Behavioral/Systems/Cognitive

Long-Term Effects of Brief Acute Stress on Cellular Signaling and Hippocampal LTP

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In a previous study, we reported that a brief exposure to swim stress transforms an electrically induced, protein synthesis-independent early long-term potentiation (early LTP) into a protein synthesis-dependent late LTP (“reinforcement of LTP” in the hippocampal dentate gyrus (DG)) (Korz and Frey, 2003). This transformation depends on activation of mineralocorticoid receptors (MRs) by corticosterone, and on intact basolateral amygdala (BLA) function. Here, we demonstrate that a brief swim experience results in lasting changes in levels of hippocampal cellular signaling molecules that are known to be involved in the induction of late LTP. Within the DG, MRs were rapidly upregulated, whereas glucocorticoid receptor (GR) levels were elevated with a 3 h delay. Levels of phosphorylated mitogen-activated protein kinase 2 (pMAPK2) and p38 MAPK, as well as phosphorylated calcium/calmodulin-dependent protein kinase II (pCaMKII) were enhanced shortly after swim stress and remained elevated until 24 h, whereas levels of phosphorylated cAMP response element-binding protein (pCREB) remained unchanged. MR and GR were upregulated with a longer delay within the CA1 region, whereas levels of pMAPK2 and p38MAPK were rapidly increased, but the former returned to basal levels after 3 h. Levels of pCREB and pCaMKII were maintained in an enhanced state after swim stress. DG-LTP reinforcement requires a serotonergic but not dopaminergic heterosynaptic receptor activation that probably mediates the BLA-dependent modulation of LTP under stress. Thus, molecular alterations induced by specific stress resemble late LTP-related molecular changes. These changes, in interaction with stress-specific heterosynaptic processes, may support the transformation of early LTP into late LTP. The results contribute to the understanding of the rapid consolidation of cellular and possibly systemic memories triggered by stress.

Key words: CaMKII; MAP kinase; early LTP; late LTP; memory; stress; glucocorticoid receptors; mineralocorticoid receptors

Introduction

Long-term potentiation (LTP) is considered to be a cellular model for learning and memory formation. Frey and Morris (1997) suggested a novel associative mechanism required for the consolidation of LTP as well as memory formation at the cellular level named “synaptic tagging.” A weak electrically activated synaptic input inducing early LTP sets a “synaptic tag” being active for a distinct time. Plasticity-related proteins, the synthesis of which was induced by temporally related modulatory heterosynaptic inputs (e.g., triggered by a strong tetanic stimulation of a second independent synaptic input) can be captured and processed by this tag leading to a transformation into a protein synthesis-dependent late LTP (LTP reinforcement) at the tagged, weakly stimulated synapse population.

The molecular basis of LTP in slice preparations in vitro obtained from the cornu ammonis 1 region (CA1) of the rat hippocampus is intensively studied (for review, see Sweatt, 2001; Lisman et al., 2002, Kelleher et al., 2004b). However, the molecular mechanisms in vivo are less well understood, particularly for the dentate gyrus (DG) of the hippocampus. A regulation of a variety of genes encoding signal transduction molecules (Hervoni et al., 1998) and of immediate early genes involved in transcriptional processes has been identified (Abraham et al., 1991; French et al., 2001; Rodriguez et al., 2005) (for review, see Abraham and Williams, 2003). Upregulation of the phosphodiesterase PDE4B3 (Ahmed et al., 2004), α-calcium/calmodulin-dependent protein-kinase II (αCaMKII) (Davis et al., 2000), and proteins involved in spine (Yamazaki et al., 2001) and synapse morphology (Kato et al., 1997) after electrical induction of late LTP in the DG in vivo has also been reported.

Little is known about the molecular mechanisms involved in the modulation of DG-LTP by behavioral experience and stress (Abraham and Williams, 2003), although it has been pointed out that in the DG, acute stress and LTP converge on similar neuronal mechanisms (Shors and Dryer, 1994) resulting in a rapid consolidation of associative memories (Shors, 2001).

