Marsicano and Lafenetre 2009; Hill et al., 2010b). Bidirectional and functional relationships between glucocorticoids and the endocannabinoid system have been demonstrated. For example, stress is known to produce rapid changes in endocannabinoid signaling in stress-responsive brain regions. In turn, the endocannabinoid system plays an important role in the down-regulation and habituation of HPA-axis activity in response to repeated stress (Patel and Hillard 2008; Hill et al., 2010a; Hill et al., 2010c). Glucocorticoids also recruit the endocannabinoid system to exert rapid negative feedback control of the HPA-axis during stress (Di et al., 2003; Patel et al., 2005; Tasker et al., 2006; Evanson et al., 2010). It became increasingly clear, however, that CB1 receptors are also abundantly expressed in the BLA and other limbic regions where they modulate emotional arousal effects on synaptic transmission (Katona et al., 2001; Tan et al., 2011), neuronal firing (Pistis et al., 2004) and memory (Campolongo et al., 2009b; Ganon-Elazar and Akirav 2009; Marsicano and Lafenetre 2009; Tan et al., 2011).

In the present paper, we will first summarize the opposing effects of glucocorticoids on memory consolidation, memory retrieval and working memory. Then, we will describe how glucocorticoids affect noradrenergic activity of the BLA (and other brain regions) to selectively modulate memory of

emotionally arousing experiences. Finally, we will present a model suggesting that endocannabinoid signaling might play an essential role in mediating such rapid effects of glucocorticoids on the noradrenergic system in regulating memory of emotionally arousing experiences.

Glucocorticoid effects on different memory phases: dependence on arousal status

In this section, we describe the effects of glucocorticoids on different memory phases and their interaction with emotional arousal. There is compelling evidence from studies in both animals and humans that glucocorticoids are involved in regulating the consolidation of memory processes (Flood et al., 1978; de Kloet 2000; Roozendaal 2000; McGaugh and Roozendaal 2002; Roozendaal 2002; Het et al., 2005; Sandi and Pinelo-Nava 2007; de Quervain et al., 2009; Roozendaal et al., 2009). Memory consolidation is the process by which a fragile memory trace is transferred into stable long-term memory. However, not all information is equally well transferred into long-term memory. In fact, it is well recognized that especially emotionally arousing experiences are well retained (McGaugh 2004). There is extensive evidence that glucocorticoids, along with other components of the stress response, are critically involved in regulating memory consolidation of emotionally arousing experiences (McGaugh and Roozendaal 2002). A blockade of glucocorticoid production with the synthesis inhibitor metyrapone impairs memory consolidation (Roozendaal et al., 1996a; Maheu et al., 2004) and prevents epinephrine- and stress-induced memory enhancement (Roozendaal et al., 1996b; Liu et al., 1999). In contrast, acute systemic administration of corticosterone or synthetic glucocorticoid ligands typically enhances long-term memory consolidation when given either before or immediately after a training experience (Flood et al., 1978; Sandi and Rose 1994; Pugh et al., 1997; Roozendaal 2000; Cordero et al., 2002). Such glucocorticoid effects on memory consolidation follow an inverted U-shaped dose–response relationship: Moderate doses enhance memory, whereas lower or higher doses are typically less effective and may even impair memory consolidation (Roozendaal 2000). Optimal glucocorticoid-induced memory enhancement depends on a variety of experimental factors, including the intrinsic aversive properties of the training procedure, but is usually achieved with a dose of corticosterone ranging between 1 and 3 mg/kg (see Fig. 1A).

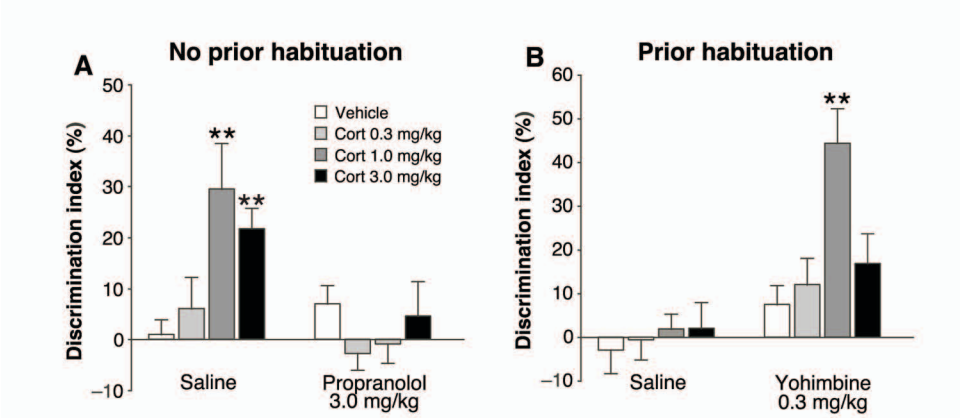

Figure 1. Glucocorticoid effects on memory consolidation for object recognition training require arousal-induced noradrenergic activation. Rats were either habituated to the training context for 7 days (prior habituation) or not habituated (no prior habituation). On day 8, they were given a 3 min training trial during which they could freely explore 2 identical objects, training was followed by systemic drug administration. Retention was tested 24 h later by placing the rats back into the apparatus for 3 min; in this trial, one object was similar to the training objects whereas the other was novel. Data represent discrimination index (%) on a 24-h retention trial, expressed as mean ± SEM. The discrimination index was calculated as the difference in the time spent exploring the novel and the familiar object, expressed as the ratio of the total time spent exploring both objects. (A) Effects of immediate posttraining administration of the β-adrenoceptor antagonist propranolol (3.0 mg/kg, s.c.) on corticosterone-induced enhancement of object recognition memory in naïve (emotionally aroused) rats. (B) Effect of coadministration of the α_2-adrenoceptor antagonist yohimbine (0.3 mg/kg, s.c.) with corticosterone on object recognition memory in habituated (emotionally non-aroused) rats. **, $P < 0.0001$ vs. vehicle. Adapted from Roozendaal et al., Proc. Natl Acad Sci USA, 2006.

Glucocorticoids are highly lipophilic and, thus, readily enter the brain and bind to mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (McEwen et al., 1968; de Kloet 2000). MRs have a high affinity for the natural steroids corticosterone and aldosterone. GRs have an approximately 10-times lower affinity for corticosterone, but show a high affinity for the synthetic ligands dexamethasone and RU 28362 (Reul et al., 1987; Sutanto and de Kloet 1987). The memory-enhancing effects of glucocorticoids appear to involve the selective activation of the low-affinity GR (Oitzl and de Kloet 1992; Roozendaal and McGaugh 1997). Such enhancing effects of glucocorticoids or GR agonists on memory consolidation have been observed in many different brain regions and with many different kinds of learning tasks, including inhibitory avoidance, contextual and cued fear conditioning, water-maze spatial and cued training, object recognition and conditioned taste aversion (Roozendaal et al., 2006a). These findings indicate that, in rodents, glucocorticoids not only enhance

memory of training on hippocampus-dependent tasks that have a strong spatial/contextual component, but also memory of recognition- and procedural training that depends on other brain regions (Miranda et al., 2008; Quirarte et al., 2009). In humans, glucocorticoid effects on memory consolidation have mostly been investigated with respect to declarative memory (Het et al., 2005).

However, glucocorticoids do not enhance memory of all experiences. Recent findings indicate that glucocorticoids enhance memory consolidation of emotionally arousing training experiences, but do not affect memory consolidation of emotionally neutral information (de Quervain et al., 2009). We investigated the importance of emotional arousal in mediating glucocorticoid effects on memory consolidation by manipulating the level of arousal during object recognition training in rats. Although no rewarding or aversive stimulation is used during object recognition training, such training induces modest novelty-induced stress or arousal (de Boer et al., 1990). However, extensive habituation of rats to the experimental context (in the absence of any objects) reduces the arousal level during the training. We found that systemic corticosterone administration does not enhance memory consolidation when the arousal level is decreased by extensive prior habituation to the training context (Okuda et al., 2004). Human studies support the findings of animal experiments that learning-associated arousal is a prerequisite for enabling the effects of glucocorticoids on memory consolidation (Abercrombie et al., 2006; Wolf 2008; de Quervain et al., 2009).

Many studies indicate that stress and glucocorticoids not only modulate the strength of newly formed memories, but also influence the remembrance of previously acquired information. In contrast to the enhancing effects of glucocorticoids on memory consolidation, these hormones typically impair memory retrieval. In the first study investigating the effects of stress and glucocorticoids on retrieval processes (de Quervain et al., 1998), we reported that 30 min after exposure to footshock stress, rats had impaired retrieval of spatial memory of a water-maze task they had acquired 24 h earlier. Interestingly, memory performance was not impaired when rats were tested either 2 min or 4 h after the stress exposure. These time-dependent effects on retrieval processes corresponded to the circulating glucocorticoid levels at the time of testing, which suggested that the retrieval impairment was directly related to stress-induced increases in adrenocortical function. In a next step, we have translated these

findings to healthy humans and found that a single administration of cortisone shortly before retention testing impaired the free recall of words learned 24 h earlier (de Quervain et al., 2000). Several further studies from different laboratories have indicated that stress exposure, glucocorticoids or the selective GR agonists dexamethasone and RU 28362 impair the retrieval of spatial or contextual memory in rats and declarative (mostly episodic) memory in humans (Wolf et al., 2001; Roozendaal et al., 2003; Buss et al., 2004; Rashidy-Pour et al., 2004; Roozendaal et al., 2004b; Het et al., 2005; Kuhlmann et al., 2005a; Kuhlmann et al., 2005b; Sajadi et al., 2007; Coluccia et al., 2008; Wolf 2008). Highly comparable to the above-described effects of glucocorticoids on memory consolidation, glucocorticoids only impair memory retrieval of emotionally arousing information or during emotionally arousing test situations (Kuhlmann et al., 2005a; Kuhlmann et al., 2005b; de Quervain et al., 2007; Smeets et al., 2008).

Glucocorticoids also impair working memory. Working memory is a dynamic process in which information is updated continuously, providing a temporary storage of information (Baddeley 1992). Evidence from lesion, pharmacological, imaging and clinical studies indicates that working memory depends on the integrity of the prefrontal cortex (Brito et al., 1982; Fuster 1991). Stress exposure is known to impair performance of rats on a delayed alternation task, a task commonly used to assess working memory in rodents (Arnsten 1998). Although basal levels of endogenous glucocorticoids are required to maintain prefrontal cortical function (Mizoguchi et al., 2004), systemic administration of stress doses of corticosterone (1-3 mg/kg) impairs delayed alternation performance in rats (Roozendaal et al., 2004c). Stress or stress-level cortisol administration is also known to impair working memory performance in human subjects during demanding tasks that require a high level of arousal (Baddeley 1992; Lupien et al., 1999; Young et al., 1999; Wolf et al., 2001; Schoofs et al., 2008).

Glucocorticoid interactions with arousal-induced noradrenergic activation in the basolateral amygdala

Why do glucocorticoids only modulate memory of emotionally arousing experiences? Our findings suggest that interactions between stress hormones and noradrenergic activity within the amygdala may be key in determining this selectivity. It is well established that emotional experiences that induce the release

of adrenal stress hormones also increase amygdala neuronal activity (Pelletier et al., 2005). Extensive evidence from our as well as other laboratories indicates that the enhancing effects of stress hormone administration on the consolidation of memory of emotionally arousing experiences involve noradrenergic activity within the amygdala. A β-adrenoceptor antagonist infused into the BLA, but not the neighboring central amygdala, blocks memory enhancement induced by a glucocorticoid administered either systemically or directly into the BLA (Quirarte et al., 1997; Roozendaal et al., 2002). It is well known that norepinephrine is released into the amygdala during emotionally arousing conditions (McIntyre et al., 2002) and considerable evidence indicates that glucocorticoids interact with training-associated noradrenergic activation within the amygdala to enhance the consolidation of memory of emotionally arousing training experiences. For example, an *in vivo* microdialysis study reported that the administration of a memory-enhancing dose of corticosterone after inhibitory avoidance training rapidly increases norepinephrine levels within the amygdala (McReynolds et al., 2010). In contrast, administration of the same dose of corticosterone to non-trained control rats does not increase norepinephrine levels in the amygdala, indicating an important role for emotional arousal in mediating such a glucocorticoid-induced facilitation of norepinephrine levels. Importantly, norepinephrine levels shortly after training were positively correlated with retention performance assessed 24 h later. Moreover, as the corticosterone-induced release of norepinephrine occurred in a rather rapid fashion (~15 min), it is likely that this effect is mediated by a nongenomic mechanism. Human studies have also provided evidence that stress hormone effects on memory enhancement for emotionally arousing experiences require concurrent amygdala and noradrenergic activity (Cahill et al., 1995; Cahill et al., 1996; Adolphs et al., 1997; Hamann et al., 1999; Canli et al., 2000). Such interactions between glucocorticoids and the amygdala noradrenergic arousal system are known to guide neural plasticity and memory in its many target regions such as the hippocampus, caudate nucleus and different cortical regions (McGaugh 2000; Roozendaal 2000; Vouimba et al., 2007; McReynolds et al., 2010).

Based on the evidence summarized above, it may be hypothesized that emotional arousal-induced increases in noradrenergic activity within the BLA are essential in enabling glucocorticoid effects on memory consolidation. Such a mechanism may then provide a direct explanation of the finding that glucocorticoids

selectively enhance memory consolidation of emotionally arousing experiences. We investigated this issue in rats trained on an object recognition task. As is discussed above, corticosterone enhances memory of object recognition training when administered to naïve rats, but is ineffective in rats that have reduced training-associated emotional arousal because of prior habituation to the experimental context (Okuda et al., 2004). As is shown in Fig. 1A, we found that, in non-habituated (i.e., emotionally aroused) rats, the β-adrenoceptor antagonist propranolol administered systemically blocks the corticosterone-induced memory enhancement (Roozendaal et al., 2006b). Propranolol infused directly into the BLA also blocks the enhancing effects of corticosterone on object recognition memory. To determine whether the failure of corticosterone to enhance memory consolidation under low-arousing conditions is due to insufficient training-induced noradrenergic activation, low doses of the α_2-adrenoceptor antagonist yohimbine, which increases norepinephrine levels in the brain, were co-administered with the corticosterone to well-habituated rats immediately after object recognition training (Fig. 1B) (Roozendaal et al., 2006b). The critical finding of this study was that such an augmented noradrenergic tone was sufficient to mimic the effects of emotional arousal in that simultaneously administered corticosterone enhanced memory consolidation (Roozendaal et al., 2006b). Further, in habituated rats, corticosterone activated BLA neurons, as assessed by phosphorylated cAMP response-element binding (pCREB) protein immunoreactivity levels, only in animals also given yohimbine (Roozendaal et al., 2006b). Such observations strongly suggest that because glucocorticoid effects on memory consolidation require noradrenergic activation within the BLA, they only modulate memory under emotionally arousing conditions that induce the release of norepinephrine.

Findings of studies investigating the neurobiological mechanism of glucocorticoid interactions with the noradrenergic system suggest that, in addition to glucocorticoid-induced release of norepinephrine from presynaptic sites, an activation of postsynaptic GRs in pyramidal neurons of the BLA may facilitate memory consolidation by potentiating the norepinephrine signaling cascade through an interaction with G-protein-mediated events (Roozendaal et al., 2002). Posttraining intra-BLA infusions of the β-adrenoceptor agonist clenbuterol or the cAMP analog 8-bromo-cAMP enhance memory consolidation

in a dose-dependent fashion (Introini-Collison et al., 1991; Liang et al., 1995; Ferry and McGaugh 1999). The GR antagonist RU 38486 infused into the BLA shortly before training shifts the dose–response effects of clenbuterol such that a much higher dose of clenbuterol is required to induce comparable memory enhancement (Roozendaal et al., 2002). In contrast, the GR antagonist does not modify the dose–response effects of 8-bromo-cAMP, indicating that cAMP acts in the BLA downstream from the locus of interaction of glucocorticoids with the β-adrenoceptor-AMP/protein kinase A (PKA) pathway. These findings strongly suggest that glucocorticoids enhance memory consolidation, in a permissive fashion, by potentiating β-adrenoceptor-cAMP/PKA efficacy in the BLA (Roozendaal et al., 2002). Findings of electrophysiological experiments in brain slices also indicate that glucocorticoids interact with the noradrenergic system in influencing neural plasticity in BLA neurons. It should be noted that these studies indicate that glucocorticoids affect noradrenergic function in a time-dependent manner (Joels et al., 2011). For example, when corticosterone was applied simultaneously with a β-adrenoceptor agonist, AMPA receptor-mediated responses in BLA neurons were rapidly facilitated, consistent with the idea that corticosterone requires concurrent noradrenergic activity to enhance memory consolidation of emotionally arousing information. However, when the corticosterone was applied several hours in advance to the β-adrenoceptor agonist, possibly genomic GR actions kick in, resulting in a potent suppression of the β-adrenoceptor agonist effect on AMPA receptor-mediated responses (Liebmann et al., 2009).

Recent findings indicate that the BLA is not the only brain region mediating glucocorticoid effects on the noradrenergic system in regulating memory consolidation. Highly comparable to the effects found in the BLA, infusions of a β-adrenoceptor antagonist or PKA inhibitor into the medial prefrontal cortex after inhibitory avoidance training prevent the memory enhancement of a GR agonist administered concurrently (Barsegyan et al., 2010). Moreover, corticosterone administered systemically immediately after inhibitory avoidance training increases PKA activity in the medial prefrontal cortex within 30 min, further supporting the view that glucocorticoid effects on facilitating noradrenergic signaling are mediated via rapid, nongenomic actions. Glucocorticoids also interact with the noradrenergic system in the insular cortex in regulating memory

consolidation. In one study (Roozendaal et al., 2010), corticosterone conjugated to a bovine serum albumin molecule (i.e., cort:BSA) was infused into the insular cortex immediately after object recognition training in non-habituated rats. This conjugate does not cross the cell membrane and thus selectively activates adrenal steroid receptors on the cell surface (Chiyo et al., 2003). This corticosterone conjugate enhanced memory consolidation of the training, and the effect was blocked by co-administration of a GR, but not MR, antagonist, thus, providing support for the view that GRs on or near the cell surface are implicated in mediating glucocorticoid effects on memory consolidation. Moreover, consistent with the view that glucocorticoids interact with the norepinephrine signaling pathway, a selective PKA inhibitor also blocked the cort:BSA-induced memory enhancement (Roozendaal et al., 2010). Immunocytochemistry revealed that cort:BSA infused after object recognition training increases pCREB levels in insular cortex neurons (Roozendaal et al., 2010).

There is now compelling evidence that the impairing effects of glucocorticoids on memory retrieval and working memory under emotionally arousing test conditions also depend on concurrent noradrenergic activity within the BLA (and other brain regions). For example, systemic administration of the β-adrenoceptor

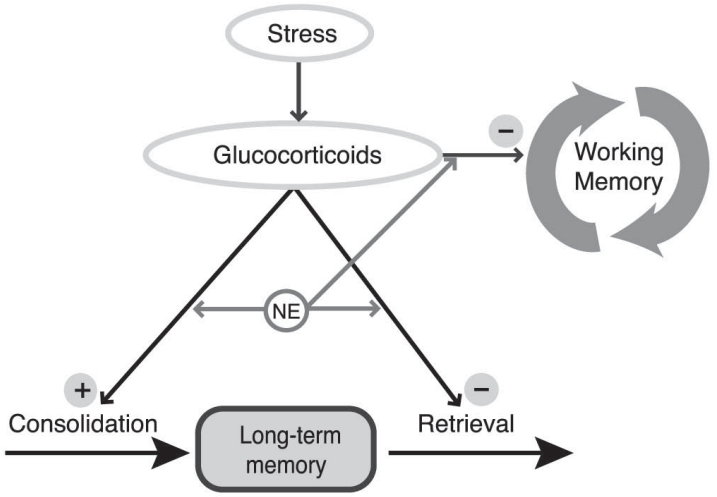

Figure 2. Effects of stress and glucocorticoids on memory functions. Glucocorticoids enhance memory consolidation, whereas they impair memory retrieval and working memory. All of these glucocorticoid effects depend on emotional arousal-induced noradrenergic activity. NE, Norepinephrine. Adapted from de Quervain et al. Front Neuroendocrinol, 2009, with permission.

antagonist propranolol, 30 min before retention testing, blocks the memory retrieval impairment of spatial/contextual information induced by a concurrent injection of corticosterone (Roozendaal et al., 2004a). A β-adrenoceptor antagonist infused into the BLA or hippocampus also prevents the retrieval-impairing effect of a GR agonist administered concurrently (Roozendaal et al., 2004b). Moreover, stimulation of β_1-adrenoceptors with systemic injections of the selective agonist xamoterol induces memory retrieval impairment comparable to that seen after corticosterone administration (Roozendaal et al., 2004b). Collectively, these findings suggest that the impairing effects of glucocorticoids on memory retrieval involve a facilitation of noradrenergic mechanisms. Given that norepinephrine is activated by emotional arousal, this could explain why emotionally arousing information or an emotionally arousing test situation is a prerequisite for enabling glucocorticoid effects on memory retrieval. In line with this view, we recently reported that the β-adrenoceptor antagonist propranolol blocks the impairing effect of cortisone on the retrieval of emotionally arousing verbal material in healthy humans (de Quervain et al., 2007). Glucocorticoid effects on working memory also depend on interactions with noradrenergic mechanisms. A β-adrenoceptor antagonist administered systemically blocks the impairing effect of corticosterone on working memory in rats (Roozendaal et al., 2004c). Furthermore, a β-adrenoceptor antagonist or PKA inhibitor infused into the mPFC blocks working memory impairment induced by a GR agonist administered concurrently (Barsegyan et al., 2010).

Thus, the findings summarized above indicate that glucocorticoids, via a nongenomically mediated mechanism, interact with arousal-induced noradrenergic activation to selectively influence memory consolidation, retrieval and working memory during emotionally arousing test situations (Fig. 2). However, they do not provide an explanation of how glucocorticoids might be able to rapidly influence noradrenergic transmission. In the next section, we will provide a brief overview of nongenomic glucocorticoid mechanisms.

Rapid nongenomic glucocorticoid effects: involvement of the endocannabinoid system

Glucocorticoids are known to modulate cellular function, including learning and memory, through both genomic (slow) and nongenomic (rapid) pathways (de Kloet

2000; Dallman 2005). Genomic glucocorticoid effects are mediated by classical steroid mechanisms involving transcriptional regulation. Glucocorticoids can influence transcription through both DNA binding-dependent and DNA binding–independent mechanisms (de Kloet 2000). Although many glucocorticoid actions suit the timeframe for a genomic mechanism, some behavioral and physiological effects of glucocorticoids, for example the above-described effects on the noradrenergic system, have a rapid onset, occurring in seconds to minutes, that is not readily compatible with transcriptional regulation. Rapid glucocorticoid actions have been reported in different limbic and brainstem structures, where they control functions ranging from learning and memory to neuroendocrine functions (Dallman 2005; Tasker et al., 2006; Haller et al., 2008; Riedemann et al., 2010). It is important to note that glucocorticoid effects on the consolidation of long-term memory might depend on an interplay between genomic and nongenomic actions (Falkenstein et al., 2000), whereas glucocorticoids' ability to temporarily impair memory retrieval and working memory might depend solely on nongenomic glucocorticoid actions. In support of this view, it has been reported that protein synthesis inhibitors fail to prevent glucocorticoid effects on memory retrieval (Sajadi et al., 2006).

Nongenomic glucocorticoid actions likely involve the activation of a membrane-associated variant(s) of the steroid receptor (Losel et al., 2003; Dallman 2005; Tasker et al., 2006; Riedemann et al., 2010). Orchinik and colleagues (Orchinik et al., 1991; Rose et al., 1993) were the first to provide evidence that glucocorticoids exert behavioral effects through the activation of a corticosteroid receptor on the neuronal membrane. In this series of experiments, glucocorticoids rapidly suppressed mating behavior in the amphibian *Taricha granulosa* (rough-skinned newt) by binding to a receptor on neuronal membranes. As mentioned, recent findings indicate that the administration of a membrane-impermeable glucocorticoid ligand, cort:BSA, into a variety of brain regions of the rat is sufficient to enhance the consolidation of long-term memory of emotionally arousing training experiences (Roozendaal et al., 2010; Lee et al., 2011) or to impair working memory (Barsegyan et al., 2010). As these cort:BSA effects are blocked by co-administration of a GR, but not MR, antagonist (Barsegyan et al., 2010; Roozendaal et al., 2010), these findings suggest a role for a membrane-associated GR in mediating rapid glucocorticoid effects on memory. Studies employing GR

immunoreactivity, at both the light- and electronmicroscopic level, provided anatomical evidence for the existence of membrane-associated GRs in neurons of the hippocampus and hypothalamus (Liposits and Bohn 1993) and postsynaptic membranes of lateral amygdala neurons (Johnson et al., 2005).

Current evidence indicates a variety of nongenomic glucocorticoid actions on neuroplasticity and memory, ranging from a rapid increase in glutamate-release probability from presynaptic sites (Karst et al., 2005) to a rapid insertion of AMPA receptor subunits in postsynaptic membranes (Groc et al., 2008). Recent evidence indicates that the endocannabinoid system also mediates some of the rapid effects of glucocorticoids. The first evidence originated from an elegant series of *in vitro* studies that examined glucocorticoid effects in hypothalamic nuclei on HPA-axis activity. In these experiments, Tasker and colleagues demonstrated that endocannabinoids mediate glucocorticoid-induced suppression of glutamate release in hypothalamic neurons of the paraventricular nucleus, which, in turn, results in a rapid inhibition of hormone secretion (Di et al., 2003; Di et al., 2005a). Biochemical analysis of hypothalamic slices treated with dexamethasone revealed a rapid increase in the endocannabinoids anandamide and 2-AG (Di et al., 2005a; Di et al., 2005b). According to their model, corticosterone first binds to a membrane-bound steroid receptor and this leads to an activation of divergent G-protein-mediated signaling pathways that culminates in the release of endocannabinoids (Di et al., 2003; Di et al., 2005a; Malcher-Lopes et al., 2006; Tasker et al., 2006; Di et al., 2009). Endocannabinoid release from the postsynaptic membrane then suppresses the release of glutamate and this may represent a mechanism by which endocannabinoids regulate HPA-axis activity. A more recent *in vivo* study by Hill et al. (2010a), examining the effect of corticosterone on tissue content of endocannabinoids in limbic brain regions, indicated surprisingly similar results. A single injection of corticosterone rapidly (within 10 min) elevated anandamide levels in both the amygdala and hippocampus.

As reviewed above, glucocorticoids are able to rapidly influence noradrenergic function to affect different memory functions. Given the now well-described role of endocannabinoids in regulating glucocorticoid effects on HPA-axis activity and the ability of glucocorticoids to rapidly recruit endocannabinoids in limbic brain regions, it is plausible that the endocannabinoid system is involved in mediating

the rapid effects of glucocorticoids on learning and memory functions. In the next section, we will first review findings indicating that endocannabinoids are involved in regulating learning and memory processes, and then we will present a model that implicates endocannabinoid signaling in regulating glucocorticoid effects on memory of emotionally arousing experiences.

Role of endocannabinoids in regulating glucocorticoid effects on memory: the model

Endocannabinoid signaling is crucial for certain forms of short- and long-term synaptic plasticity at excitatory and inhibitory synapses, and thereby contributes to various aspects of brain function, including learning and memory (Kano et al., 2009). It has long been recognized, in animals and humans, that endocannabinoids influence different memory phases. Behavioral studies in humans indicate that cannabis consumption as well as synthetic CB1 receptor agonists impair cognitive processes that subserve executive function. Executive function comprises cognitive processes such as attention, behavioral flexibility, decision-making, inhibitory control, planning, time estimation and working memory, and is crucially involved in top-down control of behavior (Egerton et al., 2006; Solowij and Michie 2007). Although the neurobiological mechanisms underlying the deleterious effects of cannabinoids on executive function are, as yet, largely unknown, accumulating preclinical findings emphasize the importance of modulatory actions on prefrontal cortical and striatal noradrenergic, dopaminergic and glutamatergic transmission (Pattij et al., 2008). Neuroimaging studies support this hypothesis and showed that changes in the activity of prefrontal, orbitofrontal and anterior cingulate cortices (among other regions) correlate with altered inhibitory processing in cannabis users (Solowij and Michie 2007). However, CB1 receptors are also highly expressed in the human limbic system, including the amygdala (Killgore and Yurgelun-Todd 2004). Animal studies confirmed the presence of CB1 receptors on GABAergic interneurons in the BLA, where they modulate synaptic transmission (Katona et al., 2001) and neuronal firing (Pistis et al., 2004). Preclinical research demonstrated a functional interaction between cannabinoids and stress in the activation of the amygdala, which may provide a context for understanding the interplay between these two systems in the regulation of memory formation for emotionally arousing events (Patel et al., 2005).

