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Endocannabinoids mediate acute fear adaptation via glutamatergic neurons independently of corticotropin-releasing hormone signaling

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Recent evidence showed that the endocannabinoid system plays an important role in the behavioral adaptation of stress and fear responses. In this study, we chose a behavioral paradigm that includes criteria of both fear and stress responses to assess whether the involvement of endocannabinoids in these two processes rely on common mechanisms. To this end, we delivered a footshock and measured the fear response to a subsequently presented novel tone stimulus. First, we exposed different groups of cannabinoid receptor type 1 (CB_1)-deficient mice ($CB_1^{-/-}$) and their wild-type littermates ($CB_1^{+/+}$) to footshocks of different intensities. Only application of an intense footshock resulted in a sustained fear response to the tone in $CB_1^{-/-}$. Using the intense protocol, we next investigated whether endocannabinoids mediate their effects via an interplay with corticotropin-releasing hormone (CRH) signaling. Pharmacological blockade of CB_1 receptors by rimonabant in mice deficient for the CRH receptor type 1 ($CRHR1^{-/-}$) or type 2 ($CRHR2^{-/-}$), and in respective wild-type littermates, resulted in a sustained fear response in all genotypes. This suggests that CRH is not involved in the fear-alleviating effects of CB_1. As $CRHR1^{-/-}$ are known to be severely impaired in stress-induced corticosterone secretion, our observation also implicates that corticosterone is dispensable for CB_1-mediated acute fear adaptation. Instead, conditional mutants with a specific deletion of CB_1 in principal neurons of the forebrain (CaMK-$CB_1^{-/-}$), or in cortical glutamatergic neurons (Glu-$CB_1^{-/-}$), showed a similar phenotype as $CB_1^{-/-}$, thus indicating that endocannabinoid-controlled glutamatergic transmission plays an essential role in acute fear adaptation.

Keywords: CB_1, corticosterone, CRF, CRH, endocannabinoids, extinction, HPA axis, rimonabant, SR141716, stress

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Endocannabinoids are fatty acid derivatives that exert their effects on emotional and motivational behavior, cognition, pain perception and neuroprotection primarily via central cannabinoid receptor type 1 (CB_1) (Piomelli 2003). They are synthesized and released on demand from postsynaptic sites and act as retrograde messengers at presynaptic terminals where they activate CB_1 and thereby suppress neurotransmitter release (Wilson & Nicoll 2002). CB_1 is expressed by different neuronal subpopulations including GABAergic and glutamatergic neurons (Marsicano & Lutz 1999). Among other functions, CB_1 was shown to mediate extinction of conditioned fear (Chhatwal *et al.* 2005; Marsicano *et al.* 2002; Suzuki *et al.* 2004), whereby it seems to be involved in habituation-like processes (Kamprath *et al.* 2006). The latter observation strikingly resembles the findings of Hillard and coworkers (Patel *et al.* 2005), who showed that endocannabinoids mediate habituation to homotypic stressors. Based on these similarities, we hypothesize that a common mechanism underlies endocannabinoid-mediated fear and stress adaptation.

Recently, Patel and Hillard (2008) proposed a mechanism for endocannabinoid-mediated stress habituation, which centrally involves the modulation of glutamatergic signaling. Upon repeated exposures to restraint stress, which results in habituation of the behavioral response, levels of the endocannabinoid 2-arachidonoyl glycerol (2-AG) in cortical brain regions were found to be increased (Patel *et al.* 2005; Rademacher *et al.* 2008). Cortical glutamate efflux, in contrast, was shown to decrease under similar circumstances (Moghaddam 2002). 2-arachidonoyl glycerol-mediated activation of CB_1 located on glutamatergic terminals might explain the decrease in glutamate signaling. Thus, it is conceivable that unrestrained glutamate release may also account for the sustained fear responses observed in CB_1-deficient mice (Kamprath *et al.* 2006; Marsicano *et al.* 2002), particularly if one considers the importance of glutamatergic transmission in the regulation of defensive behavioral responses (Millan 2003; Nordquist *et al.* 2008).

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In addition to glutamate, corticotropin-releasing hormone (CRH) was described to control behavioral stress coping, including fear and anxiety, via the hypothalamic–pituitary–adrenal (HPA) axis and/or via extrahypothalamic brain areas by activating CRH receptor type 1 (CRHR1) and type 2 (CRHR2) (for reviews, see Bale & Vale 2004; Keck *et al.* 2005; Steckler & Holsboer 1999). Interestingly, both CRH (Cota *et al.* 2003, 2007) and CRHR1 (Hermann & Lutz 2005) colocalize with CB_1 in hypothalamic and extrahypothalamic brain areas, suggesting that CB_1 may inhibit CRH signaling, accounting for the exaggerated fear responses observed in CB_1-deficient mice (Marsicano *et al.* 2002). In addition to CB_1 influencing CRH signaling, corticosterone itself can potently activate the endocannabinoid system in the hypothalamus (Di *et al.* 2003). This, in turn, inhibits glutamatergic afferences, thereby constraining HPA-axis activity and further corticosterone release.

Taken together, likely candidates in a common mechanism for endocannabinoid-mediated adaptation of fear and stress responses include glutamatergic transmission, CRH and the HPA axis with the possibility for multiple interdependences. Noteworthy, the extent of HPA-axis activation is determined by the intensity of the stressor (Armario *et al.* 1986; Hennessy & Levine 1978; Hennessy *et al.* 1979), which parallels recent findings in the endocannabinoid system, namely that the anxiolytic-like actions of endocannabinoids and their effects on stress-coping behavior seem to depend on the aversiveness of the test situation (Haller *et al.* 2004; Naidu *et al.* 2007). However, it remains to be shown that endocannabinoid-mediated fear adaptation is characterized by a similar dependency.

