CHAPTER 6

The corticotropin-releasing factor receptor 1 antagonist CP-154,526 markedly enhances associative learning and paired-pulse facilitation immediately after a stressful experience

Submitted

ABSTRACT

The neuropeptide corticotropin-releasing factor (CRF) coordinates the endocrine responses to stress as a major physiological regulator of the hypothalamic-pituitary-adrenal (HPA) axis. We assessed the effect of the non-peptidic corticotropin-releasing factor receptor 1 (CRFR1) antagonist CP-154,526 in combination with exposure to an acute stressor on hippocampus-dependent memory and hippocampal synaptic plasticity. Mice that received intraperitoneal injection of CP-154,526 before exposure to 1 hr immobilization revealed improved context-dependent fear conditioning when trained immediately after immobilization in comparison to animals which were stressed without CP-154,526 treatment. It was determined that exposure to the stressor reduced the amount of autophosphorylated Ca2+/calmodulin-dependent protein kinase II (CaMKII) in the hippocampal CA1 area. When animals were pretreated with CP-154,526, the amount of hippocampal autophosphorylated CaMKII was elevated. Electrophysiological studies in the hippocampal CA1 region of stressed animals revealed no significant effects of the CP-154,526 pretreatment on various forms of long-term potentiation (LTP) but a drastic elevation of paired-pulse facilitation (PPF). The CP-154,526-induced enhancements in fear conditioning and PPF could be prevented by the selective CaMKII inhibitor KN-62. Our results demonstrated that stress in combination with CP-154,526 pretreatment increased CaMKII activity and short-term synaptic plasticity in the mouse CA1 area which correlated with improved hippocampus-dependent memory.
INTRODUCTION

Exposure to stressful stimuli is known to affect hippocampal learning and memory. However, it appears that the duration and intensity of the stressor determine the direction of the stress effects on learning and memory and on synaptic plasticity. It was demonstrated in numerous studies that induction of hippocampal LTP by high frequency stimulation or by multiple bursts of electrical pulses delivered in a theta-related pattern is inhibited after restraint stress and inescapable tail shock (Foy et al., 1987; Garcia et al., 1997; Kim et al., 1996; Shors and Dryver, 1994). In contrast, long-term depression (LTD) observed after low frequency stimulation is known to be facilitated by such stress (Kim et al., 1996; Xu et al., 1997). In addition, several experiments suggest that stress also impairs short-term potentiation in the CA1 area of hippocampal slices prepared immediately after the stress episode (Garcia et al., 1997; Kim et al., 1996) as well as in vivo (Diamond et al., 1994). Possible explanations for the impaired synaptic efficacy after stress range from LTP saturation produced by the stress episode (Kim et al., 1996) to elevated levels of corticosterone which have been shown to decrease the magnitude of LTP and favor the induction of LTD (Kerr et al., 1994).

It has been shown that the activation of the HPA axis by stress is initiated by the action of CRF (Spiess et al., 1981; Vale et al., 1981). However, there are no data available on the contribution of the CRF receptor system to the described effects of acute stress on synaptic plasticity. In this study, we used the CRFR1 antagonist CP-154,526 (Arborelius et al., 2000; Schulz et al., 1996) to elucidate the contribution of CRF to changes in hippocampal synaptic plasticity and the possible correlation to hippocampal learning and memory in mice immediately after acute stress.

MATERIALS AND METHODS

Materials

Male Balb/c mice were obtained from Charles River (Sultzfeld, Germany). Double guide cannulae (C235) were from Plastics One (Roanoke, VA, U.S.A.). CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) was provided by Dr. Eric Ronken (Solvay Pharmaceuticals, Netherlands). KN-62 was from Calbiochem (La Jolla, CA, U.S.A.). Anti-sauvagine-30 (Ruehmann et al., 1998) and [Glu$^{11,16}$]astressin (Eckart et al., 2001) were synthesized in our laboratory as described previously. All other drugs and salts were purchased from Sigma (St. Louis, MO, U.S.A.).