We recently investigated whether the endocannabinoid system in the BLA

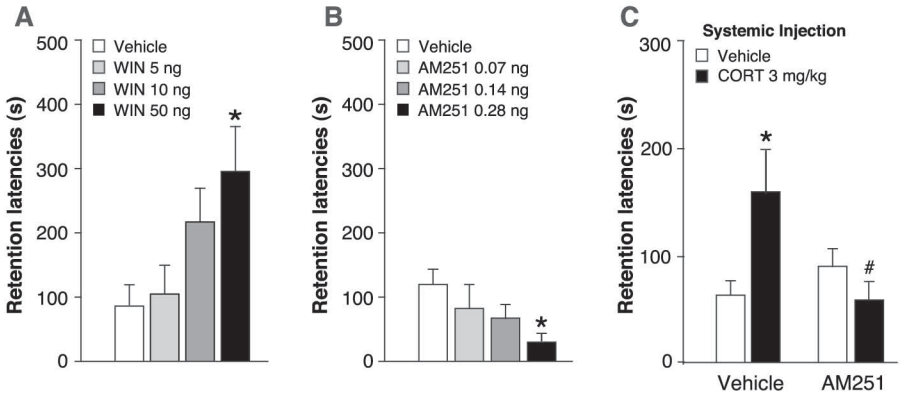

Figure 3. Endocannabinoids mediate glucocorticoid effects on memory consolidation for inhibitory avoidance training. Data represent step-through latencies (mean + SEM) on a 48-h retention test. (A) Immediate posttraining intra-BLA infusions of the CB1 receptor agonist WIN55,212-2 (WIN; 5, 10, 50 ng per 0.2 μl) enhanced memory consolidation. *, $P < 0.05$ vs. vehicle. (B) Immediate posttraining intra-BLA infusions of the CB1 receptor antagonist AM251 (0.07, 0.14, 0.28 ng per 0.2 μl) impaired memory consolidation. *, $P < 0.05$ vs. vehicle. (C) Immediate posttraining infusions of AM251 (0.14 ng per 0.2 μl) into the BLA blocked the memory enhancement induced by a subcutaneous injection of corticosterone (CORT; 3.0 mg/kg; *, $P < 0.05$ compared with the corresponding vehicle group; #, $P < 0.05$ compared with the corresponding CORT group. Adapted from Campolongo et al., Proc Natl Acad Sci USA, 2009.

influences memory consolidation of an emotionally arousing inhibitory avoidance training experience (Campolongo et al., 2009b). As is shown in Fig. 3A and 3B, intra-BLA infusion of the CB1 receptor agonist WIN55,212-2 administered immediately after the inhibitory avoidance training trial dose-dependently enhances memory consolidation, whereas that of the CB1 receptor antagonist AM251 impairs memory consolidation. These findings indicate that a manipulation of cannabinoid transmission in the BLA influences memory consolidation for emotionally arousing experiences; however, whether they also modulate memory of low-arousing training experiences has never been investigated. Consistent with these findings, others have reported that infusion of the CB1 receptor antagonist AM251 into the amygdala (Bucherelli 2006) or hippocampus (de Oliveira Alvares et al., 2005) disrupts the consolidation of long-term memory, possibly by inhibiting long-term potentiation (de Oliveira Alvares et al., 2006). Several other studies indicated an involvement of the endocannabinoid system in the extinction of fear memories (Marsicano et al., 2002; Suzuki et al., 2004; Chhatwal et al., 2005). We recently found that, similar to glucocorticoid effects on memory consolidation, endocannabinoid effects on memory for inhibitory

avoidance training follow an inverted-U shaped dose-response relationship (D. Hauer, P. Atsak, E. Eggens-Meijer, P. Campolongo, G. Schelling, R. Fornari, B. Roozendaal, unpublished observation). Accordingly, impairing effects with endocannabinoids have been described as well. For example, findings indicate that systemic administration of a much higher dose of the CB1 receptor agonist WIN55,212-2 impairs the acquisition of contextual fear conditioning (Pamplona and Takahashi 2006) and that pretraining intrahippocampal administration of

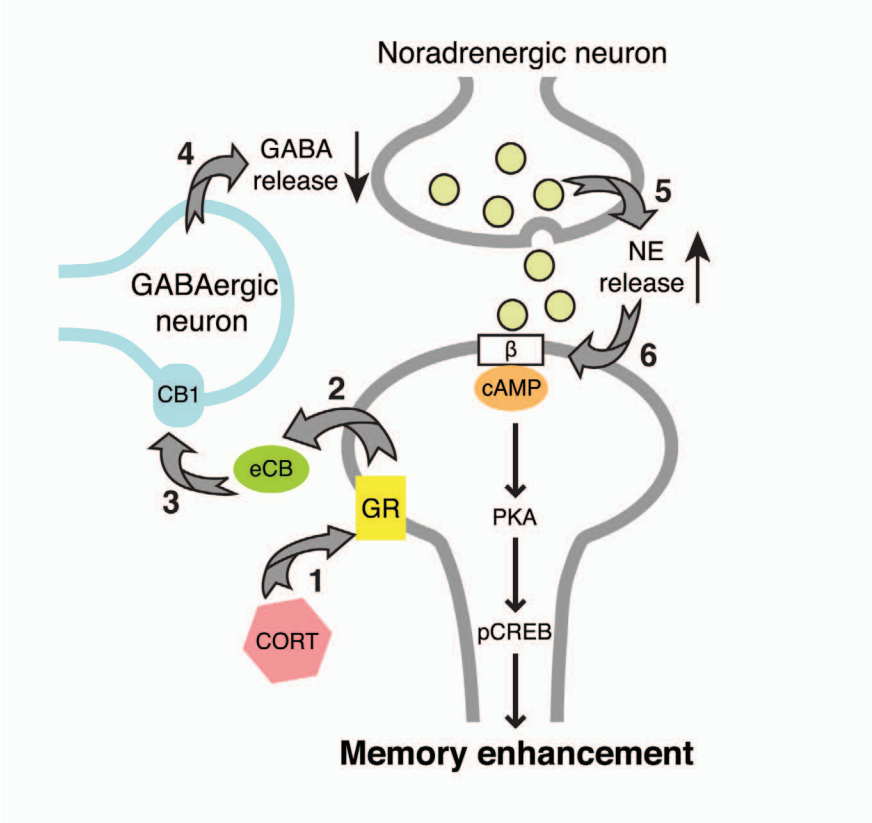

Figure 4. Model on the role of the endocannabinoid system in the BLA in mediating glucocorticoid effects on norepinephrine release in regulating memory consolidation. Corticosterone (CORT) is released during training in emotionally arousing tasks and binds to a membrane-bound glucocorticoid receptor (GR) (1), that activates a pathway to induce endocannabinoid (eCB) synthesis (2). Endocannabinoids are then released into the synapse where they bind to CB1 receptors on GABAergic terminals (3), and thereby inhibit the release of GABA (4). This suppression of GABA release subsequently disinhibits norepinephrine (NE) release (5), and this results in an activation of the postsynaptic β-adrenoceptor and the downstream cAMP/PKA/pCREB intracellular signaling pathway (6). These stress hormone effects on noradrenergic activation in the BLA are required for enhancing memory consolidation. Adapted from Hill and McEwen, Proc. Natl Acad Sci USA, 2009.

anandamide impairs memory consolidation of inhibitory avoidance training (Barros et al., 2004). Moreover, it has been reported that infusion of a CB1 receptor antagonist into the hippocampus during water-maze spatial training enhances the acquisition of this task (Robinson et al., 2008). In addition to differences in drug dosage, administration route and regimen, these opposite findings might be attributed to binding of endocannabinoids to different receptor systems, e.g., anandamide-induced activation of the transient receptor potential vanilloid type 1 receptor (TRPV1) has been reported to produce opposite effects to that of binding to the CB1 receptor (Rubino et al., 2008). Collectively, the findings appear comparable to those described for glucocorticoids in that endocannabinoids consistently impair working memory and memory retrieval, while having dose-dependent favorable effects on memory consolidation.

Given the close interaction between the endocannabinoid and glucocorticoid systems, we investigated whether endocannabinoid transmission might play a role in mediating glucocorticoid effects on memory consolidation. For this experiment, rats were trained on an inhibitory avoidance task and received immediate posttraining infusions of the CB1 receptor antagonist AM251 into the BLA together with a systemic administration of corticosterone. As is shown in Fig. 3C, intra-BLA administration of the CB1 receptor antagonist blocked the ability of corticosterone to facilitate memory consolidation of the inhibitory avoidance training (Campolongo et al., 2009b). Similarly, other researchers found that a CB1 receptor antagonist infused into the hippocampus blocked memory enhancement induced by the synthetic glucocorticoid dexamethasone (de Oliveira Alvares et al., 2010) To investigate whether this glucocorticoid effect on the endocannabinoid system is mediated by a GR on the cell surface, we performed an additional experiment. The findings of this experiment indicate that the CB1 receptor antagonist AM251 infused into the BLA blocked the memory-enhancing effects induced by concurrent infusions of either a specific GR agonist or the membrane-impermeable ligand cort:BSA (D. Hauer, P. Atsak, E. Eggens-Meijer, P. Campolongo, G. Schelling, R. Fornari, B. Roozendaal, unpublished observation). Therefore, these findings indicate that endocannabinoid transmission is required for mediating glucocorticoid effects on memory consolidation, presumably involving the activation of a GR on the cell surface.

Does the endocannabinoid system mediate the rapid effects of glucocorticoids on the noradrenergic system in selectively regulating memory of emotionally arousing experiences? Although direct evidence is lacking, it is well established that endocannabinoid effects on memory as well as on other behaviors are highly dependent on the emotional state of the animal (Patel et al., 2005; Hill et al., 2010c). We investigated whether endocannabinoid effects on memory consolidation might depend on concurrent noradrenergic activity within the BLA. Highly comparable to the above-described effects of glucocorticoids on memory consolidation, the β-adrenoceptor antagonist propranolol administered into the BLA prevented the memory enhancement induced by concurrent administration of the CB1 receptor agonist WIN55,212-2 (D. Hauer, P. Atsak, E. Eggens-Meijer, P. Campolongo, G. Schelling, R. Fornari, B. Roozendaal, unpublished observation). In an earlier paper, we already reported that systemic administration of the endocannabinoid oleoylethanolamide enhances memory consolidation of inhibitory avoidance training. As the β-adrenoceptor antagonist propranolol infused into the BLA blocks this memory enhancement (Campolongo et al., 2009a), these findings indicate that also oleoylethanolamide enhances memory consolidation via a norepinephrine-dependent mechanism in the BLA. Moreover, there is evidence that systemic or local administration of a CB1 receptor agonist increases norepinephrine levels in cortical and limbic brain regions (Oropeza et al., 2005; Page et al., 2007).

Endocannabinoids might influence noradrenergic function via a modulation of GABAergic activity. In the BLA, CB1 receptors are abundantly expressed in GABAergic interneurons (Katona et al., 2001) and activation of CB1 receptors has consistently been shown to suppress the release of GABA (Katona et al., 1999; Katona et al., 2001; Ohno-Shosaku et al., 2001) via a rapid inhibition of calcium entry into the terminals (Hoffman and Lupica 2000; Wilson et al., 2001). It is well established that the amygdala GABAergic system is involved in memory modulation. Posttraining infusions of GABA receptor antagonists into the BLA are known to enhance memory consolidation, whereas posttraining infusions of GABA receptor agonists impair memory consolidation (McGaugh and Roozendaal 2002). Importantly, the modulatory effects of GABAergic transmission on memory crucially depend on an interaction with the noradrenergic system. A β-adrenoceptor antagonist administered systemically or directly into the BLA

prevents the modulatory effects of GABAergic drugs on memory consolidation (McGaugh, 2004). Moreover, an *in vivo* microdialysis study indicated that the administration of a GABA receptor antagonist increases norepinephrine levels in the amygdala, whereas that of a GABA receptor agonist decreases norepinephrine levels (Hatfield et al., 1999). Thus, endocannabinoids might increase BLA neuronal activity by decreasing GABAergic neurotransmission, leading to increased noradrenergic activity within the BLA. Interestingly, a recent study indicated that glucocorticoids also increase the excitability of BLA neurons by decreasing the impact of GABAergic influences (Duvarci and Paré 2007).

In summary, we propose the following model of how glucocorticoids might recruit the endocannabinoid system to influence the release of norepinephrine in the BLA in regulating memory consolidation (Fig. 4): Glucocorticoids first bind to a membrane-bound receptor that activates a G-protein-coupled signaling cascade to induce endocannabinoid release. The endocannabinoids then bind to presynaptic CB1 receptors on GABAergic terminals which rapidly suppress the release of GABA. This suppression of GABAergic transmission leads to elevated norepinephrine levels, which increase noradrenergic signaling in BLA pyramidal neurons and result in a facilitation of memory consolidation of emotionally arousing experiences.

CONCLUSIONS AND FUTURE DIRECTIONS

The findings summarized above indicate that glucocorticoids enhance memory consolidation for emotionally arousing experiences and impair memory retrieval and working memory during emotionally arousing test situations via rapid interactions with arousal-induced noradrenergic mechanisms. The endocannabinoid system might play a crucial role in mediating such rapid glucocorticoid effects on the noradrenergic system. However, there are many fascinating, unanswered questions. First, our model suggests that glucocorticoids interact with the noradrenergic system via endocannabinoid signaling. Although several findings have demonstrated that endocannabinoids modulate noradrenergic transmission, it is unclear whether this is actually achieved by a suppression of GABAergic transmission, thereby increasing noradrenergic

transmission, or by a direct influence on the noradrenergic system.
Second, glucocorticoids are known to influence noradrenergic function both at presynaptic sites, enhancing the release of norepinephrine, and postsynaptically, facilitating norepinephrine effects on the cAMP/PKA signaling pathway. It is not known whether endocannabinoids mediate glucocorticoid effects on both of these mechanisms.

Third, another important unanswered question is the role of emotional arousal in regulating glucocorticoid-endocannabinoid effects on the noradrenergic system. We presented evidence that glucocorticoid administration can increase the release of norepinephrine in the BLA, but only so under emotionally arousing conditions. Where does this switch come from? Is it possible that the endocannabinoid system might represent such a switch mechanism for glucocorticoids? To explore this possibility, it would be necessary to investigate whether glucocorticoids recruit the endocannabinoid system under low-arousing experimental conditions.

Fourth, a role for the endocannabinoid system in regulating glucocorticoid effects on memory has only been investigated with respect to memory consolidation. It would be important to investigate whether the endocannabinoid system is also involved in mediating glucocorticoid effects on the impairment of memory retrieval and working memory.

Finally, glucocorticoids have been reported to increase levels of both anandamide and 2-AG (Hill et al., 2010a). The exact role of these endogenous ligands in regulating glucocorticoid effects on the formation and retrieval of emotionally influenced memory requires more extensive analysis. Further, endocannabinoid effects on memory have only been studied with respect to CB1 receptor-mediated events. But, recent evidence indicates that the CB2 receptor is also present in the brain.

ACKNOWLEDGMENTS

Research was supported by a Jan Kornelis de Cock Foundation Research Grant to PA and 'Progretti di Ricerca 2010' (Sapienza University of Rome) to PC

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Critical role of Endocannabinoid Signaling in Mediating Rapid Glucocorticoid Effects on the Noradrenergic Arousal System in Memory Consolidation

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CHAPTER 4

ABSTRACT

Glucocorticoid hormones are known to enhance the consolidation of long-term memory of emotionally arousing experiences but not that of low-arousing or neutral information. Prior studies indicated that nongenomically mediated actions of glucocorticoids on endogenous noradrenergic activation of the basolateral complex of the amygdala (BLA) induced by emotional arousal underlie this selectivity. However, the neural mechanism of how glucocorticoids might rapidly influence noradrenergic function in the context of memory consolidation remains elusive. Here, we show that the endocannabinoid system, a rapidly activated retrograde messenger system, is essentially involved in regulating glucocorticoid effects, via a glucocorticoid receptor on the cell surface, on the amygdala noradrenergic arousal system in enhancing memory consolidation. Further, glucocorticoids require endocannabinoid signaling in facilitating norepinephrine effects on neural plasticity within the BLA, as assessed by phosphorylated cAMP response element-binding immunoreactivity levels. The findings have important implications for understanding local network functions within the BLA in regulating stress hormone effects on neural plasticity and memory consolidation.

INTRODUCTION

Emotional enhancement of memory is a highly adaptive phenomenon (Christianson, 1992), but it also contributes to the development of stress-related pathologies such as anxiety and post-traumatic stress disorder (de Quervain et al., 2009). Extensive evidence indicates that glucocorticoid hormones (corticosterone in rodents, cortisol in humans) that are released from the adrenal cortex during stressful events enhance the consolidation of long-term memory of emotionally arousing experiences in animals (de Kloet et al., 1999; McGaugh and Roozendaal, 2002; Okuda et al., 2004; Sandi and Pinelo-Nava, 2007; Roozendaal et al., 2008a; Roozendaal and McGaugh, 2011) and humans (Buchanan and Lovallo, 2001; Abercrombie et al., 2006; Kuhlmann and Wolf, 2006). Yet, these hormones have little effect on memory of more mundane experiences (de Quervain et al., 2000; Buchanan and Lovallo, 2001; Okuda et al., 2004; Kuhlmann and Wolf, 2006). Findings of experiments investigating the basis underlying this selectivity indicate that glucocorticoid effects on memory consolidation depend crucially on nongenomically mediated actions, via a putative membrane-associated glucocorticoid receptor (GR), on endogenous noradrenergic activation within the basolateral complex of the amygdala (BLA) induced by emotional arousal (Quirarte et al., 1997; Roozendaal, 2000; Roozendaal, 2002; Roozendaal et al., 2006a; Hurlemann et al., 2007; Van Stegeren et al., 2007). In earlier studies, we described that glucocorticoids administered after an emotionally arousing, but not low-arousing, training experience rapidly elevate norepinephrine levels in the BLA (McReynolds et al., 2010) and facilitate norepinephrine effects on the cAMP-dependent protein kinase pathway to induce the phosphorylation of cAMP response element-binding (pCREB) protein (Roozendaal, 2002; Roozendaal et al., 2006b), whereas a blockade of β-adrenoceptors or cAMP signaling in the BLA prevents glucocorticoid-induced memory enhancement (Quirarte et al., 1997; Roozendaal et al., 2002b). However, the neural mechanism of how glucocorticoids might rapidly alter noradrenergic signaling in promoting memory consolidation received little attention so far.

The endocannabinoid system is one emerging candidate system thought to mediate some of the rapid actions of glucocorticoids in the brain (Hill and McEwen, 2009). Endocannabinoids, i.e., anandamide and 2-arachidonoylglycerol

(2-AG), are short-lived retrograde messengers that are synthesized on demand through cleavage of membrane precursors (Hashimotodani et al., 2007). They bind to the cannabinoid receptor subtype 1 (CB1 receptor) on presynaptic sites to regulate ion channel activity and neurotransmitter release (Freund et al., 2003). Although glucocorticoid effects on the endocannabinoid system have been studied almost exclusively with respect to nongenomically mediated actions of glucocorticoids on hypothalamic-pituitary-adrenal axis activity (Weidenfeld et al., 1994; Di et al., 2003; Barna et al., 2004; Patel, 2004; Tasker et al., 2006; Hill et al., 2010b; Tasker and Herman, 2011), it became increasingly clear that CB1 receptors are also abundantly expressed in the BLA (and other brain regions) where they modulate emotional arousal effects on synaptic transmission, neuronal firing and memory (Katona et al., 2001; Pistis et al., 2004; Ganon-Elazar and Akirav, 2009; Karst et al., 2010; Tan et al., 2011). The recent findings that systemic administration of corticosterone rapidly elevates endocannabinoid levels in the amygdala (Hill et al., 2010a), whereas a blockade of CB1 receptor activity in the BLA prevents corticosterone-induced memory enhancement (Campolongo et al., 2009) provided the first *in vivo* evidence in mammalian species in support of a functional interaction between these two stress systems in regulating memory processes. The current study was aimed at investigating whether such glucocorticoid-induced recruitment of the endocannabinoid system is an essential step in mediating the rapid effects of glucocorticoids on the noradrenergic arousal system in enhancing the consolidation of memory of emotionally arousing training experiences.

METHODS

Subjects. Male adult Sprague-Dawley rats (280-320 g at time of surgery) from Charles River Breeding Laboratories (Kisslegg, Germany) were kept individually in a temperature-controlled (22°C) colony room and maintained on a standard 12-h light: 12-h dark cycle (07:00-19:00 h lights on) with *ad libitum* access to food and water. Training and testing were performed during the light phase of the cycle between 10:00 and 15:00 h. All procedures were performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Surgery. Animals, adapted to the vivarium for at least 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion), and received the non-steroidal analgesic carprofen (4 mg/kg, sc; Pfizer). Oxygen (30-35%) mixed with ambient air was administered during surgery such that blood oxygenation levels would not drop below 90%. Surgery was performed according to a standardized protocol (Fornari et al., 2012). Briefly, the skull was positioned in a stereotaxic frame (Kopf Instruments), and two stainless-steel guide cannulae (15 mm; 23 gauge; Small Parts, Inc) were implanted bilaterally with the cannula tips 2.0 mm above the BLA. The coordinates were based on the atlas of Paxinos and Watson (Paxinos and Watson, 2005): anteroposterior, -2.8 mm from Bregma; mediolateral, $\pm$5.0 mm from the midline; dorsoventral, -6.5 mm from skull surface; incisor bar –3.3 mm from interaural. The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (15-mm long 00-insect dissection pins), inserted into each cannula to maintain patency, were removed only for the infusion of drugs. After surgery, the rats were administered atipamezole hydrochloride (0.25 mg/kg sc; Orion) to reverse anesthesia and were subsequently injected with 3 ml of saline to facilitate clearance of drugs and prevent dehydration. They were returned to their home cages for further recovery from anesthesia. The rats were allowed to recover for a minimum of 7 days before initiation of training and were handled 3 times for 1 min each during this recovery period to accustom them to the infusion procedure.

Inhibitory avoidance apparatus and procedure. Rats were trained and tested in an inhibitory avoidance apparatus consisting of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaugh et al., 1988). The starting compartment (30 cm) was made of opaque white plastic and well lit; the shock compartment (60 cm) was made of two electrifiable metal plates and was not illuminated. Training and testing were conducted in a sound- and light-attenuated room.

For training, the rats were placed in the starting compartment of the apparatus, facing away from the door, and were allowed to enter the dark (shock) compartment. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.60 mA, 1 s) was delivered. For

the experiment with systemic drug administration a lower footshock intensity of 0.35 mA was used. The rats were removed from the shock compartment 15 s after termination of the footshock and, after drug treatment, returned to their home cages. On the 48-h retention test, as on the training session, the latency to re-enter the shock compartment with all four paws (maximum latency of 600 s) was recorded and used as a measure of retention. Longer latencies were interpreted as indicating better retention. Shock was not administered on the retention test trial.

Drug and infusion procedures. The specific GR agonist RU 28362 (11b,17b-dihydroxy-6,21-dimethyl-17a-pregna-4,6-trien-20yn-3-one; 1, 3 or 10 ng; kindly provided by Aventis, Frankfurt, Germany), the membrane-impermeable glucocorticoid Cort:BSA (1, 3 or 10 ng; Sigma-Aldrich), the CRF-BP ligand inhibitor human/rat CRF_{6-33} (0.01, 0.1 or 1 mg; Bachem, La Jolla, CA) and the β-adrenoceptor agonist clenbuterol (1, 10 or 100 ng; Sigma-Aldrich) were infused into the BLA immediately after inhibitory avoidance training either alone or together with the selective CB1 receptor antagonist AM251 (*N*-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide; 0.14 ng; Sigma-Aldrich). The GR agonist-AM251 and clenbuterol-AM251 mixtures were first dissolved in 100% DMSO and subsequently diluted in phosphate buffer to reach a final DMSO concentration of 5%. The Cort:BSA-AM251 drug solutions were made in a vehicle containing 5% DMSO and 0.1% BSA in phosphate buffer, whereas the CRF-BP ligand inhibitor-AM251 solutions were prepared in a vehicle containing 5% DMSO in saline.

The CB receptor agonist WIN55,212-2 (*R*-(+)-(2,3-dihydro-5-methyl-3-[(morpholinyl)methyl] pyrol[1,2,3-de]-1,4-benzoxazin-6-yl)(1-naphthalenyl) methanone monomethanesulfonate; 10, 30 or 100 ng; Sigma-Aldrich) was dissolved in a vehicle containing 2% DMSO and 0.2% Triton X-100 in phosphate buffer and infused into the BLA either alone or together with the specific GR antagonist RU 38486 (17b-hydroxy-11b-(4-dimethylaminophenyl)-17a-(1-propynyl)-oestra-4,9-dien-3-one; 1 ng; kindly provided by Aventis, Frankfurt, Germany), the nonselective CRF receptor antagonist α-helical CRF_{9-41} (1 mg; Bachem) or the β-adrenoceptor antagonist propranolol (0.5 mg; Sigma-Aldrich). Drug doses were selected on the basis of previous experiments (Roozendaal et

al., 2002a; Roozendaal et al., 2002b; Roozendaal et al., 2008b; Campolongo et al., 2009; Roozendaal et al., 2010).

Bilateral infusions of drug, or an equivalent volume of vehicle, into the BLA were given immediately after inhibitory avoidance training by using 30-gauge injection needles connected to 10-μl Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 2.0 mm beyond the cannula tips and a 0.2-μl injection volume per hemisphere was infused over a period of 30 s by an automated syringe pump (Stoelting Co., Dublin, Ireland). The injection needles were retained within the cannulae for an additional 20 s following drug infusion to maximize diffusion and to prevent backflow of drug into the cannulae. The infusion volume was based on findings that drug infusions of this volume into the BLA, but not into the adjacent central nucleus of the amygdala, modulate memory consolidation (Roozendaal and McGaugh, 1997; Roozendaal et al., 2007; Campolongo et al., 2009).

Systemic drug administration: Corticosterone (3 mg/kg; Sigma-Aldrich) either alone or together with the inverse CB1 receptor agonist SR141716 (rimonabant, 5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; 1 mg/kg; Kemprotec Ltd, Middlesbrough, UK) was given subcutaneously, in a volume of 2 ml/kg, immediately after the training trial. Corticosterone and SR141716 were dissolved in a vehicle containing 5% polyethylene glycol, 5% tween-80 and 5% ethanol in saline.

Histology. Rats were anesthetized with an overdose of sodium pentobarbital and perfused transcardially with a 0.9% saline solution followed by 4% formaldehyde dissolved in water. Following decapitation, the brains were removed and immersed in fresh 4% formaldehyde. At least 24 h before sectioning, the brains were submerged in a 25% sucrose (wt/vol) solution in water for cryoprotection. Coronal sections of 50 mm were cut on a cryostat, mounted on gelatin-coated slides, and stained with cresylviolet. The sections were examined under a light microscope and determination of the location of injection needle tips in the BLA was made according to the standardized atlas plates of Paxinos and Watson (Paxinos and Watson, 2005) by an observer blind to drug treatment condition. Only rats with needle tips within the boundaries of the BLA were included in the

data analysis. Rats with extensive tissue damage at the injection needle tip were also excluded from analysis.