In the present study, we investigated the impact of the aversiveness of the previously encountered stressful experience on the involvement of endocannabinoids in fear adaptation. To this end, we applied inescapable footshocks of different intensities to different groups of CB_1-null mutant mice and their wild-type littermates and measured their fear responses to a tone on the following day. The most effective protocol was then applied to rimonabant-treated CRHR1-deficient (Timpl *et al.* 1998) and CRHR2-deficient (Coste *et al.* 2000) mice to test the hypothesis that an interplay of the endocannabinoid system and CRH signaling is responsible for the sustained fear response observed in animals with impaired CB_1 signaling. Finally, we investigated which neuronal subpopulation expressing CB_1 is involved in the previously observed phenotype by means of conditional mutants lacking CB_1 expression either in principal neurons of the forebrain (CaMK-CB_1; Marsicano *et al.* 2003) or in cortical glutamatergic neurons (Glu-CB_1; Monory *et al.* 2006). The specific deletion of CB_1 in CaMK-$CB_1^{-/-}$ affects, among others, glutamatergic and GABAergic projection neurons of cortical and subcortical brain structures, including the hypothalamus (Marsicano *et al.* 2003). Consequently, in the forebrain of these mice, expression of CB_1 is largely constricted to GABAergic interneurons. Glu-$CB_1^{-/-}$, in contrast, affects a much lower number of neurons because these mice lack CB_1 specifically in cortical glutamatergic neurons, thus maintaining CB_1 expression in subcortical brain structures (including the hypothalamus; Monory *et al.* 2006). If CB_1, indeed, mediates its fear-alleviating effects via restriction of cortical glutamate release, the phenotype of the two conditional mutant lines should resemble that observed in conventional CB_1 knockouts with germ-line deletion of the *CB_1* gene.

Materials and methods

All experiments were approved by the Committee on Animal Health and Care of the State of Bavaria (Regierung von Oberbayern, Germany) and performed in strict compliance with the European community recommendations for the care and use of laboratory animals.

Animals

Male mice at the age of 8–14 weeks were used in all experiments. All mutant mice and their respective wild-type littermate controls were generated and genotyped as described previously ($CB_1^{-/-}$, $n = 61$ and $CB_1^{+/+}$, $n = 68$: Marsicano *et al.* 2002; CaMK-$CB_1^{-/-}$, $n = 13$ and CaMK-$CB_1^{+/+}$, $n = 14$: Marsicano *et al.* 2003; Glu-$CB_1^{-/-}$, $n = 14$ and Glu-$CB_1^{+/+}$, $n = 10$: Monory *et al.* 2006; $CRHR1^{-/-}$, $n = 14$ and $CRHR1^{+/+}$, $n = 14$: Timpl *et al.* 1998; $CRHR2^{-/-}$, $n = 14$ and $CRHR2^{+/+}$, $n = 18$: Coste *et al.* 2000). All CB_1-mutant mice (CB_1, CaMK-CB_1 and Glu-CB_1 mice) were backcrossed to C57BL/6NCrl for six generations. The $CRHR1^{-/-}$ and $CRHR1^{+/+}$ mice were originally generated by Timpl *et al.* using 129/Ola and CD1 mouse strains (see Timpl *et al.* 1998 for detailed description) and not backcrossed to any mouse strain but maintained by means of heterozygous breeding pairs. The $CRHR2^{-/-}$ and $CRHR2^{+/+}$ mice (courtesy of M. Stenzel-Poore, Oregon Health & Science University, Portland, OR, USA) were backcrossed to C57BL/6J for four generations. All conventional mutant mouse lines ($CB_1^{-/-}$/$CB_1^{+/+}$; $CRHR1^{-/-}$/$CRHR1^{+/+}$ and $CRHR2^{-/-}$/$CRHR2^{+/+}$) were maintained by heterozygous breeding pairs. Only homozygous male offspring were used for the experiments. Conditional mutant mouse lines (CaMK-$CB_1^{+/+}$/CaMK-$CB_1^{-/-}$ and Glu-$CB_1^{+/+}$/Glu-$CB_1^{-/-}$) were maintained by breeding pairs consisting of Cre(−)xCB$_1$fl/fl (i.e. wild-type) mothers and Cre(+)xCB$_1$fl/fl (i.e. knockout) fathers to avoid effects of the genetic modulation on maternal care. For the experiments, the male offspring were taken from different breeding pairs with respect to closely matching birth dates (the maximum variation among birth dates was 6 weeks). Because most litters do not contain equal numbers of male mutant and wild-type offspring, most breeding pairs did not contribute equal numbers of mutant and wild-type animals to the experimental groups. However, care was taken that at least one wild-type littermate was tested together with each mutant mouse and vice versa.

All animals were reared at the animal facilities of the Max Planck Institute of Psychiatry, Munich, Germany. Animals were single housed under an inverse 12 h:12 h light–dark cycle (lights off: 0900 h) with food and water *ad libitum* for at least 14 days before starting the experiments.

Experimental procedures

Experiments were performed on two consecutive days with application of the footshock (sensitization) on day 1 and exposure to the tone on day 2. Rimonabant was applied subcutaneously (s.c.) 45 min before tone presentation on day 2. All experiments were performed during the animals' active phase between 0930 and 1700 h.

Sensitization

Mice were placed into the shock context where they received a single inescapable footshock of 2 seconds duration essentially as previously described (Kamprath & Wotjak 2004). Shock sensitization at the individual pain threshold (PT) was achieved by manually raising the shock intensity until the animal showed the first signs of pain and discomfort (jumping and/or vocalization). The respective current intensity was maintained for 2 seconds, before the current was switched off. Naïve (non-shocked) controls were not placed into the shock context.