Animals

Experiments were carried out on male Balb/c mice aged 9-12 weeks with an average weight between 20-25 g. The mice were individually housed and maintained on a 12 hr light/dark cycle (lights on at 7 a.m.) with free access to food and water. All experimental procedures were in accordance with the European Council Directive (86/609/EEC) by permission of the Animal Section Law enforced by the District Government of Braunschweig, Lower Saxony, Germany.

Hippocampal Slice Electrophysiology

Transverse hippocampal slices (400 µM) were obtained on a McIlwain tissue chopper and kept submerged (minimum of 1 hr at room temperature before recordings) in artificial cerebrospinal fluid (aCSF) solution of the following composition (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH$_2$PO$_4$, 1.5
MgSO₄, 2 CaCl₂, 24 NaHCO₃, and 10 glucose. Extracellular field potentials were recorded in a chamber maintained at 32°C with recording electrodes pulled from borosilicate glass and filled with 2 M NaCl (3-5 mΩ). All recordings were performed using a SEC-05L amplifier (npi Electronics, Germany). To record field potentials in the CA1 pyramidal cell body layer, Schaffer collaterals were stimulated with a bipolar electrode placed on the surface of the slice. At the beginning of each experiment, a stimulus response curve was established by increasing the stimulus intensity and measuring the amplitude of the population spike. Based on the input-output function, the stimulus was adjusted to elicit a population spike with an amplitude of half of the maximum and was fixed at this level throughout the experiments. Traces were stored on a computer using Pulse 7.4 software (HEKA, Lambrecht, Germany) for off-line analysis.

**Cannulation**

Double guide cannulae were implanted with the help of a stereotactic holder during 1.2% avertin anesthesia (0.02 ml/g, i.p.) under aseptic conditions as previously described (Radulovic et al., 1999; Stiedl et al., 2000). Each double guide cannula with inserted dummy cannula and dust cap was fixed to the skull by dental cement. The cannulae were placed into both lateral brain ventricles, anteroposterior (AP) –0.0 mm, lateral 1 mm, depth 3 mm or directed toward both dorsal hippocampi, AP –1.5 mm, lateral 1 mm, depth 2 mm (Franklin and Paxinos, 1997). The animals were allowed to recover for 4-5 d before the experiments started. On the day of the experiment, bilateral injections were performed using an infusion pump (CMA/Microdialysis) at a constant rate of 0.33 µl/min (final volume: 0.25 µl per side). Cannula placement was verified post hoc in all mice by injection of methylene blue. For electrophysiological experiments double cannula placement was verified by unilateral methylene blue injection just before the decapitation procedure.

**Statistics**

Statistical comparisons were made by using either Student’s t -test or ANOVA. Data were expressed as mean ± SEM. Significance was determined at the level of p ≤ 0.05.

**Drug Treatment**

CP-154,526 (100 µl per injection) in an acidic vehicle (HCl, final concentration 0.1 N) was administered (20 mg/kg, i.p.) 15 min prior to immobilization. Approximately 400 µg CP-154,526 was applied per mouse. CP-154,526 has been shown to penetrate the blood-brain barrier and to exhibit activity in vivo (Schulz et al., 1996). KN-62 was dissolved in DMSO to a concentration of 4 mg/ml. For cannula injection the stock was diluted in aCSF to a final concentration of 64 ng/µl. Mifepristone and spironolactone were dissolved in 1% EtOH and aCSF. Antisauvagine-30 was prepared as 80 pg/µl stock solution in 0.01 M HHOAc and diluted to the final concentration of 800 ng/µl in aCSF prior to injection. [Glu¹¹,¹⁶]astressin was dissolved in aCSF.

**Immobilization Stress**

An acute immobilization stress of mice consisted of taping their limbs to a Plexiglas surface for 1 hr (Smith et al., 1995).