Early LTP in vivo lasting ~4–5 h can be transformed into late-LTP by unexpected reward (Seidenbecher et al., 1997; Bgardo et al., 2003) and novelty detection (Straube et al., 2003) during distinct time windows around tetanus. These kinds of LTP reinforcement under low or moderate stress conditions were dependent on β-adrenergic activation and protein-synthesis. Reinforcement by novelty experience under high acute swim stress, however, was dependent on activation of mineralocorticoid re-

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ceptors (MRs) by corticosterone on protein synthesis and on an intact basolateral amygdala (BLA), but not on β-adrenergic activation (Korz and Frey, 2003, 2005). Increased dopamine (Inoue et al., 1994; Inglis and Moghaddam, 1999; Macedo et al., 2005; Yokoyama et al., 2005) as well as serotonin (5-HT) levels (De La Garza and Mahoney, 2004; Macedo et al., 2005; Yokoyama et al., 2005) within the BLA and other brain structures during stress have also been reported.

The present study was aimed at the identification of possible molecular and heterosynaptic mechanisms underlying the reinforcement by acute swim stress focusing on molecules known to be involved in the induction of late-LTP, and on stress sensitive transmitters.

Materials and Methods

Surgery and electrophysiological recording. All animal experiments were performed in accordance with local and national regulations with respect to animal care. Male Wistar rats (8 weeks old) were anesthetized with Nembutal (40 mg/kg, i.p.). A monopolar recording electrode (insulated stainless steel, 125 μm in diameter) was implanted stereotaxically into the hilus of the DG and a bipolar stimulation electrode into the medial perforant path of the right hemisphere. During preparation, the population-spike amplitude (PSA) was optimized by delivering test pulses. The animals were allowed at least 1 week to recover from surgery.

During recording, electrodes were connected to a swivel by a flexible cable while rats were allowed to move freely in a recording box (40 x 40 x 40 cm). The animals had ad libitum access to food and water. The responses were amplified and stored on a personal computer. Biphasic constant-current pulses (0.1 ms per one-half wave) were applied to the perforant path to evoke DG field potentials of ~40% of the maximum PSA. Because the spike is required to induce LTP, the preparation was optimized to obtain a population spike, which, however, influences the dipole of the field EPSP (fEPSP) in the hilus. Therefore, the recording of the PSA was preferred against the EPSP. A stable baseline was registered for 1 h and early LTP was induced by weak tetanic bursts (three bursts of 15 pulses of 200 Hz with 0.1 ms duration of each stimulus and 10 s interburst interval) at the same stimulus intensity as used for the test pulses. The strong tetanus consisted of 10 bursts. Initially after 2 min and then every 15 min after tetanization, five test stimuli (10 s interpulse intervals) were applied and the mean values of field potentials were stored for the subsequent 8 h. For analysis and presentation, 1 h values were averaged over every four 15 min values. The next day, four 15 min values were averaged for a 24 h value. The 2 min value controlled for the achievement of a sufficient initial potentiation. Tetanization and experimental manipulations in most experiments took place between 10:00 and 11:00 A.M. to consider the diurnal rhythm of corticosterone titers. In one experiment, the behavioral manipulation took place 4 h after tetanus (i.e., 2:00–3:00 P.M.), around the 3 h time point, with similar corticosterone levels as the controls so that an impact of corticosterone on the results of this experiment is unlikely.

Swim-stress paradigm. The water tank was a white, circular, plastic tank (1.82 m diameter, 58 cm high). The water level was 38 cm and the temperature at 25 ± 2°C but constant during a session. Water was made opaque using white latex fluid (Sakret, Gießen, Germany). Naive rats were set into the tank 15 min, 1 h, or 4 h after tetanization or 1 h before tetanization and remained there for 2 min. They were then towel-dried and transferred back to the recording chamber. Towel-treatment on rats has a minor effect on the corticosterone response that does not exceed that of handling (Korz and Frey, 2003). Before swimming, the electrodes were protected from water immersion with Vaseline. Control animals remained in the recording boxes.