Tone presentation
On the day following footshock sensitization, mice were placed into a new 'test context' that differed from the shock context in various aspects, including shape, odor, illumination and bedding (see Kamprath & Wotjak 2004 for details). After 3 min, a tone of 9 kHz and 80 dB was presented for 3 min.

Experiment 1: Interrelation between CB_1-deficiency, footshock intensity and subsequent fear response to a tone
$CB1^{-/-}$ and $CB_1^{+/+}$ were randomly assigned to five experimental groups, which differed in the intensity of the stress sensitization procedure as follows: the first group remained non-shocked (0 mA; $CB_1^{-/-}$: $n = 10$; $CB_1^{+/+}$: $n = 11$), the second group received a footshock at the individual PT ($CB_1^{-/-}$: $n = 15$; $CB_1^{+/+}$: $n = 10$), the third group received a footshock with a current intensity of 0.5 mA ($CB_1^{-/-}$: $n = 8$; $CB_1^{+/+}$: $n = 9$), the fourth group received a footshock with a current intensity of 0.7 mA ($CB_1^{-/-}$: $n = 20$; $CB_1^{+/+}$: $n = 26$) and the fifth group received a footshock with a current intensity of 1.5 mA ($CB_1^{-/-}$: $n = 8$; $CB_1^{+/+}$: $n = 12$). The extent of sensitization was assessed by measuring the freezing response to a 3-min tone at the next day. Note that the groups of mice that received a 0.7-mA shock are identical to those published before (Kamprath *et al.* 2006).

Experiment 2: Interplay between CB_1 and CRHR1 in fear adaptation following footshock sensitization
The $CRHR1^{-/-}$ and their wild-type littermates received a footshock of 1.5 mA (which proved to be the most effective in experiment 1), and the extent of sensitization was assessed by measuring the freezing response to a 3-min tone on the next day. Half of the $CRHR1^{-/-}$ mice ($n = 7$) and their wild-type littermates ($n = 7$) were treated with rimonabant (10 mg/kg, s.c.) 45 min prior to the tone presentation, and the other half ($CRHR1^{-/-}$: $n = 7$; $CRHR1^{+/+}$: $n = 7$) were treated with vehicle.

Experiment 3: Interplay between CB_1 and CRHR2 in fear adaptation following footshock sensitization
The $CRHR2^{-/-}$ and their wild-type littermates received a footshock of 1.5 mA, and the extent of sensitization was assessed by measuring the freezing response to a 3-min tone on the next day. Half of the $CRHR2^{-/-}$ mice ($n = 7$) and their wild-type littermates ($n = 9$) were treated with rimonabant (10 mg/kg, s.c.) 45 min prior to the tone presentation, and the other half ($CRHR2^{-/-}$: $n = 7$; $CRHR2^{+/+}$: $n = 9$) were treated with vehicle.

Experiment 4: Role of CB_1 expressed by principal neurons of the forebrain in fear adaptation following footshock sensitization
CaMK-$CB_1^{-/-}$ and their wild-type littermates received a footshock of 1.5 mA, and the extent of sensitization was assessed by measuring the freezing response to a 3-min tone on the next day.

Experiment 5: Role of CB_1 expressed by cortical glutamatergic neurons in fear adaptation following footshock sensitization
Glu-$CB_1^{-/-}$ and their wild-type littermates received a footshock of 1.5 mA, and the extent of sensitization was assessed by measuring the freezing response to a 3-min tone on the next day.

Behavioral analysis

The behavioral response to the tone was videotaped. Fear was assessed off-line by a trained observer who scored the freezing response of the animals unaware of the genotype or treatment condition as described before (Kamprath & Wotjak 2004). Freezing was defined as the absence of all movements except for those related to respiration.

Drug treatment

Rimonabant [SR141716; *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; kindly provided by NIMH Chemical Synthesis and Drug Supply Program (Rinaldi-Carmona *et al.* 1994)] was dissolved in vehicle solution (one drop Tween-80 in 3 ml 2.5% dimethylsulfoxide in saline) to a final concentration of 10 mg/kg and injected s.c. at 20 ml per kg body weight under light isoflurane anesthesia 45 min prior to behavioral testing. The subcutaneous route of drug administration was chosen on basis of previous experiments (Kamprath *et al.* 2006; Marsicano *et al.* 2002). The time-point of injection (45 min prior to behavioral testing) was based on the study of Petitet *et al.* (1999), who showed that rimonabant elicits its strongest effects up to 60 min after administration independent of the route of administration. The interval between injection and behavioral testing was maximized within the given temporal range to avoid possible effects of the injection stress on the outcome of the behavioral testing. The dose of rimonabant (10 mg/kg) was chosen on basis of a dose–response experiment in C57BL/6J mice, the background strain of CRHR2 mutant mice (Figure S1) and because of its higher efficiency in terms of promoting stress-induced corticosterone secretion in C57BL/6N mice (Steiner *et al.* 2008a).

Data analysis and statistics

For analysis, the total time of tone presentation was subdivided into 20-second bins with one data point representing one interval. For every interval, the duration of freezing was expressed as a percentage of the total time of the interval ('freezing time' per interval/total interval time $\times 100$). Data were analyzed by two-way or three-way analysis of variance (ANOVA) for repeated measurements as indicated in the text using STATISTICA 5.0 (StatSoft Inc., Tulsa, OK, USA) or GRAPHPAD PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA). Newman–Keuls test was used as the *post hoc* test if appropriate. Data are presented as mean ± SEM. Statistical significance was accepted if $P < 0.05$.