**Fear Conditioning**

The fear conditioning experiments were performed as previously described (Stiedl et al., 2000) using a computer-controlled fear conditioning system (TSE, 303410, Bad Homburg, Germany). Fear conditioning was performed in a Plexiglas cage (36 x 21 x 20 cm) within a constantly illuminated (12 V, 10 W halogen lamp, 100-500 lux) fear conditioning box. In the conditioning box, a high-frequency loudspeaker (Conrad, KT-25-DT, Hirschau, Germany) provided constant background noise [white noise, 68 dB sound pressure level (SPL)]. The training (conditioning) consisted of a single trial. The mouse was exposed to the conditioning context (180 sec) followed by a tone (CS, 30 sec, 10 kHz, 75 dB SPL, pulsed 5 Hz). After termination of the tone, a footshock (US, 0.7 mA, 2 sec, constant current) was delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 sec after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 hr after fear conditioning. Contextual memory was tested in the fear conditioning box for 180 sec without CS or US presentation (with background noise). Freezing, defined as the lack of movement except for respiration and heart beat, was assessed as the behavioral parameter of the defensive reaction of mice (Blanchard and Blanchard, 1969; Bolles and...
Riley, 1973; Fanselow and Bolles, 1979) by a time-sampling procedure every 10 s throughout the memory tests. In addition, activity-derived measures (inactivity, mean activity, and exploratory area) were recorded by a photo beam system (10 Hz detection rate) controlled by the fear conditioning system.

**Western Blotting**

CA1 areas of hippocampal slices were dissected out and homogenized. The insoluble material was removed by centrifugation at 15,000 x g for 10 min at 4°C. Protein concentrations were determined with the Bradford assay (BioRad, Muenchen, Germany). Equal amounts of protein for each group were separated on a 10% SDS gel and transferred to an Immobilon-P membrane using a semidy transfer apparatus. The blot was probed using an anti-active CaMKII antibody (Promega, Madison, WI) or antibody directed against total CaMKII (Chemicon, Temecula, CA) and detected with alkaline phosphatase-conjugated second antibody. Western blots were developed using the chemiluminescence method.

**RESULTS**

**Effects of CP-154,526, a selective CRFR1 antagonist, on fear conditioning and CaMKII activity**

When mice were trained immediately after 1 hr immobilization the conditioned fear to context was mildly, however not significantly, attenuated in comparison to controls (p > 0.05). Interestingly, when mice received intraperitoneal (i.p.) injections of the specific CRFR1 antagonist CP-154,526 15 min before the stress session the fear conditioning to context was significantly enhanced when compared to untreated mice. The injection of CP-154,526 alone without stressing the mice had no effect on fear conditioning (Fig. 1A). Because context-dependent fear conditioning is a hippocampus-dependent learning task (Holland and Bouton, 1999; Kim and Fanselow, 1992; Phillips and LeDoux, 1992) we next determined if intrahippocampal (i.h.) injection of CRFR antagonists immediately before immobilization could mimic the CP-154,526 effect. However, i.h. administration of either the non-selective CRFR antagonist [Glu^{11,16}]astressin (Eckart et al., 2001) or the CRFR2 antagonist antisauvagine-30 (Ruehmann et al., 1998) had no effect on conditioned fear to context (Fig. 1C). Similarly, both compounds did not significantly improve contextual fear conditioning when given intracerebroventricularly (i.c.v.) before immobilization (p = n.s.; data not shown). Previous studies have shown that the CRF-induced increase in plasma adrenocorticotropic hormone is blocked by CP-154,526 in rats (Schulz et al., 1996). We expected that the subsequent corticosterone reduction could be responsible for the observed CP-154,526 effect, and therefore, we investigated the effects of corticosteroid receptor antagonists on conditioned fear to context. However, administration (i.h.) of the specific mineralocorticoid receptor antagonist, spironolactone (Kim et al., 1998), or the specific glucocorticoid receptor antagonist, mifepristone (Kim et al., 1998), immediately before immobilization had no effect on fear conditioning in comparison to vehicle-treated animals (Fig. 1D).
CP-154,526 enhances learning and PPF after an acute stress

Figure 1. CP-154,526 enhances fear conditioning after immobilization stress. A. Mice were injected i.p. with either CP-154,526 (20 mg/kg) or vehicle (0.1 N HCl) 15 min before the 1 hr stress session and trained immediately after stress cessation. Control mice were naive, untreated mice. B, Animals preinjected with CP-154,526 or vehicle were injected i.h. with KN-62 (32 ng per mouse) immediately before the stress session. C, Mice were injected i.h. with either vehicle (aCSF), antisauvagine-30 (400 ng per mouse) or [Glu(11,16)]astressin (200 ng per mouse) immediately before the stress session. D, Mice were injected i.h. with either vehicle (1% EtOH in aCSF), mifepristone (1 ng per mouse) or spironolactone (1 ng per mouse) immediately before the stress session. Data represent mean ± SEM from 4 to 9 animals. Statistically significant differences: * p < 0.05.