Immunohistochemitry: Sixty minutes after training and systemic drug treatment, rats were perfused transcardially with ice-cold 0.01 M phosphate-buffered saline (PBS), pH 7.4, followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, postfixed overnight at 4°C, and then transferred to a 25% sucrose solution in 0.1 M PB for 3-6 days at 4°C. Frozen coronal sections at the level of the BLA were cut at a thickness of 20 µm on a cryostat and collected in Tris-buffered saline (TBS) with 0.1% sodium azide and phosphatase inhibitors (20 mM sodium fluoride and 2 mM sodium orthovanadate). Every eighth section was used for quantification. Free-floating sections were pretreated with 1% H_2O_2 for 1 h to block endogenous peroxidase and with 5% normal donkey serum (nds, Jackson ImmunoResearch) to block nonspecific binding and 0.3% Triton X-100 to increase antibody penetration. These substances were diluted in TBS with phosphatase inhibitors. Subsequently, sections were incubated with a monoclonal rabbit anti-pCREB (Ser133) antibody (Cell Signaling Technology, #9198, 1:30) in TBS containing phosphatase inhibitors, 0.3% Triton X-100 and 1% nds at 4°C for 48 h. After rinsing thorough, sections were subsequently incubated with biotinylated donkey anti-rabbit IgG secondary antibody (2 µg/ml, Jackson ImmunoResearch) for 1 h followed by incubation with avidin-biotin complex-peroxidase (1:500, ABC kit, Vector laboratories) for 1 h at room temperature. Finally, sections were stained with 0.025% diaminobenzidine, 0.2% nickel ammonium sulphate adding 0.3% H_2O_2. Sections were mounted, dehydrated and coverslipped. Omission of the primary antibody resulted in complete loss of pCREB immunoreactivity. pCREB-positive cells were blindly quantified in the BLA (2.3-3.1 mm posterior to Bregma) using an automated imaging analysis system. The selected areas were digitized by using a Sony charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (Leica, Wetzlar, Germany) at 100x magnification. Regions of interest were outlined and pCREB-positive nuclei were counted using a computer-based image analysis system (Leica Imaging System Ltd., Cambridge, UK). Data are reported as number of positive cells/0.1 mm. The number of positive cells for the experimental groups is shown relative to vehicle. As no left–right asymmetry in pCREB immunoreactivity was found, the mean of both sides was calculated.

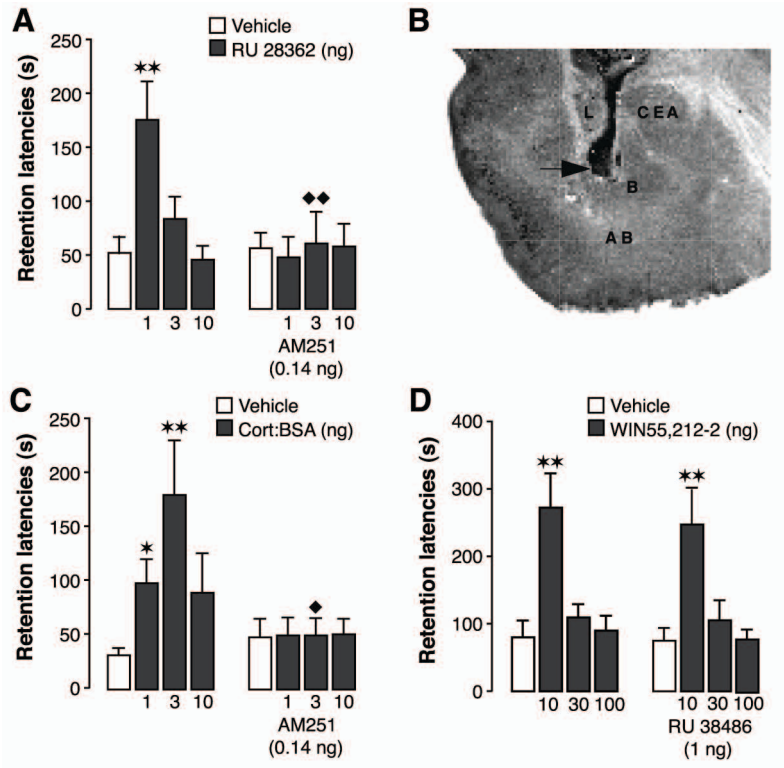

Figure 1. Glucocorticoid interactions with the endocannabinoid system of the BLA in enhancing memory consolidation of inhibitory avoidance training. (A) Step-through latencies (mean + SEM) in seconds on the 48-h inhibitory avoidance retention test of rats given immediate posttraining infusions into the BLA of the CB1 receptor antagonist AM251 (0.14 ng in 0.2 μl) together with the GR agonist RU 28362 (1, 3 or 10 ng). ✶✶, $P < 0.01$ compared with the corresponding vehicle group; ◆◆, $P < 0.01$ compared with the corresponding RU 28362 alone group (n = 9-12 per group). (B) Representative photomicrograph illustrating placement of a cannula and needle tip in the BLA. Arrow points to needle tip. L, lateral nucleus; B, basal nucleus; AB accessory basal nucleus; CEA, central nucleus of the amygdala. (C) Step-through latencies (mean + SEM) in seconds on the 48-h inhibitory avoidance retention test of rats given immediate posttraining infusions into the BLA of the CB1 receptor antagonist AM251 (0.14 ng in 0.2 μl) together with the membrane-impermeable glucocorticoid Cort:BSA (1, 3 or 10 ng). ✶, $P < 0.05$; ✶✶, $P < 0.01$ compared with the corresponding vehicle group; ◆, $P < 0.05$ compared with the corresponding Cort:BSA alone group (n = 11-14 per group). (D) Step-through latencies (mean + SEM) in seconds on the 48-h inhibitory avoidance retention test of rats given immediate posttraining infusions into the basolateral amygdala of the CB receptor agonist WIN55,212-2 (10, 30 or 100 ng in 0.2μl) either alone or together with the GR antagonist RU 38486 (1 ng) ✶✶, $P < 0.01$ compared with the corresponding vehicle group; (n = 10-14 per group).

Statistics. Data are presented as mean ± SEM. Training and retention test latencies were analyzed using two-way ANOVAs with immediate posttraining infusions of different doses of the agonist and antagonist as between-subject variables. Further analysis used Fisher's *post-hoc* tests to determine the source of the detected significances, when appropriate. To determine whether learning had occurred, paired *t*-tests were used to compare the training and retention latencies. For all comparisons, a probability level of < 0.05 was accepted as statistical significance. The number of rats per group is indicated in the figure legends.

RESULTS

Involvement of the endocannabinoid system of the BLA in mediating glucocorticoid receptor agonist effects on memory consolidation.

In view of the evidence that the low-affinity GR, and not the mineralocorticoid receptor, mediates the effects of glucocorticoids on both noradrenergic activity and memory consolidation (Oitzl and de Kloet, 1992; Roozendaal, 2002; Barsegyan et al., 2010), the first experiment was aimed at delineating whether the GR is also implicated in mediating the crosstalk between glucocorticoids and the endocannabinoid system of the BLA. Therefore, rats were trained on a one-trial inhibitory avoidance task and immediately afterwards administered the specific GR agonist RU 28362 (1, 3 or 10 ng) into the BLA either alone or together with the CB1 receptor antagonist AM251 (0.14 ng). Retention of the training was tested 48 h later.

Average step-through latencies for all groups during training, i.e., before footshock or drug treatment, were 13.4 ± 0.7 s (mean ± SEM). Two-way ANOVA for training latencies showed no significant differences between groups (for all comparisons: $P \geq 0.14$; data not shown). Forty-eight-hour retention test latencies of rats administered vehicle into the BLA immediately after training were significantly longer than their response latencies on the training trial (52.7 ± 13.5 s; paired *t*-test: $P = 0.01$), indicating that the rats retained significant memory of the shock experience. As is shown in Fig. 1A, AM251 blocked the retention enhancement induced by the GR agonist RU 28362. Two-way ANOVA for 48-h retention latencies revealed a significant RU 28362 effect ($F_{3,80} = 2.98$; $P = 0.04$), a significant AM251 effect ($F_{1,80} = 4.26$; $P = 0.04$), as well as a significant interaction between both factors ($F_{3,80} = 4.07$; $P = 0.04$). RU 28362 infused alone into the BLA induced dose-

dependent enhancement of retention performance (1 ng: $P < 0.01$). The low dose of the CB1 receptor antagonist AM251 alone did not impair retention latencies, but blocked the retention enhancement induced by concurrently administered RU 28362. Retention latencies of rats treated with AM251 together with the 1 ng dose of RU 28362 were significantly shorter than those of rats given the corresponding dose of RU 28362 alone ($P < 0.01$). Thus, these findings indicate that the low-affinity GR mediates glucocorticoid effects on the endocannabinoid system in enhancing the consolidation of inhibitory avoidance memory.

Histological analysis revealed the majority of needle placements to be localized within the boundaries of the BLA as defined by Paxinos and Watson (Paxinos and Watson, 2005). Rats found to have needle tip placements outside the boundaries of the BLA were excluded from data analysis. A representative photomicrograph of a needle track terminating within the BLA is shown in Fig. 1B.

Involvement of the endocannabinoid system of the BLA in regulating memory enhancement induced by a membrane-impermeable glucocorticoid.

Next, we investigated whether glucocorticoid effects on the endocannabinoid system of the BLA in regulating the consolidation of inhibitory avoidance memory involve the activation of a corticosteroid receptor on the cell surface. Emerging evidence indicates that rapid actions of glucocorticoids, including those on the noradrenergic system, are mediated via an activation of putative G-protein-coupled receptors or membrane-associated cytosolic steroid receptors (Orchinik et al., 1991; Johnson et al., 2005; Karst et al., 2005; Hill and McEwen, 2009; Barsegyan et al., 2010).

As is shown in Fig. 1C, the CB1 receptor antagonist AM251 (0.14 ng) administered into the BLA after inhibitory avoidance training blocked retention enhancement induced by concurrent infusions of the membrane-impermeable conjugate of corticosterone and bovine serum albumin (Cort:BSA; 1, 3 or 10 ng). Two-way ANOVA for step-through latencies on the training trial showed no significant differences between groups (for all comparisons: $P \geq 0.09$**;** data not shown). In contrast, two-way ANOVA for 48-h retention latencies revealed a significant Cort:BSA effect ($F_{3,92} = 2.85$; $P = 0.04$), a significant AM251 effect ($F_{1,92} = 7.33$; $P = 0.008$), as well as a significant interaction between both factors ($F_{3,92} = 2.76$; $P = 0.046$). Cort:BSA infused alone into the BLA immediately after the training induced

a dose-dependent enhancement of retention performance (1 ng: $P < 0.05$; 3 ng: $P < 0.01$). The CB1 receptor antagonist AM251 blocked the retention enhancement produced by concurrently administered Cort:BSA. Retention latencies of rats treated with AM251 together with the intermediate dose of Cort:BSA (3 ng) were significantly shorter than those of rats given that dose of Cort:BSA alone ($P < 0.05$).

Blockade of glucocorticoid receptors in the BLA does not prevent memory enhancement induced by a CB receptor agonist.

These findings indicating that CB1 receptor activity within the BLA is essentially involved in mediating glucocorticoid effects on memory enhancement, lend support for the view that glucocorticoids, via a membrane-associated GR, influence downstream endocannabinoid activity (Hill and McEwen, 2010). However, they do not reveal whether the endocannabinoid pathway is the principle route by which glucocorticoids enhance the consolidation of memory. Glucocorticoids might influence memory consolidation by concurrently activating GRs at several functional levels within the cell or local network, some that are independent of

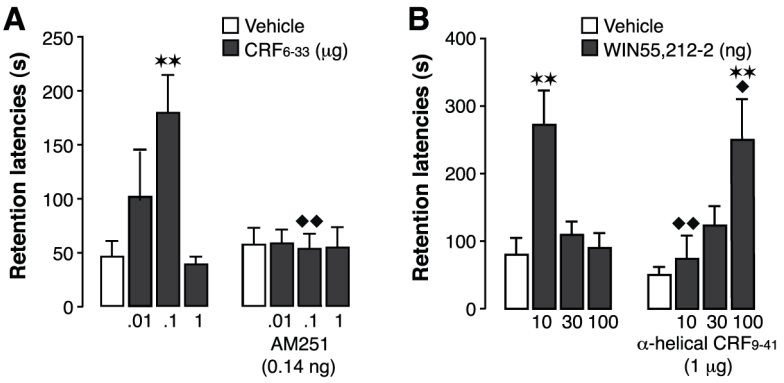

Figure 2. Interactions between CRF and the endocannabinoid system of the BLA in enhancing memory consolidation of inhibitory avoidance training. (A) Step-through latencies (mean + SEM) in seconds on the 48-h inhibitory avoidance retention test of rats given immediate posttraining infusions into the BLA of the CB1 receptor antagonist AM251 (0.14 ng in 0.2 μl) together with the CRF-BP inhibitor CRF_{6-33} (0.01, 0.1 or 1 mg). ✶✶, $P < 0.01$ compared with the vehicle group; ◆◆, $P < 0.01$ compared with the corresponding CRF_{6-33} alone group (n = 10-13 per group). (B) Step-through latencies (mean + SEM) in seconds on the 48-h inhibitory avoidance retention test of rats given immediate posttraining infusions into the basolateral amygdala of the CB receptor agonist WIN55,212-2 (10, 30 or 100 ng in 0.2 μl) either alone or together with the CRF receptor antagonist α-helical CRF_{9-41} (1 μg). ✶✶, $P < 0.01$ compared with the vehicle group; ◆, $P < 0.05$; ◆◆, $P < 0.01$ compared with the corresponding WIN55,212-2 alone group (n = 10-14 per group).

endocannabinoid activity (e.g. via a translocation of the ligand-receptor complex to influence gene transcription in the nucleus) (Oitzl et al., 2001). To investigate the significance of the endocannabinoid pathway in contributing to the memory-modulatory effects of glucocorticoids, we examined whether an activation of endocannabinoid signaling with the full CB receptor agonist WIN55,212-2 enhances the consolidation of inhibitory avoidance memory under a condition when other GR-mediated actions are pharmacologically blocked.

As is shown in Fig. 1D, the GR antagonist RU 38486 (1 ng) infused into the BLA immediately after inhibitory avoidance training did not preclude the retention enhancement induced by concurrently administered WIN55,212-2 (10, 30 or 100 ng). Two-way ANOVA for training latencies showed no significant differences between groups (for all comparisons: $P \geq 0.56$; data not shown). In contrast, two-way ANOVA for 48-h retention latencies revealed a significant WIN55,212-2 effect ($F_{3,91} = 13.65$; $P < 0.0001$), but no significant GR antagonist effect ($F_{1,91} = 0.33$; $P = 0.57$) or interaction between both factors ($F_{3,91} = 0.04$; $P = 0.99$). WIN55,212-2 infused alone induced dose-dependent enhancement of retention performance. The 10 ng dose of WIN55,212-2 significantly enhanced retention ($P < 0.01$), whereas retention latencies of animals given the higher (30 or 100 ng) doses failed to reach significance. As with WIN55,212-2 administered alone, the 10 ng dose of WIN55,212-2 infused together with the GR antagonist induced significant enhancement of retention latencies ($P < 0.01$). Thus, these findings indicate that a GR-mediated activation of downstream endocannabinoid signaling appears to be a major route of how glucocorticoids affect memory consolidation.

Is the endocannabinoid system uniquely involved in mediating glucocorticoid effects on memory consolidation?

Glucocorticoids are certainly not the only hormones known to enhance memory consolidation of emotionally arousing experiences. Other stress-activated response systems such as peripheral catecholamines and central corticotropin-releasing factor (CRF) are also potent modulators of memory consolidation (Roozendaal and McGaugh, 2011). Moreover, it is known that the memory-enhancing effects of both catecholamines and CRF depend on noradrenergic activity within the BLA (Roozendaal and McGaugh, 2011). To investigate whether the stress-activated CRF system enhances memory consolidation also via an

interaction with the endocannabinoid system, the next experiment examined whether the CB1 receptor antagonist AM251 (0.14 ng) administered into the BLA after inhibitory avoidance training blocks the memory-enhancing effect of concurrently administered CRF_{6-33} (0.01, 0.1 or 1 mg). We previously reported that CRF_{6-33}, which has a high affinity for the CRF-binding protein (CRF-BP) and is devoid of any intrinsic activity at the CRF receptor (Sutton et al., 1995), enhances memory consolidation by displacing CRF from the CRF-BP complex and increasing the 'free' concentration of endogenous CRF (Behan et al., 1995; Roozendaal et al., 2008b).

As is shown in Fig. 2A, intra-BLA infusions of AM251 blocked CRF_{6-33}-induced retention enhancement. Two-way ANOVA for training latencies showed no significant differences between groups (for all comparisons: $P \geq 0.32$; data not shown). In contrast, two-way ANOVA for 48-h retention latencies revealed a significant CRF_{6-33} effect ($F_{3,86} = 3.42$; $P = 0.02$), a significant AM251 effect ($F_{1,86} = 4.50$; $P = 0.04$), as well as a significant interaction between both factors ($F_{3,86} = 3.69$; $P = 0.02$). CRF_{6-33} infusions alone induced a dose-dependent enhancement

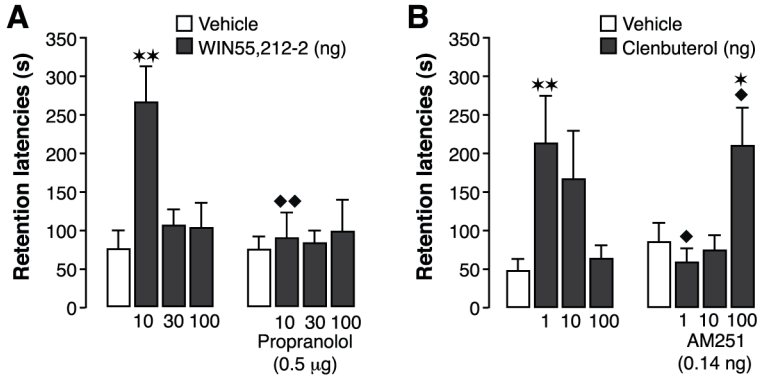

Figure 3. Interactions between the endocannabinoid and noradrenergic systems of the BLA in enhancing memory consolidation of inhibitory avoidance training. (A) Step-through latencies (mean + SEM) in seconds on the 48-h inhibitory avoidance retention test of rats given immediate posttraining infusions into the BLA of the β-adrenoceptor antagonist propranolol (0.5 mg in 0.2 μl) together with the CB1 agonist WIN55,212-2 (10, 30 or 100 ng). ✶✶, $P < 0.01$ compared with the corresponding vehicle group; ◆◆, $P < 0.01$ compared with the corresponding WIN55,212-2 alone group (n = 9-12 per group). (B) Step-through latencies (mean + SEM) in seconds on the 48-h inhibitory avoidance retention test of rats given immediate posttraining infusions into the BLA of the CB1 receptor antagonist AM251 (0.14 ng in 0.2 μl) together with the β-adrenoceptor agonist clenbuterol (1, 10 or 100 ng).✶, $P < 0.05$; ✶✶, $P < 0.01$ compared with the corresponding vehicle group; ◆, $P < 0.05$ compared with the corresponding clenbuterol alone group (n = 9-12 per group).

of retention performance (0.1 mg: $P < 0.01$). The CB1 receptor antagonist AM251 infused alone into the BLA immediately after the training trial did not impair retention, but blocked the retention enhancement induced by concurrently administered CRF_{6-33}. Retention latencies of rats treated with AM251 together with the intermediate dose of CRF_{6-33} (0.1 mg) were significantly shorter than those of rats given the corresponding dose of CRF_{6-33} alone ($P < 0.01$).

To determine whether the endocannabinoid system is located downstream from the CRF receptor, in the second part of the experiment, we investigated whether the nonselective CRF receptor antagonist α-helical CRF_{9-41} (1 μg) administered into the BLA after inhibitory avoidance training altered the memory-enhancing effect of concurrently administered WIN55,212-2 (10, 30 or 100 ng) (Fig. 2B). Two-way ANOVA for training latencies showed no significant differences between groups (for all comparisons: $P \geq 0.08$; data not shown). In contrast, two-way ANOVA for 48-h retention latencies showed a non-significant α-helical CRF_{9-41} effect ($F_{1,87} = 0.37$; $P = 0.54$), but a significant WIN55,212-2 effect ($F_{3,87} = 4.31$; $P = 0.007$) and interaction between conditions ($F_{3,87} = 8.13$; $P < 0.0001$). WIN55,212-2 infused alone induced dose-dependent enhancement of retention performance. The

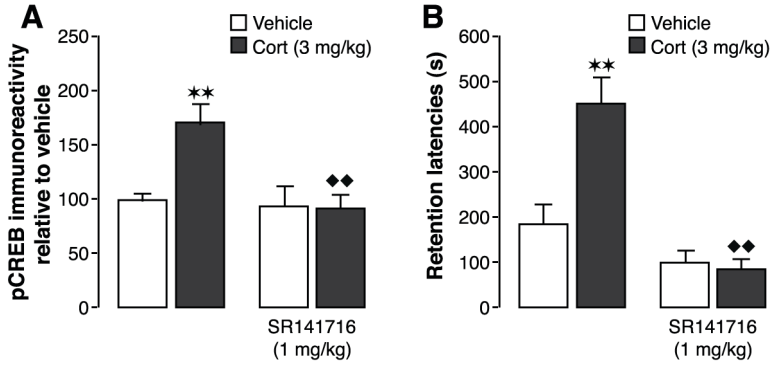

Figure 4. Endocannabinoids enable the glucocorticoid-induced enhancement of BLA neuronal activity and IA memory. **(**A) Number of pCREB immunopositive cells in BLA of rats given vehicle (5 % ethanol) or CORT (3mg/kg) alone or together with SR141716 (1 mg/kg). ✶✶, $P < 0.01$ compared with the corresponding vehicle group; ◆◆, $P < 0.01$ compared with the corresponding CORT alone group (n = 5 - 6 per group). (B) Step-through latencies (mean + SEM) in seconds on the 48-h inhibitory avoidance retention test of rats given immediate posttraining systemic treatment of either vehicle (5% ethanol) or CORT (3 mg/kg) alone or together with SR141716 (1 mg/kg). ✶✶, $P < 0.01$ compared with the corresponding vehicle group; ◆◆, $P < 0.01$ compared with the corresponding CORT alone group (n = 10 per group).

10 ng dose of WIN55,212-2 significantly enhanced retention ($P < 0.01$), whereas retention latencies of animals given the higher (30 or 100 ng) doses failed to reach significance. In rats also given α-helical CRF_{9-41}, the lowest dose of WIN55,212-2 (10 ng) failed to enhance retention, and a 10-times higher dose of WIN55,212-2 (100 ng) was necessary to induce significant retention enhancement ($P < 0.01$). Thus, these findings indicating that CRF receptor blockade, unlike GR blockade, shifted the dose-response effects of WIN55,212-2 on retention enhancement demonstrate that the endocannabinoid system is not just located downstream from the CRF receptor. These findings provide further evidence for a selective and specific role of endocannabinoid transmission as a rapid mediator of glucocorticoid actions.

Interactions between the endocannabinoid and noradrenergic systems of the BLA on memory enhancement of inhibitory avoidance.

The findings described so far clearly show that endocannabinoid signaling is critically involved in regulating the memory-modulatory effects of glucocorticoids. However, we have not yet explored whether such endocannabinoid activity is an intermediary step in mediating glucocorticoid effects on the noradrenergic arousal system. To address this issue, we first investigated whether the endocannabinoid and noradrenergic systems of the BLA interact in regulating memory consolidation.

The first experiment examined whether the β-adrenoceptor antagonist propranolol (0.5 mg) infused into the BLA immediately after inhibitory avoidance training blocked the memory enhancement induced by concurrent infusions of the CB receptor agonist WIN55,212-2 (10, 30 or 100 ng). As is shown in Fig. 3A, two-way ANOVA for 48-h retention latencies revealed significant WIN55,212-2 ($F_{3,75} = 4.33$; $P = 0.007$) and propranolol effects ($F_{1,75} = 4.64$; $P = 0.03$) as well as a significant interaction between conditions ($F_{3,75} = 3.15$; $P = 0.03$). In agreement with the above-mentioned findings, WIN55,212-2 administered posttraining into the BLA induced dose-dependent enhancement of retention performance (10 ng: $P < 0.01$). Propranolol infused alone into the BLA immediately after the training trial did not impair retention performance, but blocked the retention enhancement induced by concurrently administered WIN55,212-2. Retention latencies of rats treated with propranolol together with the 10 ng dose of WIN55,212-2 were

significantly shorter than those of rats given the corresponding dose of WIN55,212-2 alone ($P < 0.01$). Thus, these findings indicate that, as with glucocorticoids, endocannabinoid effects on memory consolidation enhancement depend on concurrent noradrenergic activity within the BLA.

Previously, we reported that a blockade of GRs in the BLA with its antagonist RU 38486 shifted the dose-response effects of the β-adrenoceptor agonist clenbuterol such that a much higher dose of clenbuterol was required to induce comparable memory enhancement (Roozendaal et al., 2002). The second part of the experiment investigated whether blockade of CB1 receptors in the BLA with posttraining infusions of AM251 (0.14 ng in 0.2 μl) induced a similar shift in the dose-response effects of clenbuterol (1, 10 or 100 ng). As is shown in Fig. 3B, relative to vehicle controls, immediate posttraining infusions of clenbuterol into the BLA induced dose-dependent enhancement of retention performance. The lowest dose (1 ng) enhanced retention ($P < 0.05$), whereas higher doses of clenbuterol (10 or 100 ng) were ineffective. Two-way ANOVA for retention latencies showed non-significant clenbuterol ($F_{3,75} = 1.55$; $P = 0.21$) or AM251 effects ($F_{1,75} = 0.32$; $P = 0.57$), but a significant interaction between conditions ($F_{3,75} = 6.13$; $P = 0.0009$). AM251 administered alone did not affect retention latencies, but shifted the dose-response effects of clenbuterol. When AM251 was co-infused with clenbuterol, the lowest dose of clenbuterol (1 ng) failed to enhance retention performance, and a 100-times higher dose of clenbuterol was necessary to induce significant retention enhancement ($P < 0.01$). These findings indicating that a blockade of endocannabinoid activity decreases the sensitivity of the BLA to the memory-enhancing effects of noradrenergic stimulation are thus highly comparable to the effects of GR blockade within the BLA (Roozendaal et al., 2002b).

Endocannabinoid activity is necessary for enabling glucocorticoid-norepinehrine interactions on BLA neuronal activity.

Our findings indicating that a blockade of either glucocorticoid or endocannabinoid signaling reduces the sensitivity of the BLA to the memory-enhancing effect of noradrenergic stimulation suggest that corticosterone, via an endocannabinoid mechanism, interacts with training-induced noradrenergic activation at a postsynaptic level in increasing BLA neuronal activity (Roozendaal et al., 2006b). To assess postsynaptic BLA neuronal activity, in the next experiment

we determined immunoreactivity for the phosphorylated form of the transcription factor CREB (pCREB) protein. Noradrenergic stimulation induces pCREB activation (Roseboom and Klein, 1995; Davies et al., 2004) and several findings have implicated CREB phosphorylation in the amygdala in the modulation of memory consolidation (Silva et al., 1998; Kida et al., 2002; Bozon et al., 2003; Josselyn et al., 2004; Han et al., 2007). In a previous study we reported that corticosterone administration increased the number of pCREB-positive neurons within the BLA, but only when there was sufficient noradrenergic activity (Roozendaal et al., 2006b). To determine whether endocannabinoid activity is required for mediating the facilitating effect of corticosterone on norepinephrine-induced pCREB immunoreactivity, in the present study the inverse CB1 receptor agonist SR141716 (rimonabant; 1 mg/kg) was administered subcutaneously together with corticosterone (3 mg/kg) immediately after inhibitory avoidance training, and the number of neurons expressing immunoreactivity for pCREB within the BLA was assessed 1 h later. Other rats were administered SR141716 and corticosterone after inhibitory avoidance training and their retention latencies were tested 48 h later.

As is shown in Fig. 4A, two-way ANOVA for the number of pCREB-positive cells in the BLA 1 h after inhibitory avoidance training and drug administration revealed a significant corticosterone effect ($F_{1,18} = 6.21$, $P = 0.02$), a significant SR141716 effect ($F_{1,18} = 8.50$, $P = 0.001$) and as well as a significant interaction effect between these two factors ($F_{1,18} = 6.53$, $P = 0.02$). Post-hoc comparison tests indicated that corticosterone induced a significant increase in the number of pCREB-positive cells in the BLA ($P < 0.01$, relative to vehicle), whereas SR141716 completely blocked this corticosterone-induced increase in pCREB expression. SR141716 administration alone did not induce any changes in pCREB expression within the BLA. As is shown in Fig. 4B SR141716 also blocked the enhancing effect of corticosterone on 48-h inhibitory avoidance retention performance. Two-way ANOVA for retention latencies indicated a significant corticosterone ($F_{1,36} = 9.09$, $P = 0.004$) and SR141716 effects ($F_{1,36} = 27.16$, $P = 0.0001$) as well as a significant interaction between these two parameters ($F_{1,36} = 10.97$, $P = 0.0021$). Collectively, these findings indicate that endocannabinoids mediate the effects of corticosterone on pCREB expression within the BLA and memory enhancement via a modulatory influence on postsynaptic noradrenergic signaling mechanisms.