Results

Experiment 1: Fear-alleviating effects of endocannabinoids depend on the intensity of the previously encountered footshock

To investigate whether CB_1 mediates the adaptation of the fear response in an aversiveness-dependent manner, we applied inescapable footshocks of different intensities to different groups of CB_1-deficient mice ($CB_1^{-/-}$) and their wild-type littermates ($CB_1^{+/+}$). One day later, we assessed the behavioral responses of all groups of mice to the same novel stimulus, an 80-dB tone. Both $CB_1^{-/-}$ and $CB_1^{+/+}$ showed an increase in freezing to the tone with increasing shock intensities (Fig. 1; statistics not shown). Significant genotype differences became evident only after application of a footshock of 0.7 mA (genotype: $F_{1,44} = 9.7$, $P = 0.003$; genotype × interval: $F_{8,352} = 3.5$, $P < 0.001$; Fig. 1) or 1.5 mA (genotype: $F_{1,18} = 10.6$, $P = 0.004$; genotype × interval: $F_{8,144} = 3.6$, $P < 0.001$; Fig. 1) but not in the case of lower footshock intensities (statistics not shown). A significant decrease in the development of the freezing response over the 3-min tone presentation was observed in all groups of $CB_1^{+/+}$ which experienced a footshock, while $CB_1^{-/-}$ shocked with 1.5 mA failed to reach significance ($F_{8,56} = 1.8$, $P = 0.09$, one-way ANOVA), in contrast to their wild-type littermates ($F_{8,88} = 6.5$, $P < 0.0001$). Thus, the strongest footshock protocol (1.5 mA) yielded the most

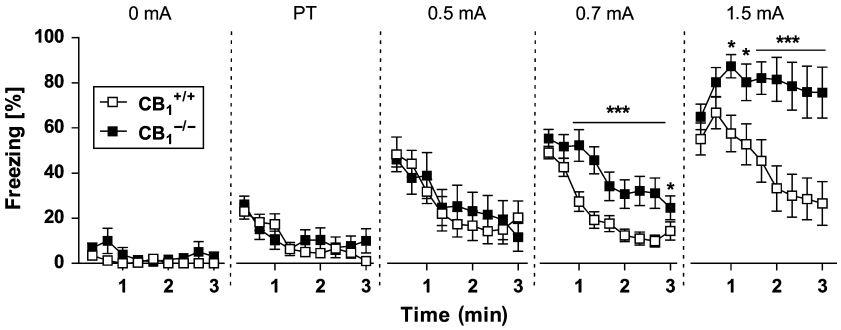

Figure 1: Fear-alleviating effects of endocannabinoids depend on the intensity of the aversive encounter. The freezing responses of both $CB_1^{-/-}$ (■) and $CB_1^{+/+}$ (□) to a 3-min tone presented 24 h after application of an inescapable footshock showed a clear dependency on the intensity of the previously encountered footshock. CB_1 deficiency caused a sustained freezing response after application of an inescapable footshock of high-shock intensities (0.7 and 1.5 mA) only. No significant differences could be detected in the freezing responses of naïve mice (0 mA) and of mice that were shocked either at the individual PT (approximately 0.25 mA) or with 0.5 mA. Data are displayed in 20-second bins. $*P < 0.05$, $***P < 0.001$ (for sample sizes, see *Materials and methods*). Note that the groups of mice that received a 0.7-mA shock are identical to those published in Kamprath *et al.* (2006) (copyright 2006 by the Society for Neuroscience).

pronounced genotype effects. Non-shocked mice showed very little freezing (0 mA, statistics not shown; Fig. 1).

Experiment 2: Interplay between CB_1 and CRHR1 in fear adaptation following footshock sensitization

As both the endocannabinoid system and the CRH signaling play important roles in the adaptation to stressful events, and recent literature suggests an interplay between both systems, our next aim was to investigate whether CRHR1 is involved in CB_1-mediated fear adaptation following sensitization with high footshock intensities. To this end, we used $CRHR1^{-/-}$, which were shown, among others, to be strongly impaired in stress-induced corticotropin (ACTH) and corticosterone responses (Timpl *et al.* 1998). Thus, pharmacological blockade of CB_1 in $CRHR1^{-/-}$ would be expected to be ineffective if CB_1-mediated acute fear adaptation depends on CRHR1 signaling or on stress-induced ACTH or corticosterone release. Based on the previous experiment, a footshock of 1.5 mA was applied to $CRHR1^{+/+}$ and $CRHR1^{-/-}$. On the next day, the freezing response of the animals to the tone was measured. Forty-five minutes prior to tone exposure, half of the animals for each genotype were treated with the CB_1-antagonist rimonabant (10 mg/kg, s.c.) and the other half with vehicle. A three-way ANOVA (drug, genotype and interval) showed that, similar to a genetic CB_1 deficiency, the CB_1 antagonist rimonabant caused an increased freezing response (drug: $F_{1,24} = 8.8$, $P = 0.007$), which was independent of the genotype (drug × genotype: $F_{1,24} = 0.002$, $P = 0.96$; Fig. 2). A significant drug × interval interaction ($F_{8,192} = 2.4$, $P = 0.016$) points to a delayed fear adaptation in rimonabant-treated animals that was independent of the genotype (drug × interval × genotype: $F_{8,192} = 1.56$, $P = 0.14$). Interestingly, $CRHR1^{-/-}$, in general, showed a stronger freezing response than their wild-type littermates (genotype: $F_{8,48} = 18.8$, $P = 0.0002$), irrespective of the treatment (drug × genotype: $F_{1,24} = 0.002$, $P = 0.96$).