In various forms of hippocampus-dependent memory, the involvement of the multifunctional CaMKII has been demonstrated (Cammarota et al., 1998; Silva et al., 1992). Therefore, with the help of Western blots, we evaluated CaMKII activation in the CA1 area of mice that had experienced 1 hr immobilization with and without CP-154,526 treatment. We used antibodies that selectively recognize the CaMKII autoinhibitory domain phosphorylated at threonine 286 (Thr286). This phosphorylation correlates with the activation of the enzyme. Acute stress resulted in a modest reduction of activated CaMKII, whereas the CP-154,526 pretreatment caused a substantial activation of CaMKII compared to controls (Fig. 2A). Mice received i.h. injection of the selective CaMKII inhibitor KN-62 after the CP-154,526
injection to further explore whether elevated hippocampal CaMKII activity might be involved in the observed improved fear conditioning after CP-154,526 treatment prior to immobilization stress. Now the learning improvement was completely prevented when compared to animals that were treated with CP-154,526 alone before the stress session (Fig. 1B). Similarly, KN-62 antagonized the CP-154,526 effect when injected i.c.v. (p < 0.05; data not shown). Again, activated CaMKII levels corresponded to enhanced learning ability. CaMKII activity was reduced when the CP-154,526 treatment was followed by KN-62 injection. Vehicle injection had no effect on CaMKII activity (Fig. 2B).

Figure 2. CP-154,526 injection increases the amount of active CaMKII following acute stress. A, Representative CaMKII Western blots of area CA1 subregions from control (non-stressed) mice, mice that were subjected to 1 hr immobilization and decapitated immediately after end of the stress session and from mice that were pretreated with CP-154,526 (i.p.; 20 mg/kg) 15 min before immobilization. Detection was performed using an antibody specific for Thr286-phosphorylated CaMKII (upper lane), or an antibody recognizing total CaMKII (lower lane). B, Mice were injected with CP-154,526 (i.p.; 20 mg/kg) and KN-62 (i.h.; 32 ng per mouse) (left), CP-154,526 (i.p.; 20 mg/kg) and aCSF (i.h.; vehicle for KN-62) (middle) and 0.1 N HCl (i.p.; vehicle for CP-154,526) and aCSF (i.h.; vehicle for KN-62) (right) before the 1 hr stress session and decapitated immediately after end of the stress session. Results are representative of four independent Western blots.
Effects of immobilization and CP-154,526 treatment on short-term and long-term synaptic plasticity

We examined the synaptic transmission in acute hippocampal slice preparation to detect possible electrophysiological correlates for the behavioral findings. A PPF study, in which double pulses were applied to the Schaffer collaterals at intervals of 10-200 ms, showed no difference in the CA1 pyramidal cell region in slices from stressed animals in comparison to slices from control animals. The ratio of the second response to the first response was significantly enhanced in slices from animals that were pretreated with CP-154,526 before the immobilization session. This enhancement was completely abolished when the CP-154,526 treatment was combined with an i.c.v. injection of KN-62 (Fig. 3A).

We next investigated the effect of stress and CP-154,526 on long-term synaptic transmission in the CA1 area from hippocampal slices. After stimulation of the Schaffer-collateral pathway by two 100 Hz (1 s) trains, LTP was not significantly different in slices from stressed animals compared to LTP induced in slices from animals that were pretreated with CP-154,526 before immobilization (Fig. 3B). Similarly, LTP induced by theta burst stimulation (TBS), consisting of 10 x 100 Hz bursts, was not significantly different in slices from stressed animals, non-stressed controls or CP-154,526-pretreated and subsequently stressed animals (Fig. 3C). In contrast to the moderate inhibitory effect of stress on LTP induced by the standard 100 Hz tetanus protocol, we found modest facilitation of LTP after acute stress when LTP was induced by TBS, consisting of 5 x 100 Hz bursts. However, there was no significant difference in the degree of LTP between slices obtained from vehicle-treated and CP-154,526-treated mice (Fig. 3D).