Protein biochemistry. The animals used for the protein biochemistry experiments were all swim naive. Fifteen minutes after swim stress the animals were killed and the right hippocampus isolated in ice-cold carbonate buffer containing 100 mM Tris-HCl, pH 7.4 containing proteinase inhibitors (Roche Diagnostic, Mannheim, Germany) and phosphatase inhibitor cocktail 1 (Sigma, St. Louis, MO). Sample protein concentrations were established using the Bradford method (Bradford, 1976). From these values, 20 μg of total protein lysates were mixed with a commercial protein loading mix (Roth-load; Carl Roth, Karlsruhe, Germany) and then subjected to SDS-PAGE (4% stacking gel, 5–20% resolving gradient gel). Electrophoresis was run with constant current (22 mA) for 3 h with constant cooling. The fractionated proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Bucks, UK) for immunoblotting. Membranes were blocked for 60–120 min with nonfat milk [5% (w/v)] dissolved in Tris-buffered saline containing 0.01% (v/v) Tween 20 (TBST) (Fluka, Ne-Ulm, Germany; Sigma), pH 7.4, then incubated overnight with gentle shaking at 4°C with primary antibody. For all analyses, single antibodies were tested and then membranes were completely stripped [two 30 min washes in strip buffer containing 0.5 M glycine, 0.1% (v/v) SDS, and 1% (v/v) Tween 20, pH 2.2, and then three 10 min washes with TBST] and retested to confirm no residual activity. The antibodies for cAMP response element binding protein (CREB) assays were anti-phospho-ser-133-CREB mouse monoclonal IgG antibody and anti CREB rabbit polyclonal IgG. The antibodies for CaMKII assays were anti-phospho-thr 286-CaMKII mouse monoclonal IgG antibody and anti-CaMKII mouse monoclonal IgG. The antibodies for mitogen-activated protein kinase (MAPK) assays were anti-phospho MAP kinase (Erk) 1/2 mouse monoclonal IgG and anti-MAP kinase 1/2 rabbit IgG from Abcam Biotechnology (Cambridge, UK) and from Zymed (San Francisco, CA). For hormone analysis, all experiments were performed in accordance with local and national regulations with respect to animal care. Male Wistar rats (8 weeks old) were anesthetized with Nembutal (40 mg/kg, i.p.). A monopolar recording electrode (insulated stainless steel, 125 μm in diameter) was implanted stereotaxically into the hilus of the DG and a bipolar stimulation electrode into the medial perforant path of the right hemisphere. During preparation, the population-spike amplitude (PSA) was optimized by delivering test pulses. The animals were allowed at least 1 week to recover from surgery.
levels were evaluated with Mann–Whitney U tests by comparing the values of the experimental groups of each time point with the controls. All tests were two-tailed and the level of significance was set at \( p < 0.05 \).