Experiment 3: Interplay between CB_1 and CRHR2 in fear adaptation following footshock sensitization

Data of experiment 2 indicate that acute effects of CB_1 on fear adaptation occur independent of CRHR1 signaling and stress-induced corticosterone release. However, the effects of CRH on stress coping depend also on another receptor, CRHR2 (for review, see Bale & Vale 2004). Thus, the various ligands of the CRH family might interact with the endocannabinoid system during the acute fear response via CRHR2. Therefore, we applied a footshock of 1.5 mA to $CRHR2^{+/+}$ and $CRHR2^{-/-}$ and measured their freezing response to the tone on the next day 45 min after treatment with rimonabant (10 mg/kg, s.c.) or vehicle. A three-way ANOVA (drug, interval and genotype) showed that, similar to $CRHR1^{-/-}$, the CB_1 antagonist rimonabant caused an increased freezing response (drug:

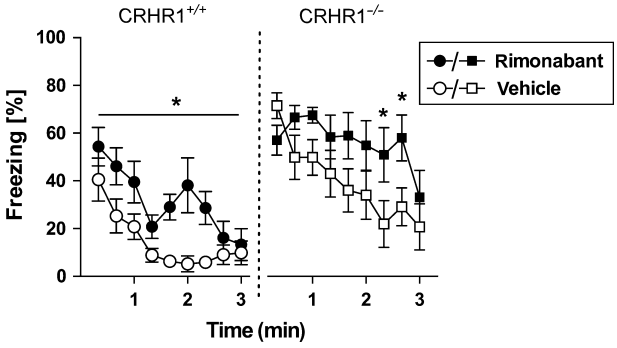

Figure 2: The CB_1-antagonist rimonabant caused increased freezing in both $CRHR1^{+/+}$ and $CRHR1^{-/-}$. $CRHR1^{+/+}$ (●, ○) and $CRHR1^{-/-}$ (■, □) received a footshock of 1.5 mA, followed by exposure to a 3-min tone 24 h later. Before tone presentation, mice were treated either with 10 mg/kg, s.c. rimonabant (●, ■) or with vehicle (○, □). Data are displayed in 20-second bins. $*P < 0.05$ (for sample sizes, see *Materials and methods*).

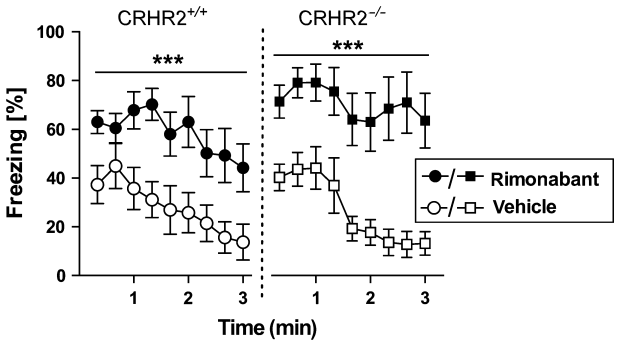

Figure 3: The CB_1-antagonist rimonabant caused increased freezing in both $CRHR2^{+/+}$ and $CRHR2^{-/-}$. $CRHR2^{+/+}$ (●, ○) and $CRHR2^{-/-}$ (■, □) received a footshock of 1.5 mA, followed by exposure to a 3-min tone 24 h later. Before tone presentation, mice were treated either with 10 mg/kg, s.c. rimonabant (●, ■) or with vehicle (○, □). Data are displayed in 20-second bins. $***P < 0.001$ (for sample sizes, see *Materials and methods*).

$F_{1,28} = 26.6$, $P < 0.0001$), which was independent of the genotype (drug × genotype: $F_{1,28} = 0.86$, $P = 0.36$; drug × interval × genotype: $F_{8,224} = 0.94$, $P = 0.48$; Fig. 3). Noteworthy, no significant genotype differences could be detected between $CRHR2^{+/+}$ and $CRHR2^{-/-}$ (genotype: $F_{1,28} = 0.57$, $P = 0.46$; genotype × interval: $F_{8,224} = 0.95$, $P = 0.48$).

Experiment 4: CB_1 deficiency in principal forebrain neurons leads to impaired fear adaptation following footshock sensitization

To investigate which neuronal subpopulation expressing CB_1 is mediating fear adaptation following footshock stress, we next tested CaMK-$CB_1^{-/-}$, a conditional mutant line, which lacks CB_1 expression in principal neurons of the forebrain (Marsicano *et al.* 2003). The specific CB_1 deletion in these mice includes, among others, CB_1 expressed by glutamatergic neurons of cortical and subcortical brain structures and by GABAergic projection neurons. Based on the results of experiment 1, we subjected these animals to the strongest protocol, that is application of a footshock of 1.5 mA and measured the freezing response to a tone 24 h later. CaMK-$CB_1^{-/-}$ showed a significant difference in the freezing response compared with their wild-type littermate controls (genotype: $F_{1,25} = 4.5$, $P = 0.04$; genotype × interval: $F_{8,200} = 3.9$, $P < 0.001$; Fig. 4). This difference was characterized by the feature that CaMK-$CB_1^{-/-}$ were not able to decrease their freezing response over the course of the 3-min tone presentation ($F_{8,96} = 1.2$, $P = 0.29$; one-way ANOVA), in contrast to their wild-type littermates ($F_{8,104} = 6.5$, $P < 0.0001$).