DISCUSSION

We showed here that pretreatment of mice with the selective non-peptidergic CRFR1 antagonist CP-154,526 enhanced context-dependent fear conditioning after immobilization stress which correlated with elevated PPF in the CA1 area of hippocampal brain slices. Both, elevated short-term synaptic transmission as well as increased conditioned fear to context were prevented by inhibition of CaMKII with KN-62. CP-154,526 treatment caused an increase in CaMKII activity after stress but had no detectable effect on various types of LTP. A similar observation is made in mutant mice expressing CaMKII-Asp286 which is more active in the absence of Ca2+ than CaMKII-Thr286 (Mayford et al., 1995). The mutant mice exhibit normal LTP in response to stimulation at 100 Hz. In contrast to our results, Mayford et al. (1995) did not observe significant differences in PPF between these transgenic and wild-type animals whereas in our experiments increased PPF was prevented by inhibition of CaMKII activity. On the other hand, mice heterozygous for a αCaMKII mutation exhibit decreased PPF, highly reduced contextual fear conditioning, but normal LTP in the CA1 region (Silva et al., 1996). PPF is a short-lasting (< 1 s) enhancement in synaptic strength that is thought to be mediated by a presynaptic mechanism (Kamiya and Zucker, 1994). Activation of protein kinases in presynaptic terminals, particularly CaMKII was shown to
correlate with neurotransmitter release (Nichols et al., 1990). For example, in rat striatum the enhanced dopamine release after repeated amphetamine treatment is mediated by increased CaMKII activity (Kantor et al., 1999).

**Figure 3.**

**A,** Plot of paired-pulse facilitation (amplitude of population spike response to second stimulus divided by amplitude of population spike response to first stimulus) in the CA1 pyramidal cell region. Hippocampal slices were prepared from non-stressed control mice (n = 16), stressed mice (immediately after 1 hr immobilization stress, n = 15) and stressed mice which were pretreated with either CP-154,526 (i.p.; 20 mg/kg; 15 min before stress, n = 13) or CP-154,526 and KN-62 (i.c.v.; 32 ng per mouse; 0 min before stress; n = 5). There were no significant effects when vehicle for KN-62 and CP-154,526 was injected alone. Control versus stressed mice: p < 0.05; control versus CP-154,526 + stressed: p < 0.0001; control versus CP-154,526 + KN-62 + stressed: p = 0.18; stressed versus CP-154,526 + stressed: p < 0.05; stressed versus CP-154,526 + KN-62 + stressed: p = 0.17; CP-154,526 + stressed versus CP-154,526 + KN-62 + stressed: p < 0.005.

**B,** LTP induced by two 100 Hz, 1 s trains (10 s interval): 211% ± 18% (control; n = 6); 136% ± 14% (stressed mice; n = 6); 145% ± 13% (CP-154,526 + stressed; n = 7). Control versus CP-154,526 + stressed: p < 0.05; control versus CP-154,526 + KN-62 + stressed: p = 0.18; stressed versus CP-154,526 + stressed: p < 0.05; stressed versus CP-154,526 + KN-62 + stressed: p = 0.673.

**C,** LTP induced by theta burst stimulation (TBS) consisting of 10 x 100 Hz bursts (10 diphasic pulses per burst, 200 ms interburst interval): 133% ± 14% (control; n = 5); 129% ± 12% (stressed mice; n = 6); 161% ± 23% (CP-154,526 + stressed; n = 4). There were no significant differences between groups.