**Results**

**The effects of swim duration**

The first set of experiments addressed the question of whether different lengths of swim (i.e., the modulation of the stress experience) have different effects on LTP reinforcement, for instance, in the way that increased swim duration results in increased potentiation. We found a significant effect in the time course of LTP between the control, 2-min-swim, and 10-min-swim groups (\( F_{1,110} = 6.93; p < 0.001 \)). LTP reinforcement could be observed in animals that swim 1 h before and 15 min, 1 h, or 4 h after application of the weak tetanus and controls (\( F_{1,110} = 6.93; p < 0.001 \)). LTP reinforcement could be observed in animals that swim 1 h before (Fig. 2A) after (\( p < 0.001 \)) and 15 min (Fig. 2B) after (\( p = 0.023 \)) or 1 h (Fig. 2C) after (\( p = 0.002 \)) the weak tetanus, as compared with the (sampled) control group, whereas animals that swim 4 h after (Fig. 2D) after the tetanus showed no difference (\( p > 0.1 \)) in comparison with controls. In addition, in the 15 min (\( p = 0.03 \)) and 1 h (\( p = 0.02 \)) after-tetanus groups, as well as in the 1 h (\( p = 0.001 \)) before-tetanus group, LTP was reinforced as compared with the 4 h after-tetanus experimental group, whereas between the three first experimental groups, no difference could be noted. Thus, the functional time window that corresponds to the time window of elevated corticosterone levels is 1 h around tetanus. We noted a slightly increased potentiation in the 1 h before-tetanus swim group (Fig. 2A) that may be related to the cellular changes induced by the stress window; however, the initial potentiation is not statistically significantly different from controls.

**Molecular changes induced by swim stress**

Because of the high affinity of the MR for corticosterone, the receptors are usually saturated even at basal levels of the hormone. To address the question how the activation of the MR can then function as a stress-related cellular signal, we tested a possible regulation on the receptor level after swim stress. In addition, we tested key molecules involved in the induction of late-LTP such as CaMKII and MAPK2 or in mediating stress effects such as CREB and p38 MAP kinase. Two hippocampal subregions were analyzed by Western blotting.

**Dentate gyrus**

As indicated in Figure 3A, we found a rapid increase of MR in the dentate gyrus 15 min after swim stress (\( p = 0.021 \)). The levels remained elevated over 24 h as compared with controls (\( p < 0.05 \) each). The glucocorticoid receptor (GR) levels were also elevated, but with a delay of at least 1 h (Fig. 3B). The levels of the activated forms of both MAP kinases (p42/p44) were enhanced (Fig. 3C,D) but the level of the stress-related p38 MAPK was immediately increased up to the 3 h time point (\( p < 0.05 \)), whereas p42 MAPK increased with a 1 h delay until the 6 h time point. The levels of phosphorylated CaMKII were increased immediately after swim stress and remained elevated over 24 h (Fig. 3E). Surprisingly we found no enhancement of phosphorylated CREB (pCREB) after swim stress (Fig. 3F).

**CA1 area**

In contrast with the DG, we found a delayed increase of MR after 1 h and a similar delay of GR with a significant increase after 3 h, as compared with the DG (Fig. 4A,B). The levels of phosphorylated 42/44 and 38 MAP kinases were potentiated immediately after swim stress and returned to basal levels within 24 h (Fig. 4C,D). Similar to the dentate gyrus, pCaMKII levels were imme-

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**Figure 1.** A. LTP after a 2 min (\( n = 9 \)) and a 10 min (\( n = 7 \)) swim, 15 min after application of a weak tetanus. Swimming animals showed significantly prolonged LTP as compared with non-swimming controls (\( n = 10 \)). B. Time course of serum corticosterone levels after a 2 min swim. Levels are significantly increased 15 min and 8 h and significantly reduced 24 h after swimming (\( n = 6 \) for controls; \( n = 8 \) for each experimental group). Given are the means and SEM. Asterisks indicate significant differences for the respective time points as compared with controls. The data set in A (15 min group) was partially adopted from Korz and Frey (2003) and complemented additional experiments.
diately increased and remained enhanced over 24 h (Fig. 4E). Again, in contrast with the DG, we found increased levels of pCREB after 1 h in the CA1 (Fig. 4F).

Neurotransmitters involved in swim stress reinforced LTP
LTP reinforcement was mediated by serotonergic (Fig. 5A) but not dopaminergic (Fig. 5B) activation. A significant difference in LTP between dopamine and serotonin receptor antagonist-treated animals and the two control groups could be noted ($F_{(3,30)} = 3.01$; $p < 0.05$). Post hoc tests revealed a significant difference in LTP between animals treated with a serotonin receptor antagonist and the (time matched) controls ($p = 0.021$ and 0.019, respectively), and with the dopamine receptor antagonist treated group ($p = 0.018$). No significant difference was detectable between the other groups. Furthermore, the serotonergic effect is stress specific, as indicated by an electrically induced late LTP that was independent of serotonergic activation (Fig. 5C).