Experiment 5: CB_1 deficiency in cortical glutamatergic neurons leads to impaired fear adaptation following footshock sensitization

In CaMK-$CB_1^{-/-}$, CB_1 expression in the forebrain is restricted to GABAergic interneurons, that is a relatively high number of

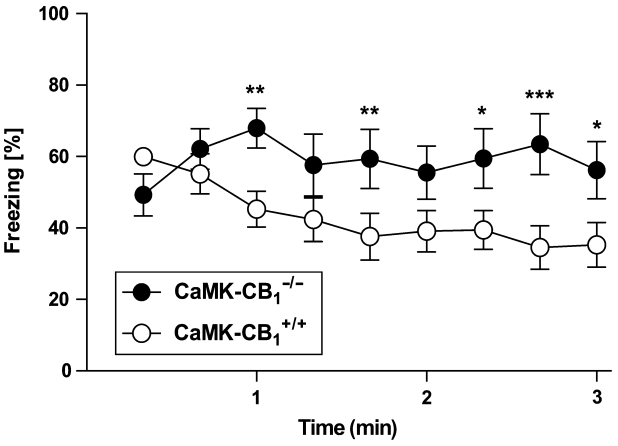

Figure 4: Sustained freezing responses in CaMK-$CB_1^{-/-}$. CaMK-$CB_1^{-/-}$ (●) and CaMK-$CB_1^{+/+}$ (○) received a footshock of 1.5 mA, followed by exposure to a 3-min tone 24 h later. Data are displayed in 20-second bins. $*P < 0.05$, $**P < 0.005$, $***P < 0.001$ (for sample sizes, see *Materials and methods*).

neurons lack CB_1 expression (Monory *et al.* 2006, 2007). To further narrow down which neuronal subpopulation expressing CB_1 mediates fear adaptation following footshock stress, we tested Glu-$CB_1^{-/-}$, another conditional mutant line, which lacks CB_1 expression specifically in cortical glutamatergic neurons (Monory *et al.* 2006). In contrast to CaMK-$CB_1^{-/-}$, the specific CB_1 deletion in Glu-$CB_1^{-/-}$ does not include CB_1 expressed by hypothalamic neurons. Similarly to $CB_1^{-/-}$ and CaMK-$CB_1^{-/-}$, Glu-$CB_1^{-/-}$ showed a stronger freezing response to the tone than their wild-type littermate controls (genotype: $F_{1,22} = 4.3$, $P = 0.049$; Fig. 5). Although we failed to observe a significant genotype × interval interaction ($F_{8,176} = 1.2$, $P = 0.30$), one-way ANOVAs performed

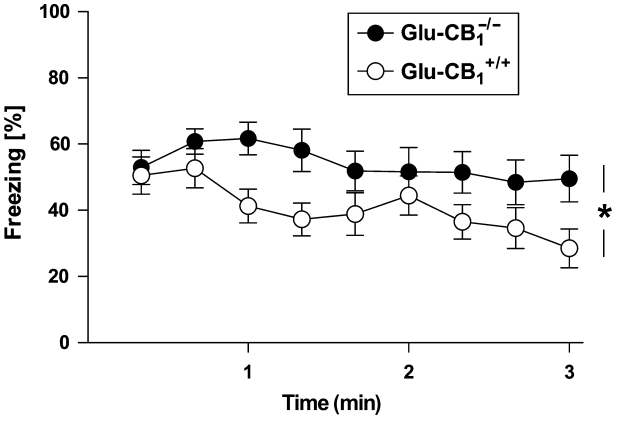

Figure 5: Sustained freezing responses in Glu-$CB_1^{-/-}$. Glu-$CB_1^{-/-}$ (●) and Glu-$CB_1^{+/+}$ (○) received a footshock of 1.5 mA, followed by exposure to a 3-min tone 24 h later. Data are displayed in 20-second bins. $*P < 0.05$ (for sample sizes, see *Materials and methods*).

separately per genotype showed that Glu-$CB_1^{-/-}$ ($F_{8,104} = 1.5$, $P = 0.17$), unlike their wild-type littermates (one-way ANOVA: $F_{8,72} = 2.9$, $P = 0.007$), were not able to significantly decrease their freezing response over the course of the 3-min tone presentation.

It is of note that, after application of a 1.5 mA footshock, none of the CB_1-deficient mouse lines ($CB_1^{-/-}$, CaMK-$CB_1^{-/-}$ and Glu-$CB_1^{-/-}$) were able to decrease their freezing responses over the course of the 3-min tone presentation, whereas the respective wild-type mice did show a decline in freezing. Although this decline of the freezing response appears to be steeper in $CB_1^{+/+}$ (wild-types) than in the wild-types of the conditional mutant lines, CaMK-$CB_1^{+/+}$ and Glu-$CB_1^{+/+}$, these differences between the CB_1-deficient mouse lines failed to reach statistical significance (line: $F_{2,65} = 2.88$, $P = 0.063$) in a three-way ANOVA (genotype, line and interval) and might relate to differences in the genetic background and variations between experiments. In contrast, the three-way ANOVA showed a significant genotype effect ($F_{1,65} = 20.5$, $P < 0.0001$) and a significant genotype × interval interaction ($F_{8,520} = 6.8$, $P < 0.00001$), which were independent of the line (genotype × line: $F_{2,65} = 1.8$, $P = 0.17$; genotype × line × interval: $F_{16,520} = 1.3$, $P = 0.20$).

Discussion

The present study shows that CB_1 only controls acute fear adaptation in the aftermath of highly aversive encounters. This process depends neither on intracerebral CRH signaling nor on stress-induced activation of the HPA axis, but on CB_1-controlled cortical glutamatergic projections.