DISCUSSION

The present study was undertaken to investigate whether the endocannabinoid system of the BLA mediates the rapid effects of glucocorticoid hormones onto the noradrenergic system in regulating the consolidation of memory of emotionally arousing experiences. The interest of this question stems from previous work, in animals and humans, indicating that glucocorticoid administration selectively enhances the consolidation of memory of emotionally salient training experiences (Buchanan and Lovallo, 2001; Okuda et al., 2004; Abercrombie et al., 2006; Kuhlmann and Wolf, 2006) because of a critical interaction with arousal-induced noradrenergic activation within the BLA (Roozendaal et al., 2006a; Van Stegeren et al., 2007). However, as these effects are too rapid to be mediated via genomic glucocorticoid actions, the neurobiological processes underlying the glucocorticoid influence on noradrenergic activity remained to be determined.

Although it has long been recognized that steroid hormones primarily exert their effects on neuronal function through their ability to modulate gene transcription in the nucleus (de Kloet et al., 1999), an array of physiological and behavioral effects of glucocorticoids have been documented to occur in a fashion that cannot be explained by genomic regulation (Dallman, 2005; Atsak et al., 2012). These findings have prompted the hypothesis that glucocorticoids possess membrane-associated receptors through which nongenomic signaling may evoke rapid effects on physiology and behavior. The present finding that the CB1 receptor antagonist AM251 administered into the BLA after inhibitory avoidance training blocked the memory–enhancing effect of the GR agonist RU 28362 or of the membrane-impermeable ligand Cort:BSA indicates that endocannabinoid activity within the BLA is essentially involved in regulating the memory-enhancing effects of glucocorticoids. This glucocorticoid action most likely involves the activation of a GR on the cell surface, launching a G-protein-dependent signaling cascade that induces the synthesis of endocannabinoid ligands (Di et al., 2003; Johnson et al., 2005; Barsegyan et al., 2010; Hill and McEwen, 2010). Multiple lines of research indicate that the endocannabinoid system of the BLA, with anandamide possibly as its central component (Busquets-Garcia et al., 2011), is importantly involved in neural plasticity mechanisms related to emotionally salient learning and memory (Marsicano et al., 2002; Ganon-Elazar and Akirav, 2009; Karst et al.,

2010). Previously, we reported that pharmacological activation of CB1 receptors in the BLA with WIN55,212-2 induces dose-dependent enhancement of inhibitory avoidance memory (Campolongo et al., 2009). The present finding that a pharmacological blockade of GRs in the BLA with its antagonist RU 38486 did not inhibit this WIN55,212-2 effect indicates that the endocannabinoid system, at least from a functional perspective, is located downstream from the GR site of action. Prior evidence indicating that systemic injection of corticosterone induces a rapid, i.e., within 10 min, elevation in endocannabinoid levels in the amygdala (Hill et al., 2010) supports this conclusion and further reinforces the nongenomic nature of this effect. On a broader level, these findings reiterate the growing notion of the endocannabinoid system as a rapid mediator of responses to stress and stress hormones (Tasker et al., 2006; Ganon-Elazar and Akirav, 2009) and are also consistent with findings of *in vitro* electrophysiological recording studies demonstrating that glucocorticoids rapidly suppress glutamate release onto parvocellular neurons in hypothalamic regions, in controlling hypothalamic-pituitary-adrenal-axis activity, through a mechanism that involves postsynaptic activation of a membrane-bound GR and the synthesis of endocannabinoid ligands (Di et al., 2003).

To investigate whether endocannabinoid signaling is uniquely involved in regulating glucocorticoid effects on memory consolidation, we also examined possible interactions between CRF and the endocannabinoid system. Our findings indicate that a blockade of endocannabinoid signaling within the BLA also prevented the enhancing effects of the CRF-BP ligand inhibitor CRF_{6-33} on inhibitory avoidance memory. Although it is possible that an activation of CRF receptors, as with GRs, directly evokes endocannabinoid signaling in the BLA, evidence for this view is lacking. Moreover, as CRF receptor blockade altered the memory-enhancing effect of WIN55,212-2 administration, these findings indicate that, unlike with GRs, the endocannabinoid system is not located downstream from the CRF receptor. More likely, glucocorticoids interact with the memory-enhancing effects of CRF via an endocannabinoid mechanism. Consistent with other evidence that the glucocorticoid and CRF systems are intimately linked (Sawchenko, 1987; Pavcovich and Valentino, 1997; Ma and Aguilera, 1999; Helmreich et al., 2001), we previously reported that the GR antagonist RU 38486 infused into the BLA blocked the enhancing effect of CRF_{6-33} administration on

the consolidation of inhibitory avoidance memory (Roozendaal et al., 2008b). Also the present finding that the CRF receptor antagonist α-helical CRF_{9-41} infused into the BLA shifted the dose-response effects of the CB receptor agonist WIN55,212-2 such that a 10-times higher dose of WIN55,212-2 was required to induce memory enhancement is highly comparable to our prior finding indicating that the CRF receptor antagonist induces a near identical shift in the dose-response relationship of the GR agonist RU 28362 (Roozendaal et al., 2008b). Such findings that manipulation of either GR or CB1 receptor has a common impact on the memory-enhancing effects of CRF provide further evidence for a close functional relationship between glucocorticoids and the endocannabinoid system in the BLA in regulating memory enhancement.

Our finding that immediate posttraining infusions of the β-adrenoceptor antagonist propranolol blocked the enhancing effect of the CB receptor agonist WIN55,212-2 on inhibitory avoidance memory indicates that endocannabinoids enhance the consolidation of memory via an interaction with the noradrenergic system. These findings, together with the evidence that synthetic cannabinoids, as with corticosterone (McReynolds et al., 2010), alter the release of norepinephrine in the BLA and other brain regions (Oropeza et al., 2005; Page et al., 2007), indicate that the endocannabinoid system is a likely candidate for mediating the rapid effects of glucocorticoids onto stimulating the release of norepinephrine from presynaptic sites. However, our finding that the CB1 receptor antagonist AM251 infused into the BLA shifted the dose-response effects of the β-adrenoceptor agonist clenbuterol such that a 100-times higher dose of clenbuterol was required to induce memory enhancement suggests that these two neuromodulatory systems also interact at the postsynaptic level. This finding, which is highly comparable to prior evidence that GR antagonism in the BLA induces a near identical shift in the dose-response effects of clenbuterol (Roozendaal et al., 2002b), suggests that glucocorticoids, through an endocannabinoid mechanism, might increase the excitability of BLA pyramidal neurons (Duvarci and Paré, 2007), rendering them more sensitive to the memory-enhancing effects of postsynaptic noradrenergic stimulation (Roozendaal et al., 2002b; Roozendaal et al., 2006b).

To address this issue, we investigated whether the enhancing effects of glucocorticoids on neural plasticity mechanisms within the BLA, as assessed by

the number of pCREB-positive neurons, after inhibitory avoidance training require concurrent endocannabinoid signaling. It is now well established that CREB phosphorylation in the BLA depends on arousal-induced noradrenergic activity (Roseboom and Klein, 1995; Davies et al., 2004) and is critically involved in the modulation of memory consolidation (Silva et al., 1998; Kida et al., 2002; Bozon et al., 2003; Josselyn et al., 2004). In a previous study we reported that corticosterone administered systemically immediately after an emotionally arousing training experience increased the number of pCREB-positive neurons within the BLA, but that corticosterone did not alter the number of pCREB-positive neurons when administered after a low-arousing training experience (Roozendaal et al., 2006b). However, the combined administration of corticosterone and the noradrenergic stimulant yohimbine significantly increased pCREB immunoreactivity in BLA pyramidal neurons under low-arousing conditions (Roozendaal et al., 2006b), indicating that corticosterone interacts with the noradrenergic system in increasing pCREB activity. Our current finding that blocking CB1 receptors with its inverse agonist SR141716 prevented the corticosterone-induced pCREB activation in the BLA after inhibitory avoidance training, as well as blocked the memory-enhancing effects of corticosterone, provides further evidence for the view that the endocannabinoid system is essentially involved in mediating rapid glucocorticoid effects on the facilitation of norepinephrine-induced neural plasticity mechanisms within the BLA in regulating memory consolidation. Findings of electrophysiological experiments in brain slices are consistent with the view that glucocorticoids interact with the noradrenergic system in influencing neural plasticity in BLA neurons. However, these studies further indicate that glucocorticoids affect noradrenergic function in a highly time-dependent fashion (Joels et al., 2011). Whereas corticosterone applied simultaneously with a β-adrenoceptor agonist induced a rapid facilitation of AMPA receptor-mediated responses, corticosterone given several hours in advance to the β-adrenoceptor agonist, possibly because of genomic GR actions, resulted in a potent suppression of the β-adrenoceptor agonist effect (Liebmann et al., 2009). An involvement of the endocannabinoid system in regulating these rapid and/or slow glucocorticoid effects on noradrenergic function has not been investigated.

A possible scenario is that the ensuing release of endocannabinoids influences noradrenergic function via an inhibition of GABAergic transmission (Campolongo

et al., 2009; Hill and McEwen, 2009; Atsak et al., 2012). Within the BLA, CB1 receptors are found predominantly on local inhibitory GABAergic interneurons (Katona et al., 2001; McDonald and Mascagni, 2001). A recent study indicated that CB1 receptors are particularly enriched in axon terminals of cholecystokinin (CCK)-positive interneurons and form invaginating perisomatic synapses with pyramidal neurons in the BLA (Yoshida et al., 2011). CCK-positive interneurons have been proposed to function as fine-tuning devices for the cooperation of pyramidal neurons, which are sensitive to the emotional and motivational state of the animal (Freund et al., 2003). Various studies have reported that activation of CB1 receptors regulates long-term depression at inhibitory synapses and decreases GABAergic synaptic transmission (Katona et al., 1999; Ohno-Shosaku et al., 2001; Hajos and Freund, 2002). Such a suppression of GABAergic transmission can account for changes in noradrenergic signaling at both pre- and postsynaptic sites. On the one hand, extensive evidence indicates that inhibition of local GABAergic circuits in the BLA enhances memory consolidation (Brioni et al., 1989; Castellano et al., 1989; McGaugh, 2004) by increasing the release of norepinephrine from presynaptic sites (Hatfield et al., 1999). On the other hand, GABAergic antagonists are known to act postsynaptically to increase the excitability of BLA pyramidal neurons (Azad et al., 2004; Pistis et al., 2004; Zhu and Lovinger, 2005; Yoshida et al., 2011). These findings, together with the evidence that glucocorticoids and CRF enhance the excitability of BLA pyramidal neurons by decreasing the impact of inhibitory GABAergic influences (Rainnie et al., 1992; Duvarci and Paré, 2007), support the hypothesis that the nongenomically mediated actions of glucocorticoids on the noradrenergic system require a rapid increase in endocannabinoid signaling within the BLA to effectively shut off local inhibitory GABAergic interneurons. Such a suppression of GABAergic activity might then result in increased noradrenergic signaling in BLA pyramidal neurons and an enhanced consolidation of long-term memory of emotionally arousing experiences.

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Glucocorticoids Interact With the Hippocampal Endocannabinoid System in Impairing Retrieval of Contextual Fear Memory

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Proc Natl Acad Sci USA (2012) 109:3504-3509.

CHAPTER 5

ABSTRACT

There is extensive evidence that glucocorticoid hormones impair the retrieval of memory of emotionally arousing experiences. Although it is known that glucocorticoid effects on memory retrieval impairment depend on rapid interactions with arousal-induced noradrenergic activity, the exact mechanism underlying this presumably non-genomically mediated glucocorticoid action remains to be elucidated. Here, we show that the hippocampal endocannabinoid system, a rapidly activated retrograde messenger system, is involved in mediating glucocorticoid effects on retrieval of contextual fear memory. Systemic administration of corticosterone (0.3–3 mg/kg) to male Sprague-Dawley rats 1 h before retention testing impaired the retrieval of contextual fear memory, without impairing the retrieval of auditory fear memory or directly affecting the expression of freezing behavior. Importantly, a blockade of hippocampal CB1 receptors with AM251 prevented the impairing effect of corticosterone on retrieval of contextual fear memory, whereas the same impairing dose of corticosterone increased hippocampal levels of the endocannabinoid 2-arachidonoylglycerol. We further found that antagonism of hippocampal ß-adrenoceptor activity with local infusions of propranolol blocked the memory retrieval impairment induced by the CB receptor agonist WIN55,212-2. Thus, these findings strongly suggest that the endocannabinoid system plays an intermediary role in regulating rapid glucocorticoid effects on noradrenergic activity in impairing memory retrieval of emotionally arousing experiences.

INTRODUCTION

It is well established that glucocorticoid (GC) hormones, released from the adrenal cortex during stressful episodes, can modulate different memory processes (de Kloet et al., 1999; McGaugh and Roozendaal, 2002; Sandi and Pinelo-Nava, 2007; Roozendaal et al., 2009). Although most studies focused on GC effects on the acquisition and consolidation of memory, extensive evidence also indicates that acutely elevated GC levels at the time of retention testing impair the retrieval of memory of spatial and contextual training (de Quervain et al., 1998; de Quervain et al., 2000; Het et al., 2005; Kuhlmann et al., 2005a; Schwabe et al., 2011). As a glucocorticoid receptor (GR) agonist infused into the hippocampus prior to retention induces comparable memory retrieval impairment (Roozendaal et al., 2003; Roozendaal et al., 2004b), such findings suggest that GC effects on memory retrieval depend, at least in part, on activation of GRs in the hippocampus. Findings of studies of human subjects are consistent with those of animal studies and indicate that exogenous GC administration or exposure to a psychosocial stressor shortly before retention testing impairs retrieval of declarative (mostly episodic) information (Kirschbaum et al., 1996; de Quervain et al., 2000; Kuhlmann et al., 2005b) and reduces hippocampal activity (de Quervain et al., 2003). Moreover, previous findings indicate that emotionally arousing information is especially sensitive to the retrieval-impairing effects of GCs (Kuhlmann et al., 2005a) and that emotional arousal during the test situation enables GC effects on memory retrieval (Kuhlmann and Wolf, 2006). Findings of recent clinical studies suggest that the administration of stress doses of GCs may have therapeutic value by attenuating the re-experiencing of highly traumatic memories in patients who have post-traumatic stress disorder (PTSD) and other anxiety disorders (Aerni et al., 2004; Schelling et al., 2004; Schelling et al., 2006; de Quervain et al., 2009).

Our previous finding that GCs interact with arousal-induced noradrenergic activity in impairing the retrieval of hippocampus-dependent memory (Roozendaal et al., 2003; Roozendaal et al., 2004a; Roozendaal et al., 2004b; Roozendaal et al., 2006) might explain why GCs selectively impair memory retrieval of emotionally arousing or traumatic experiences (Kuhlmann et al., 2005a). However, it is not understood how GCs interact with the noradrenergic system in influencing memory retrieval, as these effects appear to be too rapid to act via the classical genomic mode of

action of GCs (de Quervain et al., 1998; Roozendaal et al., 2003; Roozendaal et al., 2004a; Roozendaal et al., 2004b; Roozendaal et al., 2006; McReynolds et al., 2010; Schutsky et al., 2011). Findings of recent studies investigating the cellular mechanism underlying the rapid effects of GCs suggest a possible involvement of the endocannabinoid system (Tasker et al., 2006; Hill and McEwen, 2009; Evanson et al., 2010; Hill et al., 2010b). Endogenous ligands for cannabinoid CB1 receptors, i.e., anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are synthesized on demand through cleavage of membrane precursors and serve as retrograde messengers at central synapses (Kano et al., 2009). They bind to G-protein-coupled CB1 receptors at presynaptic sites to regulate ion channel activity and neurotransmitter release (Freund et al., 2003). It is now well established that stress and GCs can induce rapid changes in endocannabinoid signaling in stress-responsive brain regions (Patel and Hillard, 2008; Hill and McEwen, 2010). Although these effects have been mostly studied with respect to non-genomically mediated effects of GCs on hypothalamic-pituitary-adrenocortical (HPA) axis activity (Tasker et al., 2006; Evanson et al., 2010; Hill and McEwen, 2010), CB1 receptors are also abundantly expressed in the hippocampus, basolateral amygdala and other brain regions where they modulate synaptic transmission, neuronal firing and memory (Freund et al., 2003; Campolongo et al., 2009; Marsicano and Lafenetre, 2009; Akirav, 2011).

We previously reported evidence that GCs interact with the endocannabinoid system within the basolateral amygdala in enhancing the consolidation of memory of emotionally arousing training experiences (Campolongo et al., 2009; Atsak et al., 2012). In the present study, we investigated whether the endocannabinoid system is involved in mediating GC-induced memory retrieval impairment. We focus here on retrieval of contextual fear memory, as we first found that a systemic injection of corticosterone (CORT) administered shortly before retention testing impairs the retrieval of contextual, but not auditory, fear memory. Further, in view of the extensive evidence indicating that GC effects on memory retrieval depend on arousal-induced noradrenergic activity, we also examined whether endocannabinoids interact with the noradrenergic system within the hippocampus in impairing retrieval of contextual fear memory.

METHODS

Subjects. Male adult Sprague-Dawley rats (280-330 g at time of surgery) from Charles River Breeding Laboratories (Kisslegg, Germany) were kept individually in a temperature-controlled (22 °C) colony room and maintained on a standard 12-h light: 12-h dark cycle (07:00-19:00 h lights on) with *ad libitum* access to food and water. All behavioral procedures were performed during the light cycle between 10:00 and 15:00 h. All procedures were in compliance with the European Community's Council Directive on the use of laboratory animals of November 24, 1986 (86/609/EEC) and were approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Surgery. Animals, adapted to the vivarium for at least 1 week, were anesthetized with a mixture of ketamine (37.5 mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion) and surgery was performed according to a standardized protocol (Fornari et al., 2012). Briefly, the skull was positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and two stainless-steel guide cannulae (11 mm; 23 gauge; Small Parts, Inc, Miami Lakes, FL) were implanted bilaterally with the cannula tips 1.5 mm above the dorsal hippocampus (anteroposterior, -3.4 mm from Bregma; mediolateral, +1.8 mm from the midline; dorsoventral, 2.7 mm below skull surface; incisor bar –3.3 mm from interaural) (Paxinos and Watson, 2005). The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (11-mm long 00-insect dissection pins), inserted into each cannula to maintain patency, were removed only for the infusion of drugs. After surgery, the rats were administered atipamezole hydrochloride (2.5 mg/kg; Orion) to reverse anesthesia and were subsequently injected with 3 ml of saline to facilitate clearance of drugs and prevent dehydration. The rats were allowed to recover for 10 days before initiation of training and were handled 3 times for 1 min each during this recovery period to accustom them to the infusion procedure.

Fear Conditioning. After handling days were completed, all rats were habituated to the training context for 5 min, without shock exposure. On the next day, animals were trained on either the contextual or auditory fear conditioning task. For contextual fear conditioning, each rat was placed in the fear conditioning apparatus (in cm: 24 width x 25 depth x 34 height) and exposed to 5 footshocks (1.4

mA, 1 s, 1 min inter-trial interval) after 2 min of baseline. Twenty-four hours later, rats were re-exposed to the fear conditioning context for 5 min. For auditory fear conditioning, animals were exposed to 5 tones (80 dB, 4 kHz, 10 s) co-terminating with footshock (1.4 mA, 1 s). Twenty-four hours later, animals were tested in a different context with tone trials only (5 trials, each 10 s) after 3 min of baseline. Control groups were habituated to the training apparatus and, 24 h later, trained on the contextual fear conditioning task; however, on the retention test day, they were tested for 5 min in a different but previously habituated context. Freezing behavior was analyzed with Behafreeze software (http://www.pmbogusz.net/software/) and some of the groups were also analyzed manually blind to drug treatment as a quality control.

Endocannabinoid Quantification. After rapid decapitation, the hippocampus was dissected and lipid extraction was performed according to a standardized protocol as explained in the SI Methods (Vogeser et al., 2006). For endocannabinoid measurements, automated on-line solid phase extraction using column switching with subsequent direct transfer to high-performance liquid chromatography and a tandem mass spectrometry system was applied. Pure solutions were used for calibration. The method is linear within the calibration ranges. All liquid chromatography mass spectrometry (LC-MS) analyses were carried out using an 1100 LC system (binary pump and autosampler, Agilent, CA, USA) coupled to an API 4000 mass spectrometer (Applied Biosystems, CA, USA) and equipped with a Turbo-Ion-Spray (ESI) source. Because in biological matrices, 2-AG (including its deuterated analog) rapidly isomerizes to 1-AG (Vogeser and Schelling, 2007), we quantified 2-AG as the sum of both isomers.

Drug and infusion procedures. All systemic and local drug manipulations were made 1 h before retention testing. For the first experiment, different doses of CORT (0.3, 1, 3 mg/kg; Sigma-Aldrich), dissolved in 5% ethanol, were administered subcutaneously. CORT doses were based on previous findings (de Quervain et al., 1998; Cai et al., 2006). For the second experiment, the selective CB1 receptor antagonist AM251 (0.35 ng in 0.5 µl per side; Sigma-Aldrich) was infused into the dorsal hippocampus together with a subcutaneous injection of either an impairing dose of CORT (3 mg/kg) or vehicle. AM251 was first dissolved in 100% DMSO and subsequently diluted in phosphate buffer to reach a final DMSO concentration of

2%. For the third experiment, the CB receptor agonist WIN55,212-2 (10 or 30 ng in 0.5 µl per side; Sigma-Aldrich) either alone or together with the ß-adrenoceptor antagonist propranolol (1.25 mg; Sigma-Aldrich) was dissolved in a vehicle containing 2% DMSO and 0.2% Triton X-100 in phosphate buffer and infused into the dorsal hippocampus. For the last experiment, norepinephrine (1 or 3 µg in 0.5 µl per side: Sigma-Aldrich) either alone or together with AM251 (0.35 ng; Sigma-Aldrich), dissolved in 2% DMSO in phosphate buffer, was infused into the dorsal hippocampus.

Bilateral infusions of drug, or vehicle into the dorsal hippocampus were given by using 30-gauge injection needles connected to 10-µl Hamilton microsyringes by polyethylene (PE-20) tubing. The injection needles protruded 1.7 mm beyond the cannula tips and a 0.5 µl injection volume per hemisphere was infused over a period of 50 s by an automated syringe pump (Stoelting Co., Dublin, Ireland). The injection needles were retained within the cannulae for an additional 20 s to prevent backflow of drug into the cannulae.

Histology. Rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.; Sigma-Aldrich) and perfused transcardially with a 0.9% saline (wt/vol) solution followed by 4% formaldehyde (wt/vol) dissolved in water. Brains were removed and after cryoprotection in 25% sucrose, coronal sections of 50 mm were cut on a cryostat, mounted on gelatin-coated slides, and stained with cresyl violet. The location of the injection needle tips in the dorsal hippocampus was examined under a light microscope according to the standardized atlas plates of Paxinos and Watson (Paxinos and Watson, 2005) by an observer blind to drug treatment condition. Rats with injection needle placements outside the hippocampus or with extensive tissue damage at the injection needle tips were excluded from analysis.

Statistics. Data are expressed as mean ± SEM. Overall freezing scores on the retention test trials of the contextual and auditory fear conditioning tasks were analyzed with one-way or two-way ANOVAs, when appropriate. Endocannabinoid levels were analyzed with one-way ANOVA. To investigate the effect of time (for contextual fear conditioning) or tone trial (for auditory fear conditioning) on the freezing response, freezing retention scores were analyzed with repeated-

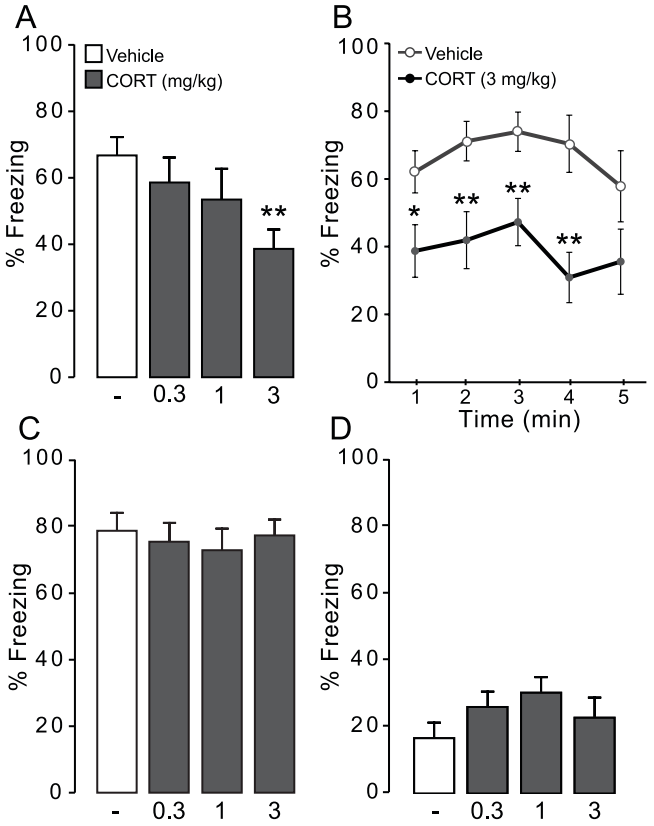

Figure 1. Effect of systemic corticosterone administration on retrieval of fear memory. (A) Systemic CORT (0.3, 1, 3 mg/kg) treatment administered 1 h before retention testing dose-dependently impairs retrieval of contextual fear memory. Results represent mean ± SEM, **p < 0.01 vs. vehicle (n = 11-13/group). (B) Effect of systemic CORT (3mg/kg) treatment on freezing during retrieval of contextual fear memory analyzed in 1-min time bins. Results represent mean ± SEM, *p < 0.05; **p < 0.01 vs. vehicle (n = 11-13/group). (C) Systemic CORT (0.3, 1, 3 mg/kg) treatment given 1 h before retention testing does not impair retrieval of auditory fear memory. Results represent mean ± SEM (n = 8/group). (D) Effect of systemic CORT (0.3, 1, 3 mg/kg) administration on basal freezing levels in a non-training context. Results represent mean ± SEM (n = 10 - 15/group).

measures ANOVA with time bin (1 min each) or tone trial as within-subject factor. Freezing scores during the training session of the contextual and auditory fear conditioning tasks were always analyzed with repeated-measures ANOVA with shock trial as within-subject factor. The analyses were followed by Fisher's LSD multiple-comparison tests, when appropriate. P values of less than 0.05 were

considered statistically significant. The number of rats per group is indicated in the figure legends.

RESULTS

Systemic corticosterone administration dose-dependently impairs retrieval of contextual, but not auditory, fear memory

This experiment investigated whether CORT administered systemically 1 h before retention testing impaired retrieval of contextual and auditory fear memory. During training, different groups of animals acquired the contextual ($F_{7,301} = 81.62$; $p < 0.0001$) and auditory ($F_{7,196} = 61.56$; $p < 0.0001$) fear conditioning tasks, as indicated by progressively increasing freezing scores during shock trials. Further, the groups that were assigned to receive control or drug treatments subsequently did not differ in acquisition performance (contextual fear conditioning: $F_{3,43} = 1.60$; $p = 0.20$; auditory fear conditioning: $F_{3,28} = 0.82$; $p = 0.96$; Table S1). Twenty-four hours later, rats received a systemic injection of either vehicle or different doses of CORT (0.3, 1, 3 mg/kg) 1 h before retention testing on the contextual and auditory fear conditioning tasks. As is shown in Fig. 1A, one-way ANOVA indicated that CORT treatment induced a dose-dependent reduction in overall percent freezing during retention testing on the contextual fear conditioning task ($F_{3,43} = 2.98$; $p = 0.04$). Fisher's post-hoc analysis revealed that the 3 mg/kg dose of CORT, but not lower doses, significantly decreased freezing levels ($p < 0.01$ compared to vehicle). We further analyzed whether freezing levels of rats administered the 3 mg/kg dose of CORT were lower throughout the retention test or whether CORT facilitated the extinction of fear during the retention test session. Repeated-measures ANOVA for freezing levels in five consecutive 1-min time bins (CORT 3 mg/kg and vehicle groups only) demonstrated a significant effect of CORT treatment ($F_{1,23} = 12.22$; $p = 0.001$), but not of time ($F_{4,92} = 1.69$; $p = 0.15$) or interaction between CORT treatment and time ($F_{4,92} = 0.65$; $p = 0.62$), suggesting that freezing levels did not change over the course of the retention test and, thus, that freezing of the CORT 3 mg/kg group was lower than that of the vehicle group throughout the test (Fig. 1B). In contrast to contextual fear memory, systemic CORT treatment did not alter freezing levels during retention on the auditory fear conditioning task ($F_{3,28} = 0.20$; $p = 0.89$; Fig. 1C).