Discussion
Functional time windows
There is a sensitive time window of $\sim 1$ h (compare Fig. 2) around tetanus in which swim stress reinforces DG-LTP and in which corticosterone levels were upregulated, respectively. We conclude from these results that hormonal and molecular changes that are directly involved or induce required processes within this time window are of special importance for the conversion of early into late LTP. Subsequent changes may be related to other neuronal processes or daily rhythms.

Molecules involved in LTP reinforcement
Mineralocorticoid receptors
The fast upregulation of MR receptors, which have been identified to being involved in swim stress-reinforced LTP (Korz and Frey, 2003), may be based on transcriptional and nontranscriptional mechanisms. Castren et al. (1995) found a glucocorticoid-induced increase of MR mRNA and protein in cultured hippocampal neurones by direct activation of the MR promoter by corticosterone indicating, at least in part, a transcriptional regulation of MR. At this, the combination of different weak enhancer glucocorticoid-responsive elements led to an MR gene expression by both types of corticosteroid receptors. In addition to the transcriptional regulation of glucocorticoid-responsive genes by homodimers (Arriza et al., 1988; de Kloet, 1991; Karst et al., 2000), a regulatory role of GR-MR-heterodimers has been described (Trapp et al., 1994). The formation of heterodimers in vitro takes place within the nucleus and depends on the concentration of corticosterone, suggesting a particular role in transcriptional activity under stress conditions (cf. Castren et al., 1995). A MAPK-dependent nontranscriptional pathway in vivo has been described by Müller Igaz et al. (2004). They found an upregulation of CaMKII- and MAPK2-mRNAs 3 h after a one-trial aversive learning, whereas the protein levels were elevated only 24 h after training, which was explained by an early transient translational suppression of the transcripts. In our study, the translation of pre-existing MR mRNA may also initially be suppressed and then, triggered by acute corticosterone signaling, induced by stress and novelty detection. The importance of translational control by MAPK signaling in the...
formation of hippocampus-dependent long-term memory and LTP has been pointed out by Kelleher et al. (2004a).

Mitogen-activated protein kinases
PKA, PKC, and MAPK pathways are also involved in corticosterone-induced gene transcription (Budziszewska et al., 2000), stress-related synaptic plasticity (Yang et al., 2004), and fear conditioning (Sweatt, 2001; Sananbenesi et al., 2003; Revest et al., 2005). Therefore, LTP reinforcement cannot be correlated alone to transcription caused by activated MR complexes, but possibly caused by processes downstream from the interaction sites within the MAPK pathways. Both the p38 and the p42/44 MAP kinases were enhanced within 1 h after stress within the DG, making these proteins suitable as putative candidates for these interactions. Navakkode et al. (2005) found p42/44 MAPKs to have a pivotal role in the maintenance of an associative long-term depression in the CA1 region in hippocampal slice experiments in vitro. These results may favor the role of the p42/44 over the p38 isoform in our study, pointing to different region-specific functions or different effects in dependence of the preparation (in vitro/in vivo) of the MAPK isoforms.