**D,** LTP induced by TBS consisting of 5 x 100 Hz bursts: 105% ± 11% (control; n = 5); 137% ± 18% (stressed mice; n = 6); 133% ± 13% (stressed + CP-154526; n = 8). There were no significant differences between groups. LTP values are the percentage of prestimulation baseline measured 1 hr after stimulation (mean ± SEM; n = number of animals)

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There are several possible target molecules to regulate neurotransmitter release in the hippocampal CA1 area after phosphorylation by CaMKII. The v-SNARE protein synaptotagmin may play the role of a calcium sensor in exocytosis (Verona et al., 2000) and has been demonstrated to be a substrate for CaMKII in isolated synaptic vesicles (Popoli, 1993). Synapsin I, which is found exclusively in neuronal presynaptic terminals (Nayak et al., 1996) is believed to link synaptic vesicles to the presynaptic cytoskeleton and restricts the availability of those vesicles for release. The on-vesicle phosphorylation of synapsin I by CaMKII and the subsequent dissociation of the protein from the vesicle membrane may represent a prompt and efficient mechanism for the modulation of neurotransmitter release and presynaptic plasticity (Stefani et al., 1997).

The intriguing question remains how CP-154,526 enhances hippocampal CaMKII activity and hippocampus-dependent memory in combination with immobilization. Considering the i.h. injection experiments it seems unlikely that CP-154,526 was acting directly in the hippocampus because neither inhibition of hippocampal CRF receptors by [Glu11,16]astressin or antisauvagine-30 nor inhibition of hippocampal corticosteroid receptors by specific inhibitors enhanced contextual fear conditioning after immobilization as did CP-154,526. It has further been shown that i.h. injection of human/rat CRF enhances conditioned fear to context (Radulovic et al., 1999). In view of this observation, it is unlikely that the blockade of hippocampal CRF receptors by CP-154,526 elicited a similar effect. Therefore it is assumed that CP-154,526 blocked CRFR1 at one or more different non-hippocampal sites which were not reached by our local i.h. and i.c.v. injections. CP-154,526 might potentially influence hippocampal function via the amygdala which contains CRFRI protein (Radulovic et al., 1998) and mRNA (Van Pett et al., 2000) and projects to the hippocampal CA1 area (Aggleton, 1986; Pikkarainen et al., 1999). In a recent study, it was demonstrated that amygdalar lesions block stress effects on CA1 LTP with no effect in unstressed animals (Kim et al., 2001). Similarly, CP-154,526 was only effective in combination with a stressful event but did not affect synaptic plasticity in unstressed animals. It may be speculated, that CP-154,526 reduced synaptic transmission from the amygdala to the hippocampus during immobilization which might have caused the changes in hippocampal CaMKII activity. In the amygdala h/rCRF increases neuronal excitability (Rainnie et al., 1992). This increase of excitability may enhance neuronal transmission from the amygdala to the hippocampus and subsequently induce elevated Ca2+ influx in neurons of the CA1 region during exposure to immobilization. The increase of intracellular Ca2+ initially increases CaMKII autophosphorylation but also produces a delayed, phosphatase-dependent decrease in the levels of autophosphorylated CaMKII (Dosemeci and Reese, 1993), an effect most probably mediated by protein phosphatase 1 (Shen et al., 2000) or Ca2+/calmodulin-dependent protein kinase phosphatase (CaMKPase) (Ishida et al., 1998). Since h/rCRF increases neuronal excitability in the amygdala (Rainnie et al., 1992), the blockade of CRF receptors by CP-154,526 during immobilization would be presumed to prevent the enhancement of neuronal transmission from the amygdala to the hippocampus. In the presence of CP-154,526 a slower rise of intracellular Ca2+ in the hippocampus and thus, a slower activation of CaMKII is expected (Miller and Kennedy, 1986). Because CaMKPase has to be phosphorylated by CaMKII for activation, delayed activation
of CaMKII would particularly slow down activation of CaMKPase and the subsequent dephosphorylation of autophosphorylated Thr286 of CaMKII (Kameshita et al., 1999). A delayed activation of CaMKII and the subsequent delayed dephosphorylation would provide a possible explanation for elevated CaMKII activity observed in the hippocampus of CP-154,526-injected animals following immobilization.

Taken together our results indicated a central role for CRFRI outside the hippocampus in the regulation of hippocampal CaMKII activity during a period of acute stress with a decisive influence on short-term synaptic plasticity and hippocampus-dependent memory.

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REFERENCES