To further exclude the possibility that CORT treatment might directly influence the

expression of freezing, separate groups of animals were trained on the contextual fear conditioning task and, 24 h later, administered different doses of CORT (0.3, 1, 3 mg/kg) 1 h before placing them in a context that was distinctly different from the training context. CORT treatment did not affect basal freezing levels in this non-training context ($F_{3, 47} = 1.24$; $p = 0.31$; Fig. 1D). Thus, these findings indicate that CORT selectively impaired conditioned freezing during retention of contextual fear memory and did not affect freezing during retention of the auditory fear conditioning task or induced any direct deficits in the expression of freezing behavior.

Endocannabinoid signaling in the hippocampus mediates the impairing effect of corticosterone on retrieval of contextual fear memory

To investigate whether the endocannabinoid system of the hippocampus plays a role in mediating the impairing effect of CORT treatment on retrieval of contextual fear memory, bilateral infusions of the CB1 receptor antagonist AM251 (0.35 ng in 0.5 µl) were administered into the dorsal hippocampus 1 h before retention testing together with systemic injections of either vehicle or CORT (3 mg/kg). Repeated-measures ANOVA for freezing scores during training showed that all groups acquired the contextual fear conditioning task as indicated by progressively increasing freezing scores during shock trials ($F_{7,203} = 63.66$, $p < 0.0001$), without a difference in the acquisition rate between later drug groups ($F_{3,29} = 0.79$; $p = 0.50$; Table S2). As is shown in Fig. 2A, two-way ANOVA for percent freezing during 24-h retention testing revealed no significant main effects of CORT ($F_{1,29} = 1.93$, $p = 0.17$) or AM251 ($F_{1,29} = 1.76$, $p = 0.19$), but a significant interaction effect between these two treatments ($F_{1,29} = 4.61$, $p = 0.04$). Fisher's post-hoc comparison tests demonstrated that systemic CORT administration significantly reduced freezing in control rats administered vehicle into the hippocampus ($p < 0.05$). However, this effect of CORT on freezing behavior was blocked in animals administered AM251 into the hippocampus ($p < 0.05$ compared to CORT alone).

Next, we investigated whether CORT administration affected endocannabinoid tissue levels in the hippocampus. For this, rats were trained on the contextual fear conditioning task and, 24 h later, given a systemic injection of CORT (0.3, 1 or 3 mg/kg) 1 h before placing them in a non-training but previously habituated context for 5 min. Immediately afterwards, the hippocampus was dissected

for endocannabinoid measurements. As is shown in Fig. 2C and 2D, one-way ANOVA revealed that CORT treatment dose-dependently elevated hippocampal levels of the endocannabinoid 2-AG ($F_{3,47}$ = 3.15; p = 0.03), without affecting levels of AEA ($F_{3,47}$ = 0.23; p = 0.87) or other measured endocannabinoids such as oleoylethanolamide and palmitoylethanolamide (Table S3). Fisher's post-hoc analyses indicated that the highest dose of CORT (3 mg/kg), but not any of the lower and non-impairing doses, increased 2-AG levels compared to vehicle (p < 0.05). Thus, these findings indicating that CORT administration elevates 2-AG levels in the hippocampus whereas a blockade of hippocampal CB1 receptors prevents CORT effects on memory retrieval impairment suggest that hippocampal endocannabinioid signaling is critically involved in mediating the impairing effects of CORT on retrieval of contextual fear memory.

Intra-hippocampal infusion of a CB receptor agonist WIN55,212-2 impairs retrieval of contextual fear memory via an interaction with the noradrenergic system

As described above, we previously reported that GC effects on memory retrieval of emotionally arousing experiences involve an essential interaction with arousal-induced noradrenergic activity (Roozendaal et al., 2004b; de Quervain et al., 2007). Hence, in this experiment we investigated whether cannabinoid effects on memory retrieval also depend on interactions with the noradrenergic system. To address this issue, we first investigated whether bilateral microinfusions of the CB receptor agonist WIN55,212-2 (10 or 30 ng in 0.5 µl) administered into the dorsal hippocampus 1 h before the retention test impaired retrieval of contextual fear memory, and whether concurrent administration of the ß-adrenoceptor antagonist propranolol (1.25 µg) blocked the impairment. All animals acquired the contextual fear conditioning task as indicated by progressively increasing freezing scores during shock trials ($F_{7,420}$ = 108.00; p < 0.0001) without a significant difference in freezing scores between later drug groups ($F_{2,63}$ = 2.18; p = 0.12; Table S4). As is shown in Fig. 3A, two-way ANOVA for percent freezing on the retention test showed a significant WIN55,212-2 effect ($F_{2,63}$ = 3.26; p = 0.04), a significant propranolol effect ($F_{1,63}$ = 15.65; p = 0.0001) as well as a significant interaction effect between these two treatments ($F_{2,63}$ = 3.63; p = 0.03). Post-hoc analysis showed that both doses of WIN55,212-2 significantly impaired freezing levels (10 ng; p < 0.001, 30 ng: p < 0.05 compared to vehicle). However, WIN55,212-2 did not reduce

freezing levels in rats also administered propranolol. Thus, these findings indicate that, as with GCs, endocannabinoid effects on memory retrieval impairment depend on concurrent noradrenergic activity within the hippocampus.

The second part of this experiment investigated whether blockade of hippocampal CB1 receptors with AM251 (0.35 ng in 0.5 µl) would affect memory retrieval impairment induced by local infusions of norepinephrine (1 or 3 µg). During training, animals increased their freezing as shock trials progressed ($F_{7,490}$ = 137.59, $p < 0.0001$) and there were no differences in the acquisition rate between later drug groups ($F_{2,72} = 0.59$, $p = 0.56$; Table S5). As is shown in Fig. 3B, two-way ANOVA for percent freezing during 24-h retention testing revealed a significant norepinephrine effect ($F_{2,72} = 8.28$; $p = 0.0005$), but no significant AM251 effect ($F_{1,72}$= 0.33; $p = 0.86$) or interaction between norepinephrine and AM251 ($F_{2,72}$= 0.37; $p = 0.70$). Microinjection of either dose of norepinephrine into the dorsal hippocampus 1 h before retention testing significantly reduced conditioned freezing levels (1 µg; $p < 0.01$, 3 µg: $p < 0.01$ compared to vehicle). As with norepinephrine administered alone, the 3 µg dose of norepinephrine infused together with the CB1 receptor antagonist induced a significant reduction in freezing ($p < 0.05$) whereas the 1 µg dose of norepinephrine just failed to reach significance ($p = 0.08$). Infusion of this low dose of AM251 alone did not alter freezing levels ($p = 0.68$). These findings indicate that the effect of noradrenergic activation is downstream of CB1 receptor activation.

To exclude the possibility that WIN55,212-2 or norepinephrine infusions into the hippocampus might have decreased freezing during the retention test by directly affecting the expression of freezing behavior, we investigated, in separate groups of animals, the effect of intra-hippocampal infusions of the same doses of WIN55,212-2 (10 or 30 ng in 0.5 µl) or norepinephrine (1 or 3 µg in 0.5 µl) on freezing behavior during retention of auditory fear conditioning. Repeated-measures ANOVA comparing freezing levels in the WIN55,212-2-treated groups showed a significant effect of tone trial ($F_{4,108}$ = 126.63; $p < 0.0001$), but no significant effect of WIN55,212-2 ($F_{2,27} = 0.41$; $p = 0.66$) or interaction between tone trial and WIN55,212-2 treatment ($F_{8,108} = 0.84$; $p = 0.56$; Fig. S1A). Highly comparable, repeated-measures ANOVA comparing retention freezing scores of norepinephrine-treated groups showed a significant effect of tone trial ($F_{4,108}$ = 90.82; $p < 0.0001$), but no significant effect of norepinephrine ($F_{2,27} = 0.25$; $p = 0.77$)

or interaction between tone trial and norepinephrine treatment ($F_{8,108}$= 0.86; p = 0.55; Fig. S1B). Thus, these findings indicate that WIN55,212-2 or norepinephrine effects on contextual fear memory were not mediated by a general, non-specific change in the expression of freezing behavior.

DISCUSSION

The present study investigated a putative involvement of the hippocampal endocannabinoid system in regulating GC effects on the retrieval of fear memory. The interest of this question stems from previous work indicating, in both rats and healthy human participants, that GCs interact with arousal-induced noradrenergic mechanisms in impairing memory retrieval of emotionally arousing information (de Quervain et al., 1998; de Quervain et al., 2000; Het et al., 2005; Kuhlmann et al., 2005a). However, as these effects are too rapid to be mediated via genomic GC actions, the neurobiological processes underlying the GC influence on noradrenergic activity remained to be determined (Joels et al., 2011). The present findings indicate that a blockade of hippocampal CB1 receptors prevents the impairing effects of GCs on retrieval of contextual fear memory, whereas the administration of an impairing dose of CORT increases hippocampal levels of the endocannabinoid 2-AG. We further found that antagonism of hippocampal ß-adrenoceptor activity blocks the memory retrieval impairment induced by the CB receptor agonist WIN55,212-2, whereas CB1 receptor blockade fails to alter memory retrieval impairment induced by concurrent hippocampal infusions of norepinephrine. These findings suggest that the endocannabinoid system is involved in mediating GC effects on the noradrenergic system in impairing memory retrieval.

Our finding that CORT administration shortly before retention testing impaired retrieval of contextual fear memory, without affecting retrieval of auditory fear memory or baseline freezing, is consistent with previous reports indicating that GCs impair memory retrieval of hippocampus-dependent contextual fear memory and that this stress hormone effect is not directly attributable to acute fear relief or to deficits in the expression of freezing behavior (Cai et al., 2006; Schutsky et al., 2011). Moreover, our finding that CORT did not facilitate the extinction of freezing within the course of the retention test is in line with other evidence

indicating that CORT facilitates the consolidation, but not the acquisition, of fear extinction memory (Cai et al., 2006; Yang et al., 2006). Findings of several other studies investigating the effects of stress, GCs or specific GR agonists on memory retrieval of other training tasks in rats, requiring the expression of other behavioral responses, as well as those of verbal reports in healthy human subjects, support the view that GCs impair immediate and delayed recall of hippocampus-dependent memory (de Quervain et al., 1998; de Quervain et al., 2000; Roozendaal et al., 2003; Roozendaal et al., 2004b). There is extensive evidence that the hippocampus is involved in the retrieval of contextual, spatial or declarative memory and is also a primary target for stress hormones (de Kloet et al., 1999; Riedel et al., 1999; Corcoran and Maren, 2001). Moreover, prior findings indicate that direct infusions of GCs into the hippocampus impair the retrieval of spatial memory (Roozendaal et al., 2003; Roozendaal et al., 2004b) and that a single GC administration to human subjects decreases hippocampal activity during declarative memory retrieval (Oei et al., 2007). The findings of studies investigating whether GCs might also impair memory retrieval of hippocampus-independent learning tasks are consistent with our current observation that GCs appear to have little or no effect on retrieval of auditory fear memory or other hippocampus-independent memories (Kirschbaum et al., 1996; Schutsky et al., 2011). However, we cannot exclude the possibility that CORT might have impaired the retrieval of memory of some specific features of the conditioning tone (e.g., frequency, intensity, duration, et cetera) used in the present study.

Our finding that pre-test blockade of hippocampal CB1 receptors with local infusions of AM251 prevented the GC-induced impairment of contextual fear memory retrieval indicates that endocannabinoid signaling plays an important role in regulating GC effects on memory retrieval. Moreover, comparable to the effect of systemic CORT administration, intrahippocampal infusions of the full CB agonist WIN55,212-2 impaired the retrieval of contextual, but not auditory, fear memory. This selective impairment of retrieval of contextual fear memory indicates that the WIN55,212-2 administration did not non-specifically affect the expression of freezing behavior, a finding that is in accordance with other reported evidence that intrahippocampal administration of WIN55,212-2 or other cannabinoid agonists (delta-9-tetrahydrocannabinol or CP 55,940) impairs spatial memory without directly affecting the expression of behaviors that were assessed

as an index of memory (Lichtman et al., 1995; Egashira et al., 2002; Wegener et al., 2008). Moreover, we found that CORT administration, in a dose that impairs memory retrieval, increased hippocampal levels of 2-AG, but not AEA or other measured endocannabinoids, in the same time course of the retention test. These findings are consistent with previous evidence that stress and GCs rapidly alter endocannabinoid signaling in a variety of stress-responsive brain regions, including the hippocampus (Hill et al., 2010a; Hill and McEwen, 2010). Although some controversy exists in the literature, stress has been shown to mobilize 2-AG

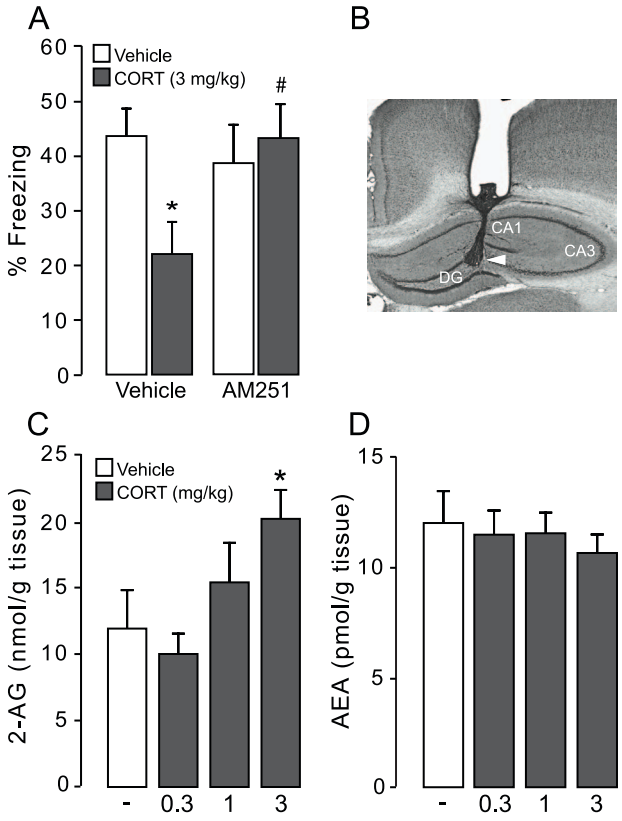

Figure 2. Role of the endocannabinoid system in regulating glucocorticoid effects on retrieval of contextual fear memory. (A) Hippocampal infusion of the CB1 receptor antagonist AM251 (0.35 ng in 0.5 µl) administered 1 h before retention testing blocks the impairment of retrieval of contextual fear memory induced by concurrent systemic CORT (3 mg/kg) treatment. Results represent mean ± SEM, *p < 0.05 vs. vehicle (n = 7-11/group). (B) Representative photomicrograph illustrating placement of cannula and needle tip in the dorsal hippocampus with subfields dentate gyrus (DG), CA1 and CA3. (C, D) Systemic CORT (0.3, 1, 3 mg/kg) treatment dose-dependently increases hippocampal 2-AG, but not AEA, in the same time window of the retention test. All results represent mean ± SEM, *p < 0.05 vs. vehicle (n = 10-15/group).

while concurrently decreasing AEA levels in the hippocampus (Patel and Hillard, 2008; Hill and McEwen, 2010). Interestingly, GR antagonists block this stress-induced increase in hippocampal 2-AG levels (Wang et al., 2012). Although it is currently unknown how GCs might increase 2-AG levels (i.e. changes in synthesis, release, uptake or degradation), the effect appears to depend on activation of a G-protein-coupled receptor and intracellular cAMP-dependent protein kinase signaling (Di et al., 2009).

Extensive evidence indicates that stress and GC effects on memory retrieval of emotionally arousing experiences depend crucially on an interaction with arousal-induced noradrenergic activity (Roozendaal et al., 2003; Roozendaal et al., 2004b; de Quervain et al., 2007; Schutsky et al., 2011). A ß-adrenoceptor antagonist administered systemically or directly into the hippocampus or basolateral amygdala in rats blocks GC effects on memory retrieval. Moreover, GCs have been shown to rapidly increase the release of norepinephrine in the amygdala after an emotionally arousing experience (McReynolds et al., 2010) in a time frame that appears incompatible with that of the classical genomic effects of GCs. The present

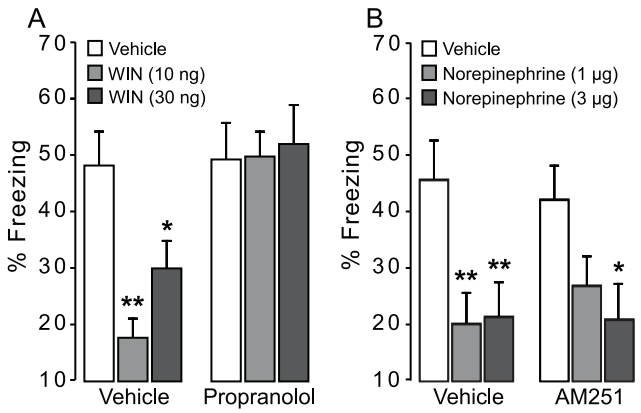

Figure 3. Endocannabinoid and norepinephrine interactions in the dorsal hippocampus on retrieval of contextual fear memory. (A) The CB receptor agonist WIN55,212-2 (WIN: 10 or 30 ng in 0.5 µl) infused into the hippocampus 1 h before the retention test impairs retrieval of contextual fear memory. Concurrent infusion of the ß-adrenoceptor antagonist propranolol (1.25 µg) blocks this WIN55,212-2-induced memory retrieval impairment. Results represent mean ± SEM, *$p < 0.05$; **$p < 0.001$ vs. vehicle (n = 10-14/group). (B) Intrahippocampal infusions of norepinephrine (1 or 3 µg in 0.5 µl) administered 1 h before the retention test, impair retrieval of contextual fear memory. Concurrent infusion of the CB1 receptor antagonist AM251 (0.35 ng) does not block this impairment. Results represent mean ± SEM, *$p < 0.05$; **$p < 0.01$ vs. vehicle (n = 11-15/group).

findings indicate that GC-induced impairment of memory retrieval is mediated, at least in part, via rapid influences on the endocannabinoid system. Moreover, our finding that the ß-adrenoceptor antagonist propranolol blocks the impairing effect of the CB receptor agonist WIN55,212-2, whereas a blockade of CB1 receptors with AM251 fails to prevent norepinephrine-induced memory retrieval impairment, indicates that norepinephrine is functionally located downstream from the endocannabinoid system. Collectively, these findings strongly suggest that endocannabinoids play an intermediary role in regulating GC effects on the norepinephrine system in impairing memory retrieval. In support of this view, previous findings indicate that the administration of a synthetic cannabinoid agonist dose-dependently increased norepinephrine levels in limbic and cortical regions (Oropeza et al., 2005; Page et al., 2007).

A possible scenario is that endocannabinoids might influence noradrenergic function via an inhibition of GABAergic transmission (Campolongo et al., 2009; Hill and McEwen, 2009; Atsak et al., 2012). Although this possibility was originally proposed for GC-induced enhancement of memory consolidation involving the basolateral amygdala (Campolongo et al., 2009), CB1 receptors are also abundantly expressed on hippocampal GABAergic terminals and to a minor extent on glutamatergic terminals (Katona et al., 1999). An activation of CB1 receptors has consistently been shown to suppress the release of GABA in the hippocampus through a Ca^{2+}-dependent depolarization-induced suppression of inhibition (DSI) (Kano et al., 2009). In support of our finding that CORT might affect memory retrieval via increased 2-AG endocannabinoid signaling, recent findings suggest that particularly 2-AG is involved in the modulation of DSI, and thus in the suppression of GABA release, in the hippocampus (Hashimotodani et al., 2008; Wang et al., 2009; Tanimura et al., 2010). Additionally, substantial evidence from pharmacological studies on memory consolidation has indicated that a blockade of GABAergic transmission with specific antagonists increases norepinephrine release from presynaptic sites (McGaugh, 2004). Based on these findings, a similar working model for GC-induced impairment of memory retrieval can be proposed: GCs first boost the release of 2-AG in the hippocampus. This endocannabinoid then binds to CB1 receptors on GABAergic interneurons to suppress the release of GABA, resulting indirectly in elevated norepinephrine levels, which, as we have shown in this study, impairs memory retrieval of salient information (Fig. S2).

As noted above, there is currently growing interest in GC influences on retrieval of memory of emotionally arousing experiences because of clinical findings indicating that GC administration to PTSD patients significantly reduces re-experiencing of highly traumatic memories and other chronic stress symptoms (Aerni et al., 2004, de Quervain et al., 2009). However, in a clinical setting the sustained use of GCs is undesirable because of the pleiotropic nature of these hormones to affect a wide array of physiological functions (e.g., immune and metabolic functions). The present finding that the hippocampal endocannabinoid system is involved in mediating GC effects on memory retrieval impairment could aid in the development of non-GC-based therapies for PTSD. Although clinical studies have not as yet investigated interactions between these two stress systems, recent findings indicate that administration of the synthetic cannabinoid nabilone to PTSD patients resulted in a highly comparable reduction of treatment-resistant daytime flashbacks and nightmares (Fraser, 2009). Moreover, PTSD is often associated with high levels of cannabis consumption (Calhoun et al., 2000), which might be related, in part, to an inadequate activation of the endogenous GC and endocannabinoid systems in these patients (Yehuda, 2009). Furthermore, based on our finding that systemic CORT administration impaired the retrieval of hippocampus-dependent contextual fear memory, without affecting the retrieval of hippocampus-independent auditory fear memory, it would seem important to also investigate whether GC or cannabinoid administration might selectively reduce the retrieval of hippocampus-dependent traumatic memories in PTSD patients.

Supporting Information

Tissue processing and lipid extraction for endocannabinoid measurement

After rapid decapitation, the hippocampus was dissected and placed in pre-cooled tubes and frozen in liquid nitrogen within 3 min of decapitation. Tissue samples were stored at -80 °C until extraction. For detection of endocannabinoids, the collected hippocampal tissue was weighed and homogenized in 2 ml polypropylene tubes (Sarstedt, Numbrecht, Germany). For lipid extraction, internal standards consisting of the stable isotope labeled endocannabinoids arachidonyl ehanolamide-d4, 2-arachidonoyl glycerol-d5, palmitoyl ethanolamide-d4,

N-arachidonoyl dopamine-d8 and arachidonoyl glycine-d8 synthesized by Roche Diagnostics, Mannheim, Germany and Cayman Europe, Tallinn, Estonia was added to the tubes. The purity of these materials was >97.2%. Then 1 ml of methyl tertiary butyl ether (Sigma-Aldrich, Germany) was added and the mixture was vortexed for 30 s and centrifuged at 12,000 × g for 6 min. The clear supernatant was transferred into clean 2 mL polypropylene tubes (Sarstedt, Numbrecht, Germany) and evaporated under N2 at 37 °C. Dried organic phases were then reconstituted in 100 µL of acetonitrile, vortexed for 30 s, centrifugated at 12,000×g for 6 min and then endocannabinoid levels were determined with the high-performance liquid

Table S1. Percentage freezing during training of contextual fear conditioning

Drug groups (mg/kg)	Shock trials				
	1	2	3	4	5
Vehicle	8 ± 5	27 ± 7	38 ± 5	51 ± 9	63 ± 8
CORT 0.3	7 ± 2	32 ± 8	43 ± 8	43 ± 8	51 ± 8
CORT 1	6 ± 3	20 ± 7	29 ± 7	54 ± 9	51 ± 11
CORT 3	2 ± 1	17 ± 7	37 ± 11	44 ± 10	59 ± 10

There was no difference in the acquisition rate between later drug groups. Data are expressed as mean ± SEM (n = 10 –15/group).

Table S2. Percentage freezing during training of contextual fear conditioning.

Drug groups		Shock trials				
		1	2	3	4	5
Vehicle	Vehicle	29 ± 9	52 ±10	54 ± 9	62 ± 8	66 ± 9
	CORT (3 mg/kg)	27 ± 15	40 ± 13	44 ± 15	44 ± 13	60 ± 13
AM251	Vehicle	31 ± 8	47 ± 12	67 ± 9	58 ± 8	67 ± 6
	CORT (3 mg/kg)	28 ± 5	44 ± 5	53 ± 5	67 ±11	81 ± 3

There was no difference in the acquisition rate between later drug groups. Values are expressed as mean ± SEM (n = 11–15/group).

Table S3. The effects of corticosterone administration before the non-training context exposure on the hippocampal tissue content of palmitoylethanolamine and oleoylethanolamine.

	PEA (pmol/g tissue)	OEA (pmol/g tissue)
Vehicle	542 ± 54	96 ± 10
CORT 0.3	539 ± 33	93 ± 10
CORT 1	521 ± 52	94 ± 8.2
CORT 3	528 ± 43	90 ± 7.1

There was no effect of CORT (0.3, 1, 3 mg/kg) administration on the hippocampal tissue content of either palmitoylethanolamide (PEA) or oleoylethanolamine (OEA). Values are expressed as mean ± SEM (n = 10–15/group).

Table S4. Percentage freezing during training of contextual fear conditioning.

Drug groups		Shock trials				
		1	2	3	4	5
Vehicle	Vehicle	25 ± 6	48 ±7	52 ± 9	57 ± 8	52 ± 8
	WIN (10ng)	11 ± 8	21 ± 10	24 ± 12	24 ± 11	42 ± 11
	WIN (30ng)	30 ± 6	51 ± 7	59 ± 8	64 ± 7	63 ± 8
Propranolol	Vehicle	14 ± 7	33 ± 8	42 ± 9	44 ± 8	40 ± 9
	WIN (10ng)	17 ± 6	24 ±7	43 ± 8	53 ±7	65 ± 8
	WIN (30ng)	34 ± 6	43 ± 7	60 ± 8	54 ± 7	56 ± 7

There was no difference in the acquisition rate between later drug groups. Values are expressed as mean ± SEM (n = 10 – 14/group).

Table S5. Percentage freezing during training of contextual fear conditioning

Drug groups		Shock trials				
		1	2	3	4	5
Vehicle	Vehicle	26 ± 7	37 ± 8	50 ± 9	57 ± 7	66 ± 6
	NE (1 µg)	19 ± 8	32 ± 9	43 ± 12	53 ± 9	61 ± 9
	NE (3 µg)	20 ± 6	32 ± 9	44 ± 10	59 ± 7	66 ± 9
AM251	Vehicle	29 ± 8	45 ± 8	60 ± 9	63 ± 7	54 ± 9
	NE (1 µg)	32 ± 7	48 ±7	53 ± 6	58 ± 6	52 ± 8
	NE (3 µg)	19 ± 5	31 ± 8	38 ± 9	50 ± 10	49 ± 10

There was no difference in the acquisition rate between later drug groups. Values are expressed as mean ± SEM (n = 11–15/group).

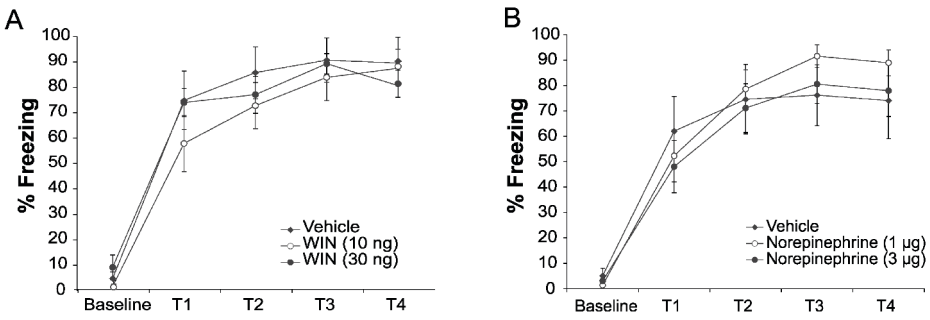

Figure S1. The effect of intrahippocampal WIN55,212-2 (WIN) or norepinephrine (NE) on freezing behavior during retention of auditory fear conditioning. (A) Effect of NE (1 or 3 µg in 0.5 µl) infused into the dorsal hippocampus one hour before the auditory fear conditioning retention during baseline (B) and tone trials (T1-T4). Results represent mean ± SEM (n = 7 – 12) (B) Effect of WIN (10 or 30 ng in 0.5 µl) infused into the dorsal hippocampus one hour before the auditory fear conditioning retention during baseline and tone trials (T1-T4). Results represent mean ± SEM (n = 9–11/group).