To investigate whether the involvement of endocannabinoids in fear adaptation depends on the aversiveness of the situation, we applied inescapable footshocks of different intensities to different groups of CB_1-deficient mice and studied their fear responses to a subsequently presented tone. In general, the intensity of the footshock directly modified the intensity of the fear response to the subsequently presented tone in a dose-dependent manner (Fig. 1; see also Kamprath & Wotjak 2004). However, the fear-alleviating effects of endocannabinoids only became evident following the two highest shock intensities (0.7 and 1.5 mA). These data underscore the general importance of intensity-response studies for testing the modulation of fear and stress responses and for evaluating the involvement of different neuronal systems in these processes. The impairments in fear adaptation observed in CB_1-deficient mice did not relate to differences in pain perception because differences between the two genotypes were neither found in the individual pain thresholds (Marsicano *et al.* 2002) nor in the initial fear responses to the subsequently presented tone (Fig. 1). Moreover, data obtained by pharmacological blockade of CB_1 before tone presentation (Figs 2 and 3) indicate that endocannabinoids are acutely involved in the adaptation of the fear response to the tone following sensitization rather than in perception of the footshock (Kamprath *et al.* 2006; Marsicano *et al.* 2002).

The finding that the endocannabinoid system mediates fear adaptation in an aversiveness-dependent manner corroborates recent findings by Haller *et al.* (2004), showing that an anxiogenic-like phenotype of CB_1-deficient mice became only detectable if the illumination of the elevated plus maze (EPM) was increased and, thus, the aversiveness of the test situation was maximized. In line with these results, treatment with the fatty acid amide hydrolase (FAAH) inhibitor URB597, which blocks degradation of the endocannabinoid anandamide, resulted in anxiolytic-like behavior in the EPM test only if a distinct strong illumination was used (Naidu *et al.* 2007) or if the animals were tested during the light phase of the circadian cycle (Moreira *et al.* 2008). Moreover, impairment of FAAH by pharmacological and genetic means led to increased active stress-coping behavior in a tail suspension test only if the aversiveness was increased by a flashlight beam focused to the animals' tail in a dimly lit room (Naidu *et al.* 2007). Our data extend those findings in that the aversiveness of the test situation determines endocannabinoid recruitment not only in terms of anxiety-related and stress-coping behavior but also in terms of behavioral fear responses.

The findings that endocannabinoid involvement in fear, anxiety and stress adaptation depends on the aversiveness of the test situation strikingly resemble the dependency of HPA-axis activation on the intensity of a stressor (Armario *et al.* 1986; Hennessy & Levine 1978; Hennessy *et al.* 1979). In the first steps of stress-induced HPA-axis activation, CRH is released from axon terminals of the hypothalamic paraventricular nucleus into the portal blood at the level of the median eminence, followed by ACTH secretion from the adenohypophysis, which subsequently triggers the release of glucocorticoids (i.e. cortisol or corticosterone) from the adrenal glands. Corticotropin-releasing hormone is also found in extrahypothalamic brain regions, where it is involved in the processing of stress responses, anxiety-like behavior and conditioned fear (Bale & Vale 2004; Keck *et al.* 2005; Steckler & Holsboer 1999). Colocalization of CB_1 and CRHR1 (Hermann & Lutz 2005) and of CB_1 and CRH (Cota *et al.* 2003, 2007) suggest a functional interplay between the two neurotransmitter systems. We therefore assumed that CB_1 might mediate its fear-alleviating effects by restraining CRH signaling within the brain. To address this point, we sensitized $CRHR1^{-/-}$ (Timpl *et al.* 1998) and $CRHR2^{-/-}$ (Coste *et al.* 2000) with a 1.5-mA footshock and treated the animals with 10 mg/kg rimonabant (s.c.) before exposure to the tone 24 h later. The rather high dose of rimonabant was chosen on the basis of a pilot experiment in C57BL6/J mice, the background strain of the CRHR2 mutant mouse strain (Figure S1). In addition, a dose of 10 mg/kg was the most efficient in promoting stress-induced corticosterone secretion in C57BL/6N mice (Steiner *et al.* 2008a). We cannot entirely rule out that the high dose of rimonabant might mediate its effects via its inverse agonist properties. However, the phenotype of CB_1-deficient mice, C57BL/6N mice treated with 3 mg/kg rimonabant (Figure S1; Kamprath *et al.* 2006) and C57BL/6J mice treated with 10 mg/kg rimonabant (Figure S1), appears strikingly similar and is most easily explained by an impairment of CB_1 signaling.

Rimonabant treatment led to impaired fear adaptation in both $CRHR1^{-/-}$ (Fig. 2) and $CRHR2^{-/-}$ (Fig. 3) and the respective wild-type controls, thus indicating that the fear-alleviating effects of endocannabinoids do not involve

intracerebral CRH systems. Although there is still the possibility for a mutual compensation of CRHR1 and CRHR2 deficiency, this seems to be rather unlikely because $CRHR1^{-/-}$ and $CRHR2^{-/-}$ show different phenotypes in anxiety-related behavior when compared with their respective wild-type littermates, with $CRHR1^{-/-}$ showing decreased anxiety (Timpl *et al.* 1998) and different lines of CRHR2-deficient mice showing either increased anxiety (Bale *et al.* 2000; Kishimoto *et al.* 2000) or no changes in anxiety at all (Coste *et al.* 2000). Moreover, pharmacological treatment showed that CRHR1 and CRHR2 seem to exert opposite effects on auditory fear conditioning (Radulovic *et al.* 1999).

Recently, Tasker and co-workers suggested a mechanism for fast glucocorticoid feedback inhibition within the hypothalamus involving endocannabinoid release (Di *et al.* 2003, 2005b), which might account for endocannabinoid-mediated stress adaptation. This model requires glucocorticoid secretion to trigger endocannabinoid release within the paraventricular nucleus of the hypothalamus that, in turn, inhibits glutamatergic afferences to the nucleus and results in CRH secretion. As a similar interaction between the corticosterone and the endocannabinoid system has been described for other parts of the hypothalamus as well (Di *et al.* 2005a), we hypothesized that corticosterone regulates endocannabinoid-controlled fear adaptation in a similar way within the fear matrix of the brain. However, because $CRHR1^{-/-}$, which are severely impaired in stress-induced corticosterone secretion (Timpl *et al.* 1998), still responded to rimonabant, this model may not apply for acute fear adaptation following footshock stress.