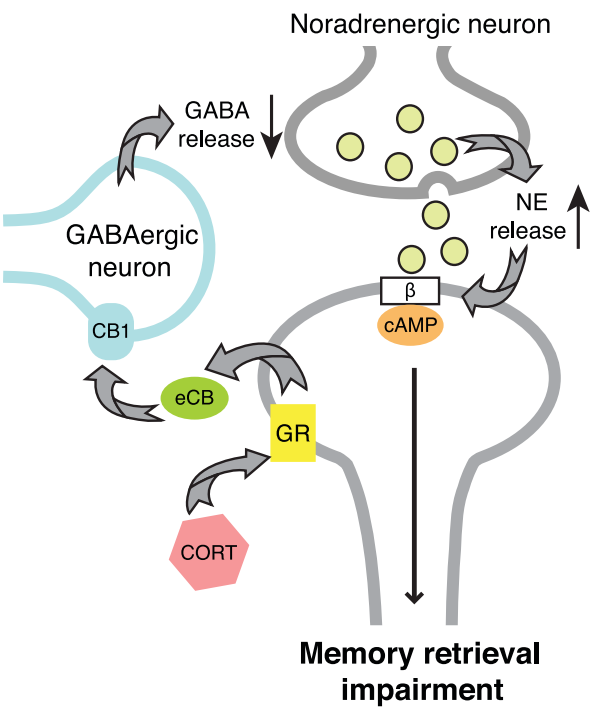

Fig. S2. Model on the role of the endocannabinoid system in the hippocampus in mediating glucocorticoid effects on the noradrenergic system in inducing memory retrieval impairment. Corticosterone (CORT) binds to a membrane-bound glucocorticoid receptor (GR) that activates a pathway to induce endocannabinoid (eCB) synthesis. Endocannabinoids are then released into the synapse, where they bind to CB1 receptors on GABAergic interneurons and thereby, inhibit the release of GABA. This suppression of GABA release subsequently disinhibits norepinephrine (NE) release, resulting in an activation of the postsynaptic β-adrenoceptor and downstream signaling pathways.

chromatography and a tandem mass spectrometry.

ACKNOWLEDGMENTS

The authors thank Jelle De Boer for assistance with data analysis and Petra Bakker, Angelika Jurdzinski, Hassiba Beldjoud and Fany Messanvi for technical assistance with the collection of tissue for endocannabinoid measurements. This research was supported by Jan Kornelis De Cock Stichting grant 6861063 (to PA) and National Science Foundation grant IOB-0618211 (to BR).

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Glucocorticoids Impair Retrieval of Recognition Memory: Role of the Endocannabinoid System

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CHAPTER 6

ABSTRACT

Considerable evidence indicates that stress exposure or glucocorticoid administration shortly before retention testing impairs the retrieval of spatial/contextual memory in rats and of declarative memory in humans. However, other findings of human studies suggest that glucocorticoids are less effective in impairing the retrieval of recognition memory. Here we investigated in male Sprague-Dawley rats the effect of corticosterone (0.3 or 1 mg/kg. sc) administration, 1 h before retention testing, on the retrieval of two different components of information acquired during an object recognition task. We found that corticosterone impaired recognition of both the training object per se and of the location of the objects during the training session, without influencing total exploration of the objects or experimental apparatus. In view of recent findings suggesting that glucocorticoid effects on memory retrieval impairment depend on rapid, nongenomically mediated interactions with the endocannabinoid system, we also investigated the involvement of endocannabinoid signaling in the effects of glucocorticoids on retrieval of these two components of object recognition memory. We found that the inverse CB1 agonist SR141716 (1 mg/kg) administered systemically before retention testing blocked the corticosterone-induced impairment of retrieval of both components of object recognition memory. Thus, these findings indicate that glucocorticoids impair the retrieval of recognition memory and that these effects require concurrent endocannabinoid signaling.

INTRODUCTION

Glucocorticoid hormones (corticosterone in rodents, cortisol in humans), released from the adrenal cortex during emotionally arousing stimulation, are known to influence different memory functions (Roozendaal, 2002; de Quervain et al., 2009; Roozendaal and McGaugh, 2011; Schwabe et al., 2011). Considerable evidence indicates that corticosterone or glucocorticoid receptor (GR) agonists administered to rats or mice shortly before retention testing impair the retrieval of previously acquired spatial and contextual memory (de Quervain et al., 1998; Rashidy-Pour et al., 2004; Roozendaal et al., 2004b; Cai et al., 2006; Schutsky et al., 2011; Atsak et al., 2012a). Likewise, human subjects treated with cortisol or cortisone before a retention test exhibit impaired free or cued recall on declarative memory paradigms (de Quervain et al., 2000; de Quervain et al., 2003; Buss et al., 2004; Het et al., 2005; Kuhlmann et al., 2005; Tollenaar et al., 2009). Few human studies also investigated the effect of glucocorticoids on retrieval of recognition memory (de Quervain et al., 2000; de Quervain et al., 2003; Tops et al., 2003; Domes et al., 2004; Buchanan et al., 2006). Findings of these studies indicate that glucocorticoids induce small and mostly nonsignificant recognition impairment. As free and cued recall of declarative information, as retrieval of spatial and contextual memory in rats, depend heavily on the hippocampus (Squire, 1992; Moser and Moser, 1998; Tulving and Markowitsch, 1998; Holt and Maren, 1999) whereas recognition memory might rely more on cortical regions (Baxter and Murray, 2001; Winters et al., 2008), the relative sparing of recognition memory could be interpreted as suggesting that glucocorticoids preferably impair the retrieval of memory of hippocampus-dependent information. The present study investigated, in rats, the effects of systemic corticosterone administration, 1 h before retention testing, on the recognition of two different components of information acquired during a single object recognition task, namely memory of the training object per se (object recognition memory) and memory of the location of the object during the training trial (object location memory). Studies on memory consolidation indicate that these two components of object recognition memory are processed by dissociable memory systems such that the representation of the object itself requires information processing within cortical regions, particularly the perirhinal and insular cortices, whereas memory regarding the location of the object within its context depends primarily on the hippocampus (Murray and Richmond, 2001;

Bermudez-Rattoni et al., 2005; Roozendaal et al., 2010) .

A second aim of this study was to investigate whether glucocorticoid effects on the retrieval of these two components of object recognition memory depend on interactions with the endocannabinoid system. As glucocorticoid effects on memory retrieval are known to have a rapid onset, but also dissipate shortly after hormone levels return to baseline (de Quervain et al., 1998; Cai et al., 2006; Schutsky et al., 2011), the temporal dynamics of this glucocorticoid effect do not appear to be compatible with their classical genomic mode of action. Many recent investigations are aimed at determining the neural mechanisms underlying such presumably nongenomically mediated actions of glucocorticoids on physiology and behavior. Several studies have now indicated that stress exposure or glucocorticoid administration can launch a G-protein-dependent signaling cascade that induces the synthesis of endocannabinoid ligands (Di et al., 2003; Di et al., 2005). Endocannabinoids are small lipid-based molecules that are rapidly produced in response to increased neuronal activity. Once released, they act as retrograde neurotransmitter and bind to G protein-coupled cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2) receptors on presynaptic sites to influence different brain functions, including neural plasticity, learning and memory, and mood (Kano 2009, Freud 2003; Campolongo et al., 2009; Atsak et al., 2012a). In a recent study, we demonstrated that the endocannabinoid system within the hippocampus is essentially involved in mediating the rapid effects of glucocorticoids on retrieval of contextual fear memory (Atsak et al., 2012b). Systemic corticosterone administration elevated hippocampal levels of the endocannabinoid 2-arachidonoylglycerol, whereas a blockade of hippocampal CB1 receptors prevented the impairing effect of glucocorticoid administration on retrieval of contextual fear memory. The present study investigated whether antagonism of CB1 receptors with a systemic injection of its inverse agonist SR141716 1 h before retention testing blocks also the impairing effects of corticosterone on retrieval of these two components of object recognition memory.

METHODS

Animals. Male adult Sprague–Dawley rats (350–430 g at time of training) from Charles River Breeding Laboratories (Kisslegg, Germany) were kept individually in a temperature-controlled (22°C) colony room and maintained on a standard 12-h light: 12-h dark cycle (07:00-19:00 h lights on) with *ad libitum* access to food and water. Training and testing were conducted during the light phase of the cycle between 10:00 and 16:00 h. All procedures were performed in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and were approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Drug treatment. The adrenocortical hormone corticosterone (0.3 or 1 mg/kg; Sigma-Aldrich) was dissolved either alone or together with the inverse CB1 receptor agonist SR141716 (rimonabant, 5 - (4 - chlorophenyl) - 1 - (2,4 - dichlorophenyl) – 4 - methyl – *N* - (piperidin – 1 - yl) - 1*H* - pyrazole – 3 -carboxamide; 1 mg/kg; Kemprotec Ltd, Middlesbrough, UK) in a vehicle containing 5% polyethylene glycol, 5% Tween-80 and 5% ethanol in saline and administered subcutaneously, in a volume of 2 ml/kg, 1 h before the retention test. Doses of corticosterone and SR141716 were selected on the basis of prior studies (de Quervain et al., 1998; Hauer et al., 2011; Atsak et al., 2012a).

Object recognition and object location retrieval tasks. The experimental apparatus was a gray open-field box (40 cm × 40 cm × 40 cm) with a sawdust-covered floor, placed in a dimly illuminated room. The objects to be discriminated were white glass light bulbs (6 cm diameter and 11 cm length) and transparent glass vials (5.5 cm diameter and 5 cm height). All rats were handled for 5 days for 1 min each immediately preceding the training day. On the training trial, the rat was placed in the experimental apparatus and allowed to explore two identical objects (A1 and A2) placed 5 cm away from the corners of the apparatus for 10 min. To avoid the presence of olfactory trails, sawdust was stirred and the objects were thoroughly cleaned with 70% ethanol between rats. Rat's behavior was recorded by using a video camera positioned above the experimental apparatus. The time spent exploring the two objects was taken as a measure of object exploration. Retention was tested 24 h after the training trial. Independent groups of rats were tested for

memory of the training object per se (i.e., object recognition memory) and for memory of the location of the objects during the training (i.e., object location memory). For object recognition memory testing, one copy of the familiar object (A3) and a new object (B) were placed at the same location as stimuli during the training trial. For object location memory testing, one copy of the familiar object (A3) was placed in the middle of the box and the other copy of the familiar object (A4) was placed in the same location as during the training trial. All combinations and locations of objects were used in a balanced order to reduce potential biases due to preference for particular locations or objects. The rat was placed in the experimental apparatus for 3 min and the time spent exploring each object and the total time spent exploring both objects were scored. Exploration of an object was defined as pointing the nose to the object at a distance of <1 cm and/or touching it with the nose. Turning around, climbing or sitting on an object was not considered as exploration. A discrimination index was calculated as the difference in time exploring the novel and familiar object (or location), expressed as the ratio of the total time spent exploring both objects. Rats showing a total exploration time < 5 s on either training or testing were excluded. To assess the rats' exploratory behavior of the experimental apparatus on the retention test, the floor of the apparatus was divided into four imaginary squares and the total number of crossings between quadrants was determined.

Plasma corticosterone levels. Corticosterone levels were determined in parallel groups of rats that were injected with corticosterone (0.3 or 1 mg/kg) and/or SR141716 (1 mg/kg) and sacrificed 1 h later under pentobarbital (100 mg/kg) anesthesia. Trunk blood was collected in tubes containing 0.5 M EDTA and samples were centrifuged at 1,900×g at 4°C for 10 min. Plasma was stored at -20°C and analyzed for corticosterone using a radioimmunoassay, according to a previously described protocol (Lin et al., 2008).

Statistics. All data are expressed as mean ± SEM. Data were analyzed by two-way analysis of variance (ANOVA) with corticosterone (3 levels) and SR141716 (2 levels) as between-subject variables. Further analyses used Fisher post-hoc tests to determine the source of the significance, when appropriate. One-sample *t* tests were used to determine whether the discrimination index was different from zero. A probability level of < 0.05 was accepted as statistically significant. The number

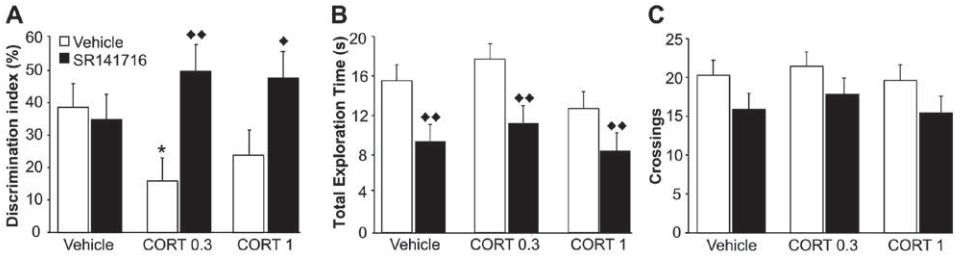

Figure 1. Corticosterone impairs retrieval of object recognition memory and SR141716 blocks this effect. (A) Discrimination index (%) of rats that were administered either CORT (0.3 or 1 mg/kg) alone or together with SR141716 (1 mg/kg) 1 hr before the object recognition retention test. (B) Total time spent (s) exploring both objects during the object recognition retention test. (C) The quadrant crossings in the experimental apparatus during object recognition retention test. ◆, $p < 0.05$; ◆◆, $p < 0.001$ SR141716 groups compared to the corresponding vehicle, *, $p < 0.05$ corticosterone compared to vehicle. All data is presented as mean ± SEM (n = 9 -12/group).

of rats per group is indicated in the figure legends.

RESULTS

Systemic SR141716 treatment blocks corticosterone-induced retrieval impairment of object recognition memory

This experiment investigated whether corticosterone (0.3 or 1 mg/kg) administered systemically 1 h before retention testing, 24 h after training, impairs retrieval of object recognition memory and whether concurrent blockade of CB1 receptors with SR141716 (1 mg/kg) prevents the corticosterone effect.

Training trial: The average time spent exploring the two identical objects on the 10-min training trial was 29.5 ±1.4 s. Two-way ANOVA indicated no differences between groups that were subsequently assigned to receive corticosterone and/or SR141716 (corticosterone: $F_{1,55} = 0.05$, $P = 0.94$; SR141716: $F_{1,55} = 0.01$, $P = 0.93$; corticosterone x SR141716: $F_{1,55} = 2.61$, $P = 0.08$).

Retention trial: As is shown in Fig. 1A, one-sample *t* test revealed that the discrimination index of vehicle-treated rats was significantly different from zero ($t_{10} = 7.20$, $P < 0.0001$), indicating that control rats readily discriminated the novel object on the 24-h retention test. Two-way ANOVA for discrimination index showed no main effect of corticosterone treatment ($F_{1,55} = 0.14$, $P = 0.86$), but a significant effect of SR141716 ($F_{1,55} = 8.18$, $P = 0.005$) as well as a significant

interaction effect between these two treatments ($F_{1,55}$ = 3.31, P = 0.04). Fisher post-hoc comparison tests indicated that corticosterone in a dose of 0.3 mg/kg, but not 1 mg/kg, significantly impaired the discrimination index (P < 0.05 compared to vehicle). Further, one sample t test indicated that rats administered this lower dose of corticosterone did not show a preference for the novel object (t_8 = 0.75, P = 0.47). Corticosterone treatment did not impair the discrimination index of rats that were also administered SR141716. The discrimination index of rats administered SR141716 alone or together with either dose of corticosterone did not differ significantly from that of the vehicle group ($P \geq 0.41$).

As is shown in Fig. 1B, two-way ANOVA for total exploration of the two objects on the retention test revealed a significant SR141716 effect ($F_{1,55}$ = 16.71, P < 0.0001), but no corticosterone effect ($F_{1,55}$ = 2.61, P = 0.09) or interaction between both factors ($F_{1,55}$ = 0.24, P = 0.78). Corticosterone treatment did not affect total object exploration times, but rats treated with SR141716, irrespective of whether they had received corticosterone or not, exhibited less total exploration of the objects than rats not administered SR141716 (P < 0.001). Comparably, two-way ANOVA for drug effects on exploration of the experimental apparatus, assessed by the number of quadrant crossings on the retention test, revealed a significant SR141716 effect ($F_{1,55}$ = 6.11, P = 0.01), but no corticosterone effect ($F_{1,55}$ = 0.56, P

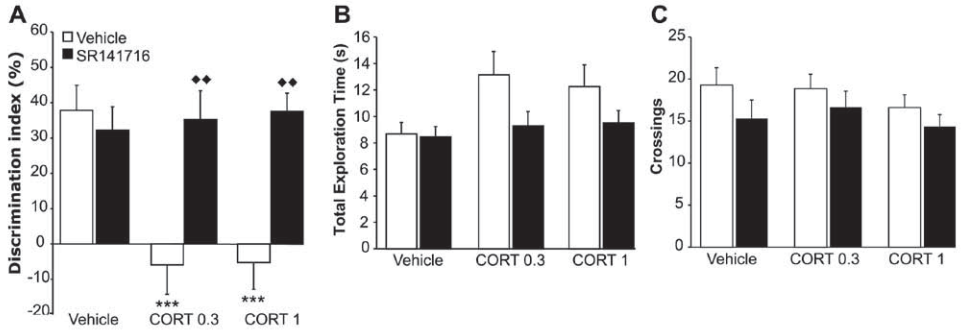

Figure 2. Cortciosterone impairs retrieval of object location memory and SR141716 blocks this effect. (A) Discrimination index (%) of rats that were administered either CORT (0.3 or 1 mg/kg) alone or together with SR141716 (1 mg/kg) 1 hr before the object location memory retrieval. (B) Total time spent (s) exploring both objects during the object location retention test. (C) The quadrant crossings in the experimental apparatus during object location retention test. All data is presented as mean ± SEM. ◆◆, p < 0.001 SR141716 compared to the corresponding vehicle, ***, p <0.0001 corticosterone groups compared to vehicle. All data is presented as mean SEM (n = 9 - 14/group).

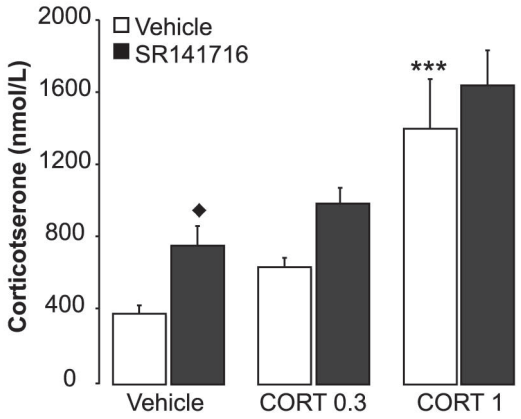

Figure 3. Plasma corticosterone levels of the rats 1 hr after the administration of CORT (0.3 or 1 mg/kg) alone or together with SR141716 (1mg/kg). All data is presented as mean ± SEM.◆, $p < 0.05$ compared to corresponding vehicle, ***, $p < 0.0001$ corticosterone groups compared to vehicle. All data is presented as mean ± SEM (n = 9 -15/group).

= 0.57) or interaction between both treatments ($F_{1,55} = 0.01$, $P = 0.98$), indicating that SR141716 treatment also reduced general exploration of the experimental apparatus (Fig. 1C).

Systemic SR141716 treatments blocks corticosterone-induced retrieval impairment of object location memory

This experiment investigated, in different groups of rats, whether corticosterone (0.3 or 1 mg/kg) administered systemically 1 h before retention testing impairs retrieval of object location memory and whether concurrent blockade of CB1 receptors with SR141716 (1 mg/kg) prevents this corticosterone effect.

Training trial: The average total exploration time of the two identical objects on the 10-min training trial was 33.7 + 1.6 s. Two-way ANOVA indicated no effect of later corticosterone treatment ($F_{2,61} = 2.47$, $P = 0.09$), later SR141716 treatment ($F_{1,61} = 0.12$, $P = 0.72$) or interaction between these two treatments ($F_{1,61} = 1.89$, $P = 0.16$).

Retention trial: As is shown in Fig. 2A, one-sample *t* test revealed that the discrimination index of rats administered vehicle 1 h before the retention test was significantly different from zero ($t_9 = 4.90$, $P < 0.001$), indicating that these rats discriminated the object placed in the novel location on the 24-h retention test. Two-way ANOVA for discrimination index revealed significant main effects of corticosterone ($F_{2,61} = 4.20$, $P = 0.02$) and SR141716 ($F_{1,61} = 17.47$, $P < 0.0001$)

as well as a significant interaction between these two treatments ($F_{1,61}$ = 6.18, P = 0.004). Fisher post-hoc tests indicated that both doses of corticosterone (0.3 and 1 mg/kg) significantly impaired the discrimination index (for both doses: P < 0.0001 compared to vehicle). In addition, one-sample t tests indicated that the discrimination index of rats treated with corticosterone did not differ significantly from zero (0.3 mg/kg: t_{13} = - 0.70, P = 0.49, 1 mg/kg: t_{12} = - 0.66, P = 0.51). Highly comparable to the findings of the first experiment, SR141716 treatment blocked the impairing effect of corticosterone on retrieval of object location memory. The discrimination index of rats administered SR141716 together with either dose of corticosterone was significantly higher than those of rats administered the corresponding dose of corticosterone alone (for both doses: P < 0.001) and did not differ significantly from that of vehicle control rats ($P \geq 0.62$).

As is shown in Fig. 2B, two-way ANOVA for the total time spent exploring the two objects on the retention test revealed a significant SR141716 effect ($F_{1,61}$ = 5.26, P = 0.02), but no corticosterone effect ($F_{2,61}$ = 2.72, P = 0.08) or interaction effect ($F_{2,61}$ = 1.15, P = 0.32). Further, as is shown in Fig. 2C, two-way ANOVA for exploration of the experimental apparatus, as assessed by the number of quadrant crossings during the test session, indicated a significant SR141716 effect ($F_{1,61}$ = 4.20, P = 0.04), but no corticosterone effect ($F_{2,61}$ = 1.07, P = 0.38) or interaction between these two treatments ($F_{2,61}$ = 1.17, P = 0.83) (Fig. 1D). Thus, consistent with the findings of the first experiment, whereas corticosterone did not affect exploration, SR141716 treatment induced a general reduction in the exploration of both objects and experimental apparatus.

Effects of corticosterone and SR141716 treatment on plasma corticosterone levels

Fig. 3 shows plasma corticosterone levels assessed 1 h after corticosterone (0.3 or 1 mg/kg) and SR141716 (1 mg/kg) administration. Two-way ANOVA indicated significant effects of corticosterone ($F_{2,54}$ = 28.00, P < 0.0001) and SR141716 treatments ($F_{1,54}$ = 8.99, P = 0.004), but no interaction effect ($F_{2,54}$ = 0.20, P = 0.81). Post-hoc analyses revealed that corticosterone administration dose-dependently elevated plasma corticosterone levels. Plasma levels induced by the higher dose of corticosterone (1 mg/kg) were significantly higher than those of vehicle-treated rats (P < 0.0001), whereas plasma corticosterone levels induced by the 0.3 mg/kg dose of corticosterone just failed to differ significantly from those of vehicle

controls ($P = 0.06$). SR141716 per se significantly elevated plasma corticosterone levels ($P < 0.05$ vs. vehicle) and corticosterone treatment further increased these levels. Plasma corticosterone levels of rats administered SR141716 together with the 1 mg/kg dose of corticosterone were significantly higher than those of rats treated with SR141716 only ($P < 0.05$) and did not differ from those of rats administered corticosterone (1 mg/kg) alone ($P = 0.24$).

DISCUSSION

The present study investigated the effects of glucocorticoid administration on the retrieval of two different components of object recognition memory in rats. We found that corticosterone administered 1 h before retention testing impaired the retrieval of both object recognition and object location memory, without influencing total exploration time of the objects or general exploration of the experimental apparatus. Furthermore, a blockade of CB1 receptors with co-administration of SR141716 prevented the impairing effects of corticosterone on retrieval of both components of object recognition memory.

Prior studies investigating stress and glucocorticoid effects on memory retrieval indicated that high circulating levels of glucocorticoids at the time of retention testing impaired the recall of hippocampus-dependent spatial/contextual information in animals and of declarative information in humans (Roozendaal, 2002; de Quervain et al., 2009). An essential involvement of the hippocampus, which expresses high levels of adrenal steroid receptors (McEwen, 2001), in regulating glucocorticoid effects on memory retrieval of spatial and contextual information was further demonstrated by findings of pharmacological studies in rats indicating that the administration of corticosterone or selective GR agonists directly into the dorsal hippocampus shortly before retention testing induced comparable memory retrieval impairment (Roozendaal et al., 2003; Roozendaal et al., 2004a; Schutsky et al., 2011). Furthermore, human neuroimaging studies indicated that cortisone administered shortly before recall testing reduced activity of the parahippocampal gyrus, an effect that was associated with impaired cued recall of words (de Quervain et al., 2003; Oei et al., 2007). In contrast, the few human studies that investigated the effect of cortisol on recall of recognition memory reported finding only modest and nonsignificant impairment (de Quervain et al.,

2000; de Quervain et al., 2003; Buchanan et al., 2006). Collectively, these findings led to the suggestion that glucocorticoids might preferably impair the retrieval of hippocampus-dependent memories.

Our current findings indicating that corticosterone administration impaired not only the retrieval of object location memory but also of object recognition memory suggest that glucocorticoids might have a more general inhibitory influence on memory retrieval. As the corticosterone did not affect either total exploration of the objects or of the experimental apparatus, the impaired discrimination index is thus not caused by any general influence of corticosterone on exploratory behavior or a reduced interest of the rats in the objects. Rather, the findings are consistent with the view that corticosterone impaired neural processes underlying the retrieval of recognition memory. The corticosterone-induced retrieval impairment of object location memory is likely mediated by the hippocampus. On the other hand, cortical regions, particularly the perihinal and insular cortices, are broadly implicated in both the consolidation and recall of object recognition memory (Baxter and Murray, 2001; Winters et al., 2008). Recently, we reported a double dissociation between the effects of pharmacological activation of the insular cortex and hippocampus and the enhanced consolidation of object recognition and object location memory, respectively (Roozendaal et al., 2010). Findings of other studies indicated that posttraining administration of corticosterone or GR agonists in the insular cortex also enhance the consolidation of object recognition memory (Roozendaal et al., 2010) as well as that of memory of other training (Miranda et al., 2008; Roozendaal et al., 2010). Although prior studies have not examined the effects of glucocorticoid infusions into these cortical regions on memory retrieval, it seems likely that the systemically administered corticosterone might have acted directly upon the insular and/or perirhinal cortex in inducing retrieval impairment of object recognition memory (Winters et al., 2008). However, some lesion studies suggested also an involvement of the hippocampus in object recognition memory (Broadbent et al., 2004). Although very large lesions, destroying 75-100 % of the hippocampal volume, were necessary to induce impairment on an object recognition task, we cannot completely exclude the possibility that the hippocampus has a role in regulating glucocorticoid effects on the retrieval of both components of recognition memory. Nevertheless, our findings indicating glucocorticoid-induced recognition impairment appear to

differ with the small and nonsignificant recognition deficits observed in human studies. As recognition memory paradigms in humans are typically rather simple compared to free or cued recall tasks and glucocorticoids are known to induce impairment of recall performance only when the task is sufficiently demanding (Diamond et al., 1999; de Quervain et al., 2000; de Quervain et al., 2003; Buchanan et al., 2006), the discrepancy between the relative absence of a glucocorticoid effect on recognition memory in humans and our findings is possibly explained by a difference in the demand of the tasks employed.