The $CRHR1^{-/-}$ showed an increased freezing response to the tone following an inescapable footshock compared with their wild-type littermates, irrespective of the treatment. This observation was unexpected, taking into consideration the increased exploratory activity and the reduced anxiety-related behavior previously reported in these animals (Timpl *et al.* 1998). The data of the present study suggest that CRHR1 is differentially involved in fear and anxiety. In contrast to fear-related paradigms, in which the animal is confronted with an inescapable stressor, the stressor used in anxiety paradigms is avoidable, and risk assessment, that is approach of the stressful situation, is tested. As different neural circuits are involved in controllable vs. uncontrollable stress (Herry *et al.* 2007; Kavushansky *et al.* 2006), CRHR1 might also be differentially involved. Alternatively, compensatory changes in other transmitter systems (e.g. upregulation of vasopressin expression; Muller *et al.* 2000) might be responsible for the increased freezing response of $CRHR1^{-/-}$. Finally, taking into consideration that injections of corticosterone at different learning phases of fear conditioning resulted in a decreased freezing response (Cai *et al.* 2006; Skorzewska *et al.* 2007), the attenuated HPA-axis response in $CRHR1^{-/-}$ with strongly impaired corticosterone release might lead to an increased freezing response not only after conditioning but also after sensitization paradigms. Corticosterone might mediate its fear-alleviating effects, at least in part, via triggering endocannabinoid release. The phenotype of an increased fear response following sensitization in vehicle-treated $CRHR1^{-/-}$ compared with vehicle-treated $CRHR1^{+/+}$ would then relate to impaired corticosterone-induced endocannabinoid signaling in the mutants (Fig. 2). However, the behavioral differences between $CRHR1^{-/-}$ and $CRHR1^{+/+}$ persisted despite rimonabant treatment (Fig. 2), thus rendering it unlikely that corticosterone-induced endocannabinoid signaling via CB_1 is crucially involved in the phenotype of $CRHR1^{-/-}$.

The question remained as to which transmitter systems are involved in CB_1-controlled fear adaptation during highly aversive encounters. Cannabinoid receptor type 1 was shown to be expressed widely throughout the brain by different neuronal subpopulations including GABAergic, glutamatergic and serotonergic neurons (Haring *et al.* 2007; Marsicano & Kuner 2008; Marsicano & Lutz 1999). To investigate whether glutamatergic transmission is involved in CB_1-mediated fear adaptation, we applied the strongest footshock protocol, which was found to yield the strongest effects in $CB_1^{-/-}$ (Fig. 1), to CaMK-$CB_1^{-/-}$ mutants. These mutants lack CB_1 expression in principal forebrain neurons, including glutamatergic and GABAergic projection neurons, but sparing CB_1 expression in GABAergic interneurons. CaMK-$CB_1^{-/-}$ showed a similar impairment of fear adaptation as $CB_1^{-/-}$ (Fig. 4). Consequently, an involvement of CB_1-expressing GABAergic interneurons in this behavioral modulation appears to be rather unlikely. To further narrow down the neuronal subpopulation involved, Glu-$CB_1^{-/-}$ were subjected to the same protocol with similar effects as observed in $CB_1^{-/-}$ and CaMK-$CB_1^{-/-}$ (Fig. 5). These results indicate that CB_1-expressing cortical glutamatergic neurons are involved in endocannabinoid-mediated fear adaptation following inescapable footshock stress. According to a mechanism proposed by Patel and Hillard (2008), endocannabinoid-regulated cortical glutamatergic transmission plays an essential role in the habituation of repeated exposure to a stressor. Although our behavioral paradigm did not involve repeated exposures to the tone, we observed within-session habituation of the behavioral response. Consequently, the mechanism suggested by Patel and Hillard (2008) might partially account for acute fear adaptation, that is endocannabinoid-regulated cortical glutamatergic transmission might play a central role, whereas increases in 2-AG synthetic capacity via upregulation of the synthesizing enzymes, which is also part of the suggested model, may not apply for the current paradigm of acute fear adaptation, but for the pronounced genotype and drug effects observed after repeated tone presentations (Kamprath *et al.* 2006). Recently, we could show that Glu-$CB_1^{-/-}$ were impaired in behavioral stress coping in a forced swim test despite unaltered corticosterone secretion (Steiner *et al.* 2008b). This supports the notion that endocannabinoid-mediated control of both behavioral stress and fear responses may rely on cortical glutamatergic projections, independent of the activity of the CRH/HPA system.

Taken together, the present study emphasizes the importance of intensity-response studies for testing the modulation of fear responses, especially with respect to the endocannabinoid system. We showed that CB_1 mediates fear adaptation following inescapable footshocks of high intensity only. Corticotropin-releasing hormone signaling or corticosterone secretion appears to be dispensable for CB_1-mediated fear adaptation, which, in contrast, critically depends on endocannabinoid-controlled glutamatergic transmission in cortical brain structures. Endocannabinoid-mediated behavioral responses to

stress and fear share similarities in that endocannabinoids are recruited in aversiveness-dependent manner and especially involved in habituation-like processes of stress and fear responses. Moreover, cortical glutamatergic transmission appears to be modulated by endocannabinoids during stress and fear habituation, whereby the exact mechanism underlying short-term and long-term habituation still remains to be determined.

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Supporting Information

The following supporting information are available for this article.

Figure S1: Different doses of rimonabant are required in different mouse strains to cause sustained fear adaptation following sensitization.

Additional Supporting Information may be found in the online version of this article.

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