Our findings further indicate that the endocannabinoid system is essentially involved in mediating the impairing effects of glucocorticoids on retrieval of these two components of recognition memory. We found that the administration of the inverse CB1 receptor agonist SR161714 blocked the impairing effect of corticosterone on retrieval of both object recognition and object location memory. The finding that SR141716 also reduced total exploration of the objects and experimental apparatus, without directly affecting the preference for the novel object or location, is in line with previous reports indicating that CB1 blockade influences habituation and explorative behaviors (Kamprath et al., 2006). Our findings add to the increasing evidence indicating the existence of bidirectional and functional interactions between glucocorticoids and the endocannabinoid system (Patel, 2004; Hill and McEwen, 2009; Evanson et al., 2010; Hill and McEwen, 2010; Hill et al., 2010b). Prior findings show that glucocorticoids, possibly by activating a GR on the cell membrane, rapidly elevate endocannabinoid levels in specific brain regions (Hill et al., 2010a; Atsak et al., 2012b; Wang et al., 2012). The released endocannabinoids then activate CB1 receptors on presynaptic sites to affect neuronal function (Freund et al., 2003; Kano et al., 2009). CB1 receptors are abundantly present in memory-related brain regions such as the hippocampus, amygdala and cortex (Katona et al., 1999; Katona et al., 2000; Katona et al., 2001). In a previous study (Atsak et al., 2012a), we reported that endocannabinoid signaling within the hippocampus is essentially involved in mediating the impairing effects of glucocorticoids on retrieval of highly arousing contextual fear memory. Here, we extend these findings and show that endocannabinoid signaling is also involved in mediating glucocorticoid effects on retrieval of non-aversive object recognition memory.

As the endocannabinoid system is also implicated in regulating the stress response; particularly, a suppression of endocannabinid signaling is known to alter hypothalamic-pituitary-adrenal (HPA) axis activity (Patel, 2004; Steiner et al., 2008; Hill and McEwen, 2010; Hill et al., 2011), we determined plasma levels of corticosterone in parallel groups of animals 1 h following drug treatment. Our finding that SR141716 treatment alone elevated plasma corticosterone levels is consistent with previous evidence indicating that a genetic disruption or pharmacological blockade of CB1 receptor signaling also increases HPA-axis activity (Hill and McEwen, 2010). However, as SR141716-treated rats, despite having higher corticosterone levels, did not show impairment of memory retrieval on either the object recognition or object location tasks, these findings demonstrate that SR141716 did not rescue the glucocorticoid-induced memory retrieval impairment by altering HPA-axis activity and resultant circulating corticosterone levels. Thus, consistent with our previous findings indicating that local infusion of CB1 antagonist into the hippocampus blocked corticosterone effects on memory, in the present study systemic SR141716 administration likely prevented the glucocorticoid-induced memory retrieval impairment by directly blocking endocannabinoid signaling in memory-related brain regions.

In summary, the present findings that corticosterone administration impairs the retrieval of both object recognition and object location memory show that glucocorticoids might influence memory retrieval processes more generally than previously thought. Our findings also provide strong support for the view that endocannabinoid signaling is a common mediator of rapid, nongenomic glucocorticoids effects on adaptive responses to stress, behavior and memory (Hill et al., 2010a; Tasker and Herman, 2011; Atsak et al., 2012a; Atsak et al., 2012b).

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General Discussion

CHAPTER 7

CONTENTS

1. Summary

1.1. Endocannabinoids mediate nongenomic glucocorticoid effects on memory consolidation

Substantial evidence indicates that glucocorticoids, along with other components of the stress response, are critically involved in regulating the consolidation of memory of emotionally arousing experiences (Flood et al., 1978; de Kloet, 2000; Roozendaal, 2000; McGaugh and Roozendaal, 2002; Roozendaal, 2002; Het et al., 2005; Sandi and Pinelo-Nava, 2007; de Quervain et al., 2009; Roozendaal et al., 2009). Such glucocorticoid-induced enhancement of memory consolidation seems to entail the selective activation of the low-affinity glucocorticoid receptor (GR) (Oitzl and de Kloet, 1992; Roozendaal and McGaugh, 1997). Accordingly, the activation of GRs by agonists administered into many different brain regions has been reported to enhance memory consolidation of many different kinds of emotionally arousing learning experiences, including inhibitory avoidance (IA), contextual and cued fear conditioning, water-maze spatial and cued training, object recognition and conditioned taste aversion (Roozendaal et al., 2006a). Glucocorticoids are thought to modulate neurophysiology and behavior through both genomic and nongenomic pathways (de Kloet, 2000; Dallman, 2005) and recently the endocannabinoid system emerged as a potential candidate to regulate some of the nongenomic actions of glucocorticoids. A single injection of a stress dose of glucocorticoids is known to rapidly boost up endocannabinoid signaling within limbic brain regions. Previous findings indicate that elevated endocannabinoid levels within hypothalamic regions are important in mediating glucocorticoid-induced fast inhibition of the hypothalamic–pituitary–adrenal (HPA) axis (Tasker and Herman, 2011). In chapter 4, we showed that the CB1 receptor antagonist AM251 administered into the basolateral amygdala (BLA) after IA training blocked the memory–enhancing effect of the GR agonist RU 28362. These findings extend previous evidence indicating that CB1 receptor blockade in the BLA prevents the memory-enhancing effects of systemically administered corticosterone (Campolongo et al., 2009). Thus these results indicate that endocannabinoid signaling within the BLA is essentially involved in regulating the memory-enhancing effects of glucocorticoids. Furthermore, we found that a blockade of CB1 receptors in the BLA immediately after IA training also prevented the memory-enhancing effects of a coadministration of the

membrane-impermeable ligand Cort:BSA. Based on these findings, it appears that the memory-enhancing effects of glucocorticoids most likely involve the activation of a GR on the cell surface. Consistent with these findings, Tasker and colleagues reported that the activation of a membrane GR launches a G-protein-dependent signaling cascade that induces the synthesis of endocannabinoid ligands (Di et al., 2003; Evanson et al., 2010). Collectively, these data suggest that the activation of the endocannabinoid system by post-training GR agonist infusions within the BLA might have mediated the memory-enhancing effects of glucocorticoids.

Other stress-activated response systems such as central corticotropin-releasing factor (CRF) are also potent modulators of memory consolidation (Roozendaal and McGaugh, 2011). In chapter 4, we also demonstrated that the CB1 receptor antagonist AM251 administered into the BLA after IA training blocked the memory-enhancing effect of concurrently administered CRF_{6-33}, indicating that the endocannabinoid system is also required for enabling the enhancing effects of other stress-related systems on memory consolidation. However, as we discuss in that chapter, it is possible that glucocorticoids interact with the memory-enhancing effects of CRF receptor activation via an endocannabinoid-dependent mechanism.

Since arousal-induced noradrenergic activity is crucially required for enabling glucocorticoid effects on memory consolidation (Roozendaal and McGaugh, 2011), we set out to explore interactions between the endocannabinoid and noradrenergic systems to examine whether endocannabinoids mediate the rapid actions of glucocorticoids on the noradrenergic system in enhancing memory consolidation. We found that the CB agonist WIN55,212-2 infused into the BLA immediately after an IA learning experience enhanced memory consolidation; however, immediate posttraining infusions of the β-adrenoceptor antagonist propranolol blocked the enhancing effect of WIN55,212-2 on memory consolidation. Conversely, the CB1 receptor antagonist AM251 infused into the BLA, as with a GR antagonist shifted the dose-response effects of the β-adrenoceptor agonist clenbuterol such that a much higher dose of clenbuterol was required to induce memory enhancement. These findings strongly suggest that glucocorticoids, via an endocannabinoid-dependent mechanism, render

the BLA more sensitive to the memory-enhancing effects of noradrenergic stimulation.

Moreover, arousal-induced noradrenergic activity is tightly linked to an activation of the intracellular protein kinase A pathway and thereby leads to the phosphorylation of cAMP response binding element (pCREB) protein. Previous findings indicated that glucocorticoids increase pCREB activation in the BLA via an interaction with the noradrenergic system (Roozendaal et al., 2006b). Many findings support the view that an activation of the transcription factor CREB initiates gene expression important for memory formation. For example, interference with CREB activation through genetic or pharmacological manipulations leads to memory impairment, whereas an activation of CREB is associated with an enhanced memory formation (Silva et al., 1998; Bozon et al., 2003; Josselyn et al., 2004). As glucocorticoids are known to increase pCREB activation via an interaction with arousal-induced norepinephrine, we tested whether CB1 receptor antagonism would block the corticosterone-induced increase in pCREB expression within the BLA. We found that CB1 receptor blockade with a systemic injection of SR141716 prevented the increase in pCREB expression in the BLA as well as blocked the memory-enhancing effects of corticosterone. These findings clearly indicate that endocannabinoids enhance the consolidation of memory via an interaction with the noradrenergic system.

Endocannabinoids have been suggested to influence noradrenergic function via an inhibition of GABAergic transmission (Campolongo et al., 2009; Hill and McEwen, 2009; Atsak et al., 2012b). CB1 receptors are abundantly present on local inhibitory GABAergic interneurons in the BLA (Katona et al., 2001; McDonald and Mascagni, 2001). A recent study indicated that CB1 receptors are particularly enriched in axon terminals of cholecystokinin (CCK)-positive interneurons and form invaginating perisomatic synapses with pyramidal neurons in the BLA (Yoshida et al., 2011). CCK-positive interneurons have been proposed to function as fine-tuning devices for the cooperation of pyramidal neurons, which are sensitive to the emotional and motivational state of the animal (Freund et al., 2003). Various studies have reported that an activation of CB1 receptors regulates long-term depression at inhibitory synapses and decreases GABAergic synaptic transmission (Katona et al., 1999; Ohno-Shosaku et al., 2001; Freund and Hajos, 2003). Substantial evidence

demonstrates that an inhibition of local GABAergic circuits in the BLA enhances memory consolidation (Brioni et al., 1989; Castellano et al., 1989; McGaugh, 2004) by increasing the release of norepinephrine from presynaptic sites (Hatfield et al., 1999). Additionally, GABAergic receptor antagonists are known to act postsynaptically to increase the excitability of BLA pyramidal neurons (Azad et al., 2004; Pistis et al., 2004; Zhu and Lovinger, 2005; Yoshida et al., 2011). These findings, together with the evidence that glucocorticoids and CRF enhance the excitability of BLA pyramidal neurons by decreasing the impact of inhibitory GABAergic influences (Rainnie et al., 1992; Duvarci and Paré, 2007), support the hypothesis that nongenomically mediated actions of glucocorticoids on the noradrenergic system require a rapid increase in endocannabinoid signaling within the BLA to effectively shut off local inhibitory GABAergic interneurons. Such a suppression of GABAergic activity might then result in augmented noradrenergic signaling in BLA pyramidal neurons and an enhanced consolidation of long-term memory of emotionally arousing experiences.

1.2. Endocannabinoids mediate nongenomic glucocorticoid effects on memory retrieval

Opposite to the enhancing effects of glucocorticoids on the consolidation of memory, stress or pharmacologically induced increases in glucocorticoid levels exert temporary impairment of retrieval of memory of contextual/spatial information in rats and declarative information in human subjects (de Quervain et al., 1998; de Quervain et al., 2000b; Roozendaal et al., 2003; Roozendaal et al., 2004a; Roozendaal et al., 2004b; de Quervain et al., 2009). Since the hippocampus has long been implicated in the retrieval of contextual/spatial and declarative memory (Holt and Maren, 1999; Corcoran and Maren, 2001) and expresses high levels of adrenal steroid receptors (McEwen, 2001), these findings have been taken to suggest that hippocampus-dependent memories are particularly sensitive to the disruptive effects of glucocorticoids. An involvement of the hippocampus in regulating glucocorticoid effects on memory retrieval of spatial and contextual information was further shown by findings of pharmacological studies in rats indicating that corticosterone or selective GR agonists administered directly into the dorsal hippocampus shortly before retention testing induces comparable memory retrieval impairment (Roozendaal et al., 2003; Roozendaal et al., 2004a; Schutsky et al., 2011). In Chapter 5, we reported that systemic corticosterone

induces dose-dependent retrieval impairment of contextual fear memory without influencing the retrieval of hippocampus-independent auditory fear memory. The findings of other studies investigating whether glucocorticoids might impair memory retrieval on hippocampus-independent learning tasks are consistent with our current observation that glucocorticoids seem to have little or no effect on retrieval of auditory fear memory or other hippocampus-independent memories (Kirschbaum et al., 1996; Schutsky et al., 2011). Since both tasks employed the same behavior (freezing) as an index of memory, these results further strengthen the view that the glucocorticoid administration specifically impaired the retrieval of memory, rather than nonselectively altering the expression of the behavior.

Evidence indicates that glucocorticoids selectively impair the retrieval of emotionally arousing, but not neutral, information because of an essential interaction with arousal-induced noradrenergic activation. For instance, β-adrenoceptor antagonist administered systemically or directly into the hippocampus blocks the impairing effects of glucocorticoids on memory retrieval (Roozendaal et al., 2004a; Roozendaal et al., 2004b). Similarly, human studies indicated that the β-adrenoceptor antagonist propranolol blocked the impairing effect of cortisone on retrieval of emotionally arousing words (de Quervain et al., 2007). Overall these studies indicate an essential interaction between the noradrenergic and glucocorticoid systems in mediating stress effects on memory retrieval. However the induction of noradrenergic activity by glucocorticoids occurs too rapid to be mediated by the classical genomic mechanism of glucocorticoids (McReynolds et al., 2010). Recently, there has been a growing interest in endocannabinoids as a potential candidate to mediate these rapid glucocorticoid effects on noradrenergic activity (Atsak et al., 2012b). In chapter 5, we provide novel and convincing evidence for a critical involvement of hippocampal endocannabinoid signaling in mediating glucocorticoid-induced impairment of memory retrieval. We showed that a blockade of hippocampal CB1 receptors by local infusions of AM251 prevented the impairing effects of systemically administered glucocorticoids on retrieval of contextual fear memory, whereas the corticosterone induced a significant elevation in hippocampal 2-arachidonoylglycerol (2-AG), but not anandamide (AEA), levels in the same time course of retention test. In order to determine whether endocannabinoids mediate the effects of glucocorticoids on the noradrenergic system, we further examined

possible interactions between the endocannabinoid and noradrenergic systems on retrieval of contextual fear memory. We showed that the CB receptor agonist WIN55,212-2 infused into the hippocampus 1 h before retention testing impaired the retrieval of contextual fear memory; however, co-infusion of the β-adrenoceptor antagonist propranolol blocked the impairing effects of WIN55,212-2. Conversely, the CB1 receptor antagonist AM251 infused into hippocampus together with an impairing dose of norepinephrine failed to abolish the impairing effect of norepinephrine on memory retrieval. Collectively, these data indicate that endocannabinoids interact with the noradrenergic system in influencing memory retrieval and that the noradrenergic system appears to be located downstream, at least functionally, from the endocannabinoid system. Several other studies have reported that systemic or local administration of cannabinoid analogs increases the release of norepinephrine in specific brain regions (Oropeza et al., 2005; Page et al., 2007; Carvalho and Van Bockstaele, 2012). Although the mechanism of how cannabinoids boost up the release of norepinephrine is not entirely clear, one possibility is a direct effect of cannabinoid analogs on the locus coeruleus (LC) (Mendiguren and Pineda, 2004; Mendiguren and Pineda, 2006; Muntoni et al., 2006). It has been shown that the administration of the CB1 reverse agonist SR141716A causes a significant reduction of spontaneous firing of LC neurons, suggesting that the LC might be under control of an endogenous cannabinoid tone.

Endocannabinoids also influence other neurotransmitter systems, for instance, the effects on glutamate and GABA are well described (Hashimotodani et al., 2007; Kano et al., 2009). Thus, another possibility is that endocannabinoids exert their influence on the noradrenergic system indirectly by first affecting other neurotransmitter systems. For instance, within the BLA and hippocampus, CB1 receptors are predominantly located on GABAergic terminals and activation of these receptors decreases GABA release (Ohno-Shosaku et al., 2001; Freund et al., 2003; Kano et al., 2009) via a rapid inhibition of Ca^{2+} influx into the terminals (Hoffman and Lupica, 2000; Wilson et al., 2001). Furthermore, as described above, it has been reported that the amygdalar GABAergic system modulates memory consolidation (McGaugh and Roozendaal, 2002) and that an inhibition of GABAergic activity within the BLA enhances memory consolidation by increasing the release of norepinephrine (Hatfield et al., 1999). In view of this evidence, it is

suggested that endocannabinoids can stimulate the release of norepinephrine by binding to presynaptically located CB1 receptors on GABAergic terminals, thereby inhibiting the release of GABA (Campolongo et al., 2009; Hill and McEwen, 2009; Atsak et al., 2012b). Collectively, these findings strongly suggest that the endocannabinoid system might mediate glucocorticoid effects on memory retrieval via an influence on the noradrenergic system.

Very few studies investigated the effect of glucocorticoids on recall of recognition memory. In contrast to the effects seen on retrieval of declarative and contextual memory, these studies reported small and nonsignificant effects of glucocorticoids on the retrieval of this kind of memory (de Quervain et al., 2000a; de Quervain et al., 2003; Buchanan et al., 2006). Therefore, in chapter 6, we investigated the effects of systemic corticosterone administration on the recognition of two different components of information acquired during object recognition training, i.e., memory of the object per se and memory of the location of the object. We found that corticosterone, administered 1 h before retention testing, impaired the retrieval of both aspects of object recognition memory. Studies on memory consolidation indicate that these two components of object recognition memory are processed by dissociable memory systems such that the representation of the object itself requires information processing within cortical regions, particularly the perirhinal and insular cortices, whereas memory regarding the location of the object within its context depends primarily on the hippocampus (Murray and Richmond, 2001; Bermudez-Rattoni et al., 2005; Roozendaal et al., 2010). Thus, it is possible that glucocoticoids might have impaired the retrieval of these two components of object recognition memory by acting onto different brain regions. However, as some lesion studies suggested also an involvement of the hippocampus in object recognition memory (Broadbent et al., 2004), the possibility that the hippocampus takes a role in regulating glucocorticoid effects on the retrieval of both components of recognition memory cannot be excluded at the present time. Importantly, we also demonstrated that systemic blockade of CB1 receptors with SR141716 prevented the impairing effects of glucocorticoids on retrieval of both aspects of recognition memory. These results suggest that the endocannabinoid system is essentially involved in regulating the impairment of retrieval of recognition memory, possibly by influencing different memory-related brain regions.

1.3. Comparison of the two models

The findings discussed thus far demonstrate that the endocannabinoid system is crucially involved in regulating glucocorticoid effects on both memory consolidation and memory retrieval. Glucocorticoids rapidly launches the activation of the endocannabinoid system in different brain regions including the hippocampus and amygdala (Hill et al., 2010), possibly through a nongenomic mechanism involving a Gs-coupled membrane-bound receptor (Di et al., 2003; Di et al., 2005a; Di et al., 2005b; Malcher-Lopes et al., 2006; Di et al., 2009). This increased endocannabinoid signaling might mediate the modulatory effects on both memory consolidation and memory retrieval via rapid interactions with the noradrenergic system. Thus, the described models for endocannabinoid regulation of glucocorticoid effects seem to be highly similar for memory consolidation and retrieval. However, although not explored in this thesis, there might be subtle but important differences. For example, depending on the brain region investigated endocannabinoids can affect neurotransmitter systems other than GABA. Both in the hippocampus and BLA, CB1 receptors are abundantly present in GABAergic and into a minor extent glutamatergic cells and the activation of CB1 receptors can modify the release of both neurotransmitters (Katona et al., 1999; Katona et al., 2001; Azad et al., 2003; Kawamura et al., 2006; Kano et al., 2009). The glucocorticoid-induced fast inhibition of the HPA axis is mediated by an endocannabinoid-dependent inhibition of glutamatergic inputs to CRF-releasing cells within the paraventricular nucleus (Tasker and Herman, 2011). Since we did not investigate the neurotransmitter systems mediating the endocannabinoid effects on memory consolidation and memory retrieval, it remains unknown whether glucocorticoid-induced changes in endocannabinoid signaling underlying memory consolidation and memory retrieval rely predominantly on GABAergic, glutamatergic or yet another neurotransmitter systems.

Additionally, some studies suggested that different endocannabinoid ligands might mediate different physiological processes. Thus another possible unexplored distinction between the two mechanisms can be the exact endocannabinoid ligand mediating the glucocorticoid effects on memory consolidation and memory retrieval. This suggestion originated particularly from studies indicating that endogenous AEA and 2-AG ligands are differently modulated by emotional

stimuli (Marsicano et al., 2002; Busquets-Garcia et al., 2011). Some evidence indicated that stress and glucocorticoids might induce different effects on AEA and 2-AG levels in brain regions like the amygdala, hippocampus and prefrontal cortex. For instance, a single episode of stress was reported to decrease AEA levels in the amygdala without changing 2-AG levels. Taking into account the anxiolytic effects of inhibitors of the AEA-catalyzing enzyme fatty acid amide hydrolase (Kathuria et al., 2003), the decline in AEA signaling that occurs in response to stress is taken to contribute to increased emotional and anxiety-like behaviors that accompany stress exposure (Hill and McEwen, 2010). However, a single glucocorticoid injection induces a different response pattern and rapidly elevates AEA levels without altering the 2-AG levels. These results are taken to suggest that glucocorticoids might be functioning to reinstate homeostasis after stress (Hill and McEwen, 2010).

Some studies also reported that AEA, rather than 2-AG, is implicated in the effects of cannabinoids on memory consolidation. For example, systemic pharmacological manipulation of AEA tone has been implicated in regulating the modulatory effects of endocannabinoids on memory consolidation (Costanzi et al., 2004; Busquets-Garcia et al., 2011). Moreover, rats trained on an IA task had higher levels of AEA in the BLA than rats that were only exposed to the IA context. Post-training infusions of the AEA-hydrolysis inhibitor URB597 into the BLA, increasing AEA levels, resulted in an enhanced memory consolidation of IA training (Morena et al, unpublished observation). Taken together, it is thus possible that AEA might be the major endocannabinoid mediating the memory-enhancing effects of post-training infusions of GR agonist (chapter 4). In contrast, in chapter 5 we reported that the administration of an retrieval impairing dose of corticosterone shortly before memory retrieval testing selectively increased hippocampal levels of 2-AG without affecting those of AEA. Thus, such findings suggest the intriguing possibility that AEA might be predominantly involved in regulating the enhancing effects of glucocorticoids on memory consolidation, whereas 2-AG might be more involved in regulating glucocorticoid effects on memory retrieval impairment. Further, it cannot be excluded that an involvement of AEA relative to 2-AG in regulating glucocorticoid effects on different memory functions is also highly dependent on the brain region investigated.

2. Other Mediators of Nongenomic Glucocorticoid Actions

The findings presented in this thesis show that endocannabinoids, released upon activation of a membrane GR, are an important regulator of nongenomic glucocorticoid actions on different memory processes. However, there are also other known mediators of nongenomic actions of glucocorticoids. A recent study reported that activation of membrane GRs evokes the release of nitric oxide, another retrograde messenger, from pyramidal cells in the hippocampus (Hu et al., 2010). This released nitric oxide diffuses back to the presynaptic membrane and rapidly induces the release of GABA from hippocampal interneurons and hypothalamic magnocellular neurons (Di et al., 2009; Hu et al., 2010). Glucocortiocoids also enhance glutamate transmission in hippocampal CA1 pyramidal neurons in the rat (Karst et al. 2005). Since this rapid steroid effect is eliminated by pharmacological blockade of mineralocorticoid receptors (MRs) and furthermore is lost in mice with a conditional knockout of the MR in the forebrain, but not of the GR (Karst et al., 2005; Olijslagers et al., 2008), this rapid corticosteroid effect is likely mediated by a direct activation of a presynaptic membrane-associated MR. This membrane-associated MR has a 10-fold lower affinity than the regular MR which means its occupation, like of the GR, is more prevalent during stress compared to basal conditions (Kloet 2000). Different from nongenomic membrane GR effects, this fast MR-mediated effect on glutamatergic transmission has been shown to be endocannabinoid independent (Karst et al., 2010).

Evidence is accumulating in support of a postsynaptically located MR mediating fast glucocorticoids effects on various aspects of AMPA receptor trafficking (Groc et al., 2008). AMPAs are ionotropic glutamate receptors that mediate the majority of fast excitatory transmission in the brain. They are heterotetramers, comprised of a combinatorial assembly of four subunits, GluR1–GluR4, in which GluA1 and GluA2 are essentially linked to the formation of emotional memories (Wisden and Seeburg, 1993; Hollmann and Heinemann, 1994). Glucocorticoids, via an MR-dependent mechanism, have been shown to facilitate AMPA trafficking, in particular, by increasing the membrane expression and synaptic insertion of GluA2 subunits (Groc et al., 2008; Krugers et al., 2010; Joels et al., 2011). Moreover, norepinephrine also targets AMPA receptors. Considerable evidence indicates that norepinephrine facilitates the phosphorylation of the AMPA subunit GluA1

by activating protein kinase A and calcium/calmodulin kinase II/ protein kinase C (Hu et al., 2007; Joels et al., 2011). It is known that phosphorylation of GluA1 promotes AMPA-receptor trafficking to synapses and contributes to the synaptic strengthening and the induction of long-term potentiation (Barry and Ziff, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003; Malenka and Bear, 2004). These effects of norepinephrine on AMPA signaling have been proposed to underlie the mechanism of how emotions enhance memory (Hu et al., 2007). Since glucocorticoids rapidly facilitate arousal-induced norepinephrine release, they can also exert indirect effects on AMPA signaling through norepinephrine. Future experimentation is required to further address these questions.

3. Convergence of genomic and nongenomic glucocorticoid effects

Although the nongenomic effects of glucocorticoids recently became in sight again (Dallman, 2005), in the classical perspective, glucocorticoids are broadly renowned for their genomic (delayed) effects. Glucocorticoids regulate gene transcription by activating MRs and GRs, functioning either as homodimers or heterodimers, interact at a glucocorticoid-response element and recruit corepressors or coactivators, whereas GR monomers interact with stress-induced transcription factors or other proteins to dampen their transcriptional activity. The first step that leads to their ultimate effect on adaptive behavior involves the altered expression of responsive genes. Recently, a study performed with large-scale gene-expression-profiling methods identified glucocorticoid-responsive genes in the hippocampus. The activation of MRs or GRs, mimicking the physiological conditions, resulted in waves of gene regulation, with both transactivation and transrepression of responsive genes (Datson et al., 2008). Although GR binds to genomic DNA autonomously, recent data show that the binding profile of GR is pre-determined by chromatin state and thus depends on the accessibility of the chromatin (John et al., 2011). These findings indicate that glucocorticoids coordinate the expression of high- and medium-abundant genes that underlie aspects of cell metabolism, structure and synaptic transmission and through these affects many processes in different brain regions.

Through activating GRs and through rapid interactions with arousal-induced norepinephrine, glucocorticoids activate the cAMP-dependent protein kinase (PKA) pathway and subsequently increase levels of pCREB (Roozendaal et al.,

2006a). As mentioned before, pCREB is highly linked to memory formation and changes the transcription of many genes. Glucocorticoids, via a nongenomic, CREB-dependent mechanism increase histone acetylation that leads to enhanced synaptic plasticity and long-term memory formation (Vecsey et al., 2007; Roozendaal et al., 2010). In chapter 4, we showed that a blockade of endocannabinoid signaling by a specific receptor antagonist prevents the corticosterone-induced pCREB activation in the BLA. These findings suggest that membrane GR-associated release of endocannabinoids activate pCREB, possibly via an interaction with norepinephrine, that might converge with the DNA-binding capacity of nuclear GRs, ultimately resulting in genomic effects of glucocorticoids.

Nongenomic and genomic actions can still interact or converge together even if they occur in different time frame, this eventually determines glucocorticoid effects to be highly time dependent. Based on the baseline activity, which means, whether there is already a modified state by genomic actions of glucocorticoids, the direction of glucocorticoid effects on certain cellular processes can be entirely opposite. For instance, corticosterone given immediately before the induction of long-term potentiation (LTP) stimulates synaptic strengthening (Wiegert et al., 2006), whereas corticosterone applied hours earlier inhibits the induction of the same type of LTP (Diamond et al., 1992; Pavlides et al., 1993). Thus, electrophysiological data suggested that after a stressful event corticosterone quickly enhances glutamatergic transmission in the hippocampus through nongenomic mechanism and later on reinstates cellular activity through genomic GR mechanims (Karst et al., 2010). Similarly, a human imaging study reported that hydrocortisone desensitizes amygdala responsivity during the processing of fearful facial expression in a rapid fashion, while it normalizes the response slowly in a time frame compatible with its genomic effects (Henckens et al., 2010).

Some evidence indicates that the outcome of interactions between glucocorticoids and norepinephrine can also be highly time dependent. Electrophysiological data suggest that if corticosterone is given together with isoproterenol (β-adrenoceptor agonist) as similar to the physiological condition during stress, it facilitates LTP in the hippocampus (Pu et al., 2007). However if corticosterone is given some hours prior to the isoproterenol, as it can occur in certain clinical conditions or with sustained glucocorticoid therapy, then it induces a suppression of the

β–adrenoceptor-induced facilitation of LTP, possibly because genomic GR effects already kick in(Joels et al., 2011). Collectively, the actual rapid signaling might help the organism to cope with the situation in a short time frame, subsequently the genomic effects will kick in to take over and restore the activity of the circuits to reach to homeostasis (Groeneweg et al., 2011).

4. Clinical relevance of the findings: Implications for post-traumatic stress disorder and phobias

Persistent traumatic memory, characterized by intrusive recollections and re-experiencing of highly stressful experiences, is a hallmark of stress-related disorders such as post-traumatic stress disorder (PTSD) (de Quervain et al., 2009). Changes in glucocorticoid signaling, like increased sensitivity of the GR (Hauer et al., 2011) and enhanced feedback of the HPA axis (Yehuda, 2009), have consistently been reported in patients with PTSD. Further, a low cortisol response to traumatic events might reflect a higher risk for the development of PTSD (Yehuda;, 1997). In line with these observations, clinical investigations reported that the administration of stress levels of glucocorticoids following trauma or in patients with established PTSD significantly reduces re-experiencing and other chronic stress symptoms (Aerni et al., 2004; Schelling et al., 2004; Schelling et al., 2006; Surís et al., 2010). Glucocorticoids also reduce retrieval of fear memory in patients with phobia and thereby preclude stimulus-induced fear (Soravia et al., 2006; De Quervain and Margraf, 2008). Collectively, glucocorticoids are suggested to exert beneficial effects in diseases related to memory, at least in part, by impairing the retrieval of trauma-related memories, but also by facilitating the extinction of stress-related memories (Bentz et al., 2010; de Quervain et al., 2011; Atsak et al., 2012a). Despite the potential value of glucocorticoids as treatment for PTSD and phobias, in a clinical setting, the sustained use of glucocorticoids is undesirable because of the pleiotropic nature of these hormones to affect a wide array of physiological functions (e.g., immune and metabolic functions). The findings presented in this thesis indicating that the endocannabinoid system mediates glucocorticoid effects on memory retrieval impairment could aid in the development of non-glucocorticoid–based therapies for PTSD. Although clinical studies have not yet investigated interactions between these two stress systems, recent findings indicate that administration of the synthetic cannabinoid nabilone

to PTSD patients resulted in a highly comparable reduction of treatment-resistant daytime flashbacks and nightmares (Fraser, 2009). Moreover, the possible positive outcome of treatments targeting the endocannabinoid system in PTSD has been also suggested by recent data showing altered levels of plasma endocannabinoid levels (AEA, 2-AG and Oleoylethanolamide and palmitoylethanolamide) in PTSD patients (Hauer et al. unpublished observations).

5. Conclusions and Future Directions

Glucocorticoids enhance memory consolidation for emotionally arousing experiences and impair memory retrieval during emotionally arousing test situations via rapid interactions with arousal-induced noradrenergic mechanisms. The overall conclusion of the part II of the thesis is that the endocannabinoid system is playing a crucial role in mediating such rapid glucocorticoid effects on the noradrenergic system. However, there remain many unanswered questions. First, decades of research substantially reproduced the findings of animal experiments that glucocorticoids, by means of interactions with arousal-induced noradrenergic activity, modulate memory also in humans (Wolf, 2008; de Quervain et al., 2009). It is crucial to perform experiments addressing the role of the endocannabinoid system in glucocorticoid-induced effects on human memory. In chapter 6, we showed that systemic injections of a CB1 receptor antagonist prevented the impairing effects of glucocorticoids on recognition memory in rats. Future experiments that employ a similar design and drug treatment will be invaluable to compare the role of the endocannabinoid system in regulating glucocorticoid effects on memory across species.

Second, although similarities between the mechanisms of how endocannabinoids mediate glucocorticoid effects on consolidation and retrieval have been addressed in chapter 4 and 5, future experiments dissecting the possible differences between the two models will be needed. For example, there seems to be a possible functional distinction in the endocannabinoid ligands mediating the modulatory effects of glucocorticoids on memory consolidation versus memory retrieval. As mentioned before, AEA has been more implicated in memory consolidation, whereas we reported in chapter 5 that 2-AG appears to be more linked to the effects of glucocorticoids on memory retrieval. Future research explicitly addressing the involvement of different endocannabinoid ligands in

mediating glucocorticoid effects on different memory processes will be required.

Third, although several findings have demonstrated that endocannabinoids modulate noradrenergic transmission, it is unclear whether this is actually achieved indirectly by influences on GABAergic or glutamatergic transmission, or by a direct influence on the noradrenergic transmission. Future experiments examining the effects of glucocorticoids in conditional mutant mice lacking CB1 expression specifically in glutamatergic or GABAergic neurons (Marsicano and Lutz, 1999) and as well as experiments focusing on the mechanism of how manipulation of endocannabinoid signaling relates to direct changes on noradrenergic transmission will be essential.

Another crucial question is the role of emotional arousal in regulating glucocorticoid-endocannabinoid effects on the noradrenergic system. Emotional and non-emotional memory seems to be differently modulated by endocannabinoid system activation (Akirav, 2011) and endocannabinoids exert opposite effects depending on the baseline arousal level of the organisms (Campolongo et al. unpublished observations). Moreover, in our experiments we only demonstrated the recruitment of endocannabinoids by glucocorticoids to affect noradrenergic activity under arousing conditions, hence, it would be necessary to investigate whether glucocorticoids also recruit the endocannabinoid system under low-arousing experimental conditions.

Although speculative at this point, considering the alteration of circulating endocannabinoid levels in PTSD patients (Hauer et al., unpublished observations), it would be interesting to investigate whether the achieved benefits of glucocorticoid treatment in this patient group are also mediated by changes in endocannabinoid signaling. And last but not least, investigating whether the known disturbances in glucocorticoid signaling in PTSD patients are actually linked to changes in the endocannabinoid system would be valuable to understand the dynamics between these two systems as well as to develop alternative treatments targeting this interaction in pathological conditions.

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Conclusion

CONCLUSION

Stress can exert rapid as well as delayed effects at many different levels of cognition. This thesis explored the effects of stress on social cognition and memory in rats. The first part investigated the role of social communication between conspecifics in stress transmission and demonstrated that the display of distress behavior by a rat to a stressor (footshock) can induce an immediate stress reaction in another, observing rat. In particular, this part pointed out that this emphatic response in an observing animal requires prior experience with the same stressor. The underlying neuronal mechanism and the sensory modality that conveys the information between conspecifics and the contribution of various factors such as familiarity, similarity, gender of the animals and salience of the stimulus remains to be explored in future experiments. The second part examined the neurobiological mechanisms underlying the effects of stress hormones on different memory phases. The experiments of this part demonstrated that the lipid-based endocannabinoid system plays a crucial role in mediating the rapid actions of glucocorticoids on arousal-induced noradrenergic activity that are necessary for enhancing the consolidation of memory as well as impairing the retrieval of memory for emotionally arousing experiences. Future experiments dissecting the role of particular endocannabinoid ligands in mediating glucocorticoid effects on memory consolidation and retrieval as well as the role of the endocannabinoid system in regulating glucocorticoid effects on memory across species, in particular, in humans will be needed.

Samenvatting

Stress kan op velerlei manieren cognitie beïnvloeden. In dit proefschrift heb ik deze stresseffecten binnen twee verschillende cognitiedomeinen onderzocht. In hoofdstuk 2 beschreef ik dat sociale communicatie tijdens dreigend gevaar een belangrijke rol speelt bij het doorgeven van stresssignalen van de ene op de andere rat. Dit gebeurde echter alleen als beide ratten ervaring hadden met de stressor en niet als de observerende rat naief was met betrekking tot de stress-stimulus. De resultaten van dit onderzoek kunnen dienen als voorbeeld voor het concept van stress op sociale cognitie en empathie bij ratten. In het tweede gedeelte van dit proefschrift onderzocht ik hoe stress verschillende aspecten van het geheugen kan beïnvloeden. Het is algemeen bekend dat stressvolle en emotionele gebeurtenissen beter onthouden worden dan alledaagse gebeurtenissen, terwijl acute milde stress juist het oproepen van eerder aangeleerde emotioneel-gekleurde informatie uit het geheugen vermindert. Uitgebreid onderzoek heeft aangetoond dat beide effecten het gevolg zijn van de werking van hormonen die vrijkomen uit de bijnieren tijdens stress. In hoofdstuk 3 heb ik een overzicht gegeven van de complexe manier waarop een van de belangrijkste stresshormonen, glucocorticoïden (cortisol bij de mens), het geheugen beïnvloeden. Glucocorticoïden komen vrij uit de bijnierschors tijdens stress en verbeteren de consolidatie en opslag van nieuwe informatie in het geheugen terwijl ze tegelijkertijd het oproepen van eerder opgedane ervaringen uit het geheugen verminderen. Glucocorticoïden kunnen op twee manieren hersenactiviteit, en dus hersenfuncties, beïnvloeden. Enerzijds is er een langzame weg die afhankelijk is van transcriptionele activiteit op genniveau. Het duurt daarom enige uren voordat deze effecten zichtbaar worden. Anderzijds zijn er ook snelle niet-genomische effecten (waarbij geen sprake is van transcriptionele activiteit) die al na een paar minuten zichtbaar worden. Ondanks het feit dat verschillende van deze glucocorticoïd effecten op het gedrag en geheugen afhankelijk zijn van deze snelle weg, is het mechanisme daarvan niet goed begrepen. Wel is bekend dat de snelle effecten van glucocorticoïd-hormonen afhankelijk zijn van noradrenaline, een ander stresshormoon, dat vrijkomt in de hersenen tijdens emotionele gebeurtenissen. Maar hoe glucocorticoïden een interactie aangaan met noradrenaline is niet bekend. Uit recent onderzoek blijkt dat het endocannabinoïdsysteem, een snelwerkend lipide-systeem in de hersenen, medeverantwoordelijk is voor het bewerkstelligen van een aantal van deze snelle glucocorticoïd effecten. Op dit moment zijn meer dan twaalf

verschillende endocannabinoïden beschreven maar specifieke functies voor elk van hen zijn onbekend. De aanmaak van verschillende endocannabinoïd-liganden wordt aangewakkerd door neuronale depolarisatie, een toename in intracellulaire calciumconcentraties, en activering van verschillende metabotrope en excitatoire ionotrope neurotransmitter receptoren. Eenmaal vrijgekomen binden endocannabinoïden zich aan CB1 en CB2-receptoren. CB1-receptoren zijn in zeer grote dichtheden aanwezig in het centrale zenuwstelsel. Stress en glucocorticoïden kunnen de activiteit van verschillende van deze endocannabinoiden beïnvloeden. Zo is het bekend dat glucocorticoïden endocannabinoïd concentraties in de hypothalamus verhogen en op deze wijze de hypothalamus-hypofyse-bijnier-as activiteit afremmen. Deze resultaten zijn belangrijk omdat ze het eerste bewijs leverden voor een rol van het endocannabinoïdsysteem in het mediëren van snelle glucocorticoïd effecten.

In dit proefschift heb ik onderzocht of endocannabinoïden ook betrokken zijn bij de effecten van glucocorticoïden op de consolidatie en oproepen van het geheugen. In hoofdstuk 4 toonde ik aan dat endocannabinoïden een belangrijke rol spelen bij de effecten van glucocorticoïden op het verbeteren van de consolidatie van geheugen. De voornaamste conclusie van dit onderzoek is dat glucocorticoïden via het endocannabinoid systeem noradrenerge activiteit binnen het basolaterale complex van de amygdala (BLA), een belangrijk hersengebied voor de regulatie van stress effecten op leren en geheugen, beïnvloeden. Verder heb ik kunnen aantonen dat activiteit van het endocannabinoidsysteem noodzakelijk is om de effecten van glucocorticoïden op neurale plasticiteit van het BLA te bewerkstelligen. Zoals reeds hierboven beschreven, kan blootstelling aan stress of de toediening van glucocorticoid hormonen kort voor een retentietest juist het oproepen van herinneringen uit het geheugen verminderen. In hoofdstuk 5 onderzocht ik daarom een mogelijke betrokkenheid van het endocannabinoïdsysteem in deze glucocorticoïd-geïnduceerde verslechtering van het oproepen van informatie uit het geheugen. Ratten werden getraind op een contextuele angsttaak (contextual fear conditioning), waarbij ze leerden dat ze een electrische schok konden krijgen in een bepaald apparaat (= context). Systemische toediening van corticosteron, het belangrijkste glucocorticoïd-hormoon in de rat, een uur voor de retentietest verminderde het oproepen van het geheugen voor deze contextuele angsttaak, zonder rechtstreeks de expressie van angstgedrag te veranderen. Belangrijk

is dat een blokkade van CB1 receptoren in de hippocampus dit corticosteron effect volledig blokkeerde. Deze resultaten laten dus zien dat activiteit van het endocannabinoïdsysteem noodzakelijk is voor het mediëren van deze corticosteron effecten op het oproepen van emotioneel geheugen. Verder vond ik dat de toediening van corticosteron resulteerde in een verhoging van de spiegels van 2-arachidonoylglycerol, een van de meest voorkomende endocannabinoiden, in de hippocampus. Lokale toediening van WIN55,212-2, een agonist voor CB receptoren, in de hippocampus resulteerde, net als bij corticosteron, in een vermindering van het oproepen van contextueel geheugen. Ook hier lijkt de samenhang tussen glucocorticoïden en endocannabinoïden uiteindelijk uit te monden in een verandering van noradrenerge activiteit. Ik heb namelijk kunnen vaststellen dat antagonisme van ß-adrenerge activiteit in de hippocampus door lokale toediening van propranolol het effect van de CB-receptor agonist volledig blokkeerde.

Hoewel de effecten van glucocorticoïden op een vermindering van het oproepen van hippocampus-afhankelijk ruimtelijk en contextueel geheugen vaak zijn beschrijven, lijken glucocorticoïden veel minder effectief te zijn in het beïnvloeden van het oproepen van recognitiegeheugen, een vorm van geheugen dat onafhankelijk is van de hippocampus. Daarom heb ik in hoofdstuk 6 onderzocht of de toediening van corticosteron het oproepen van verschillende soorten informatie met betrekking tot een object-recognitietaak kan beïnvloeden. Corticosteron toediening kort voor de retentietest resulteerde in een vermindering van de herkenning van zowel het object zelf als de plaats waar dit voorwerp zich bevond tijdens de training. Dit experiment laat dus zien dat corticosteron mogelijk een veel algemener effect heeft op het oproepen van informatie uit het geheugen dan tot nu toe werd aangenomen. Aangezien de resultaten van hoofdstuk 5 lieten zien dat het endocannabinoïdsysteem betrokken is bij de effecten van glucocorticoïden op het oproepen van geheugen, heb ik verder onderzoek gedaan naar de betrokkenheid van endocannabinoïden in de effecten van glucocorticoïden op het ophalen van deze twee componenten van objectherkenning en vond dat systemische toediening van de inverse CB1-agonist SR141716 1 uur voor aanvang van de retentietest de effecten van corticosteron volledig blokkeerden.

Stress kan dus zowel snelle als langzame effecten uitoefenen op veel verschillende aspecten van cognitie. Dit proefschrift onderzocht de effecten van stress op sociale cognitie en geheugen bij ratten. Het eerste deel toonde aan dat uitingen van angst tijdens dreigend gevaar kunnen leiden tot een onmiddellijke stressreactie in een andere observerende rat, maar alleen als deze ervaring had met diezelfde stressor. Het onderliggende neuronale mechanisme, de sensorische modaliteit (gehoor, reuk, zicht et cetera) dat betrokken is/zijn bij de sociale transmissie van angst alsmede de rol van diverse andere factoren zoals geslacht van de dieren en saillantie van de stimulus moeten nog worden onderzocht. Het tweede deel toonde aan dat het endocannabinoïdsysteem een cruciale rol speelt bij het reguleren van de snelle effecten van glucocorticoïden op zowel de verbetering van de consolidatie van nieuwe informatie in het geheugen als de vermindering van het oproepen van bestaande herinneringen uit het geheugen. Toekomstige studies zullen moeten onderzoeken in hoeverre specifieke endocannabinoïd liganden een rol spelen bij deze diverse glucocorticoïd effecten. Verder zal moeten onderzocht worden of het endocannabinoid net zo'n een voorname rol speelt in de regulatie van stress effecten op het menselijk geheugen.

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2011 Travel Grant: IBRO 750 €
Travel Grant: NIDA 625 $

Acknowledgment

When I came here, I thought getting my title would be the most important achievement in this small town however Groningen offered me much more special experience. I want to thank to all those, no matter what, who made it special, who supported me by sharing their personal and proffessional experiences, who invited me to their outer and inner layers, who opened different horizons, who proved eventually life as well as PhD is what you make out of it!

Benno Roozendaal, I would like to thank you for your excellent mentoring. Over the 2.5 years of time, I enjoyed everyday of my work with you. It was fun, acive/ interactive, motivating, constructive and sometimes critical, overwhelming. Overall there is no one element or advice that did not benefit me. I'd like to thank also for your effort to make my working performance efficient in the limited time by listening to the plenty of problems that distracted me. I'd like to thank for taking my disagreements with a very constructive way. I also appreciate your social mentoring and your encouraging support for establishing new collaborations. Every year, hosting so many well-known researchers in Groningen was also a very valuable contribution to my PhD time. As the last but not the least, I appreciate the lab atmosphere and environment and learning and memory team that you created, I have been delighted to work in such a wonderful and supportive team and certainly that made it possible for me to create 4 chapters in 2.5 years of time. Thank you for showing me science is not only about papers but it is about how much you enjoy and have fun while producing them.

Christian Keysers, I would like to thank for your long-run and effortful mentoring. I think we have had quite some challenges to get over and scientific challenges were not the biggest ones sometimes. When the project was not going smoothly and people started to doubt whether we could ever come up with this 'rat empathy model' and actually since then some left the project, you have been the one that backed me up and gave me full support. I also appreciate for your support after my detachment from the project. Although as time passed, we had up and downs, many scientific and some personal discussions, disagreements and etc, I really felt you wanted me and supported me to make it and you, somehow, knew that I'd eventually make it. I appreciate the most your belief and trust in me. That is, I believe one of the reasons that carried me to

the end of my PhD!

I'd also like to thank to my collaborators: Marta Moita and Valeria Gazzola as well as Christel Westenbroek for their efforts in producing empathy chapter. I thank to Gustav Schelling, Patrizia Campolongo and Matthew Hill for their efforts, mentoring and support in my personal development and in the production of chapter 3, 4 and 5. I appreciate Gerry Hoogenberg for her efforts in arranging complicated issues about finances associated with my position and project as well as her mentoring over my rights and well-being, your help certainly have made it possible for me to finalize my PhD here.

I have been hosted by a lab, created by wonderful people, here my words for my lab buddies, my dear friends (alphabetic order):

Areg, my Abbott buddy! The night bird! It had been depressing to work at nights in the lab before you came in. When I was about to fall asleep in the surgery room, you always backed me up with your energy and positive attitude. Working with you or sometimes rather talking to you have been a great pleasure. I appreciate your help and I'm grateful for your time to train me in infusion methods. No matter how much work you had, you always offered me help and enjoyable long conversations.

Daniela, I have enjoyed your company a lot in Groningen but also in Munchen, Chicago, Rome and all the other places that we have been together! It has been a great pleasure to know you and work with you. Our endless paper is finishing soon (I know you don't believe me and I don't blame you). We reached many times to the point of desperacy and we totally lost our beliefs in finishing it but here finally I am telling you and this time for the last time, IT IS DONE! Thank you for your great friendship and opennes, fairness as well as for all the fun particularly in Octoberfest. I appreciate all your efforts in our work and friendship.

Fany, my dear paranym, my complain wall, my ice-tea mate! Thank you for listening me all those hours when I popped into your office on my walkway and occupied you in your busiest times. We are a good example of how opposite sides

attract each other. I enjoyed our conversations a lot, although we did not share the same ideas sometimes, this never discouraged us to share more, in reverse it created more closeness. Thank you for deep talks, ice teas, fun&dance and being my tough side sometimes :)

Erika, you are the one whom one can share many gradients of life! You can make the bad good and make the good best, ay aman! You have been my antidepressant and a neutralizer buddy! I enjoyed everything that I have done with you including 17 hrs surgeries (seriously). I'm grateful for dozens of hours of sharings about science and life and countless hugs and laughters :) Words will not be enough to express how much you I appreciate your support in the last 6 months, I said many times but I'll say it again, I could not have made it without you, girl!

Raquel, I thank you a lot for the warm-blooded latino attitude you brought into lab. Thank you for all enjoyable talks, great coctail parties and of course brazilian Bbq and all the fun! We miss you Brazilian but coming soon!!!

Romy, my office-mate, cdp unit-mate, surgery-mate, perfusion-mate, yes my mate, 6 years we have been here: Back to back, face to face, side to side. We had up and downs, ins and outs, depressed and motivated times... It has been tough but we always found a way out, we had a great rythm to fix the other! Everything has changed a lot including us and we struggled together to adapt to all these changes. Interestingly we always believed in each other more than oneself. I always wanted this day to come to tell you: whatever happened and no matter what: we have made it, we started and finished it! When you highlighted how stubborn I am, you should not have spared yourself. Let me be the first one to congratulate you for your title, girl!

Hassiba, my other dear office-mate, I want to thank you for long talks, for giving me hugs every morning when I came into office, feeding me when I forgot to eat the whole day, thank you for worrying for me and caring about me. I also appreciate the delicious dinners and offering help always. You are one of those who never stopped trying to relieve me when I was under pressure and definitely a good friend who never gave up on me no matter how cranky I was sometimes :)

Jelle (de Boer), thank you for all the techical help you provided me. I appreciate your hard work for all those months that you have been working days/nights and weekends with me. Also thanks for fun talks, I wish you good luck with your career and I hope you'll find the path that you desire and wish.

Jelle (Kleijn), well it was not so many days that we worked together but it was quite fun and fruitful. Thank you for being so kind to help me and train me in microdialysis when you were as busy as hell to run your final experiments for your own PhD. The time you spent with me is very deeply appreciated! And one day we should finally go to kite-surf together :)

Marie, I appreciate all the work that you did for the emapthy chapter. All in all, I hope it contributed to your passion in science.

I also would like to thank to our former technicians: Angelika Jurdzinski, Petra Bakker, Ellie Eggens-Meijer for their invaluable help and to former Social brain lab members, though my presence was short in the lab, I appreciate Marc, Luca, Marleen, Jojanneke, Harma for not minding to listening to my empathic rat experiments as well as to the rest part of the Anatomie, in particular to Ineke van Zanten-Timmer, Janniko Georgiadis, Peter Gerrits and Suzanne Quinten for long-run support and interest in my work.

To CDP team:
We complained a lot at the beginning and after years of effort to make things better for us, maybe we failed to show our appreciation. Here, I'd like to thank to Alex and Flip for their friendly attitude and for spending plenty of time to solve our problems, you provided us a great support to run our experiments smoothly. Thanks to Hester for long front door talks and always for fixing late, early, wrong orders for me, to Arie for teaching me many things and being so kind in approving my last minute submitted workprotocols, to Ester and Linda for spending a lot of time to provide me the information about animals. I know I gave a lot of trouble to you by asking sometimes 5 times a day: how many animals used, how many left, transfer from one dec to other, it was tough for me as well but one reason why doing the welfare evaluation for my group was not a total burden was because I had a great support from you. I'd also like to thank to our

welfare officer Catherine Thuring for putting extensive effort in improving the quality of our methods and experiments as well as for her friendly attitude and always welcoming us whenever we had a problem or question. Thanks to all our animal care takers for trying to realize all our weird wishes about cage change time, checking time, moving the animals around and etc. I want to highlight Natascha, in particular for her effort in helping us for abbott experiments, we could not have done it without you and I'd like to thank to Annemieke who sorted out a lot of mess regarding housing the animals, labels and etc. and to Ar for always finding time to sort out issues for us.

My dear friends:
Ana, my dear, my dance buddy, my hug giver! Thanks for always having time for me! I am blessed to have you! You are special - heey woman- :) roger that?

Peter, my weird friendly mate! Sometimes we cheer up and sometimes we keep quiet, sometimes when one does not find meaning talking to a stranger, one finds the one who transforms into a best buddy in a matter of seconds. Thanks for being the one who takes the one as it is, no matter how weird, how demanding and how ridicoulus it is!

Katharina&Vibor, thanks for having me insistently in the Serbian&Crotian parties and activities, even that meant for you guys to speak in english all day/ night to integrate me into the group. And thanks to you, Kaca, for convincing me that there is a life after 30!

Goda&Oof, I always felt that I don't need to talk to you guys in order to express myself. You offered me a great comfort, positive atmosphere and deep looks. I appreciated all the fun you brought into the raki parties, I can't think of having parties wihout you guys. And with you Goda, I think we shared one of the most interesting experience, though we will never know whether they will eventually build this student house in Groningen, if they do, we will know how it was decided and planned :), also thanks for giving me plenty of hugs, knowing that I need it but I could not say it...

Elena, we met late but you know what they say - better late than never - we

compensated and will compensate more!

Astro group, no, I did not forget you guys: Simona, Leon, Rajat, Matteo, Gianni, Panos, Laura who brought a lot of colours into my first Groningen years. Thanks for dinners&drinks and broadening my astrophysics knowledge dramatically (it eventually somehow helped in Neuroscience) and sorry for destroying your proffesional talks with my neuroscientific background. I'd never understand how brain looks similar to a galaxy without you guys!

Liga&Gert Jan, thanks for all the fun activities you got me into! Sushi nights, ice-skating, dance&music, bbq, the koffer and many other things. It has been great to know you guys, I really enjoyed everything that we have done together. To be continued in Nijmegen...

I'd also like to thank to Ramona, Katharina Goerlich, Kaca, Gerry, Marc, Nadir, Julien, Carlos for coloring my smoking time with enjoyable conversations.

And of course, thanks to my dear dutch teacher, Arianne, who through teaching dutch, listened to my life, my ups and downs, my hopes, excitements and desperacies! Thanks for great home-made cappuccinos and proof-reading of my dutch summary!

As last but not least:
Annem - Beni kimsenin sevmediği gibi seven, kimsenin bilmediği gibi bilen sana,
Sinan - Arafta ve cümbüşte, bulan ve düşte olan taraflarımı bilen sana,
Babam - Beni olmak istediklerim icin degil, ben olduğum için destekleyen sana,
Berfu - Her rengime kucak açan, senin de benim ödülüm olduğunu bilen sana,
Abime - Her şeyin biz de başlayıp, biz de bittiğini bilen sana,
Sanem - Paylaşmanın sınırları olmadığını bilen sana,
Ebru - Hayatın her şeye rağmen devam ettiğini bilen ve öğreten sana,
Araz - Her yolun sonunun insanın kendisine çıktığını bilen sana,
Devrim (Kaba) - Güvenilmenin sınırları olmadığını bilen sana,
Hayriye –Mesafelerin paylaşıma yenilmediğini bilen sana,
Hıdır – Zamanin paylaşıma yenilmediğini bilen, ben gelemedikçe bana gelen sana,
Ve ayrıca paard müdavimi diğer canım arkadaşlarım Can, Ozlem, Ozan, Nadir,

Devrim, Onur, Eda, Volkan, Burcu, Umut ve sana Kanat, keyifli anlar, uzun sohbetler, yemekler, partiler, gezmece-tozmaca, paylaşımlarınız, öğretileriniz, desteğiniz, kısaca hayatıma getirdiğiniz bütün renk ve derinlikler için minnettarım.

And my final words is for you, the only one that I evolved together over all these years, the one I shared the happiness and sadness, the one who knows my deepest layers, the one who strenghtened me, the one that inspired me, the one who maybe deserves the biggest credit for this work after me. It has been tough sometimes but I believe, we have had great mutual support and inspiration through our countless hours of talks and love. Many things happened and we have changed, our lives goes into different directions now and unfortunately we can't adapt as fast as we wish we would always. What remains the same, however, is what I appreciate the most. Thank you Leonardo, particularly for showing me sometimes it is necessary to stand at the top of the cliff and jump off, and build your wings on the way down!

Piray
St. Petersburg/Antalya/Groningen
August 2012