Calcium-calmodulin-dependent protein kinase
CaMKII is a crucial molecule, being involved in the induction of CA1-LTP and in memory formation (Malinow et al., 1988; Lisman et al., 2002; Ahmed and Frey 2005). However, because LTP reinforcement by swim stress depends on MR and 5-HT receptor activation, an interaction of CaMKII and one or both of these receptors has to be assumed. To our knowledge, for MR receptors, such an interaction has not yet been described. At least some evidence for an interplay between serotonin receptors and CaMKII in hippocampus-dependent learning (Moyano et al., 2004; Schiapparelli et al., 2005) and serotonin-dependent changes in CaMKII autophosphorylation in the hippocampus has been discussed (Popoli et al., 1995). Thus, CaMKII in the DG may contribute to LTP reinforcement by yet unknown mechanisms during stress.

cAMP response element-binding protein
The surprising result of the lack of enhanced pCREB within the DG may be an outcome of glucocorticoid effects. Legradi et al. (1997) report that glucocorticoids suppress CREB phosphorylation, a feedback mechanism for the inhibition of corticotropin-releasing hormone (CRH) biosynthesis in the hypothalamus. In our study, a similar, long-lasting effect seems to take place in the DG, but not the CA1. The results for the DG contrast with those of Bilang-Bleuel et al. (2002) who noted an enhancement of pCREB in the DG after swim stress. However, in this study, either stress or exposure to a cold environment were ineffective on enhancing CREB phosphorylation, pointing to a stressor-specific pCREB response. Furthermore, Shen et al. (2004) found an increase of activated p38 MAPK but not p42/44 MAPK within the hippocampus under similar swim-stress conditions. Thus, these seemingly contradictory results may be explained by the different
stress procedures and/or rat strains used. These authors forced the rats to swim for 15 min in a small (20 or 24 cm in diameter) glass cylinder, which may cause an escalated stress response, whereas in our study, the animals were allowed to explore a water arena for 2 or 10 min, inducing only a transient increase in corticosterone. The first procedure is very likely distinguished through uncontrollability, whereas the second provides the possibility to escape from the situation. Uncontrollability of a stressful situation impairs LTP, in contrast to controllable stress (Shors et al., 1989).

Neurotransmitter systems involved in LTP reinforcement

The balance of occupied MR and GR, rather than the individual dynamics of the different receptors, determine the physiological functions regulated by these receptors (de Kloet et al., 2005). In addition, monoamine neurotransmitters can modulate densities of GR and MR within the hippocampus. Whereas most studies report increased levels of hippocampal serotonin (Connor et al., 1999; Linthorst et al., 2002; Umriukhin et al., 2002) after swim stress, which may be also functional during cognitive tasks in the water maze (Richter-Levin et al., 1994), others found no change (Kirby et al., 1997). These opposing effects may be related to the specificity of central serotoninergic responses to stress and the interplay of serotoninergic transmission, corticoids, and stress to different experimental designs (Chaouloff, 2000). Additionally, an increase of 5-HT levels (De La Garza and Mahoney, 2004; Macedo et al., 2005; Yokoyama et al., 2005) and 5-HT turnover in the amygdala (Kellhner et al., 2000), as well as changes in the activity of the cortical synaptosomal serotonin transporter (Racca et al., 2005), have been observed after swim stress or conditioned fear. 5-HT has been reported to increase both the GR (Racca et al., 2005), have been observed after swim stress or controllable stress (Korz and Frey, 2005), but because shown that an intact basolateral amygdala is required for the LTP reinforcement. It has been shown that dopaminergic (Berger et al., 1976; Goldstein et al., 1996; Rosenkranz and Grace, 1999) and noradrenergic (Roozendaal et al., 2004) activation of the amygdala mediates the communication with the prefrontal cortex during stress so that a prefrontal contribution to LTP reinforcement is unlikely in our study. However, brain structure-specific activations remain to be identified. Recently, we have shown that an intact basolateral amygdala is required for the LTP reinforcement by swim stress (Korz and Frey, 2005), but because of the intracerebroventricular application of antagonists in our studies, it is still unclear whether the MR or the serotoninergic effect is required in both structures, the hippocampus and the amygdala, or in only one of these structures.

However, the present data show that a brief stressful experience induces lasting changes in cellular signaling in the DG that partly resemble electrically induced late LTP. Mechanisms triggered by these signals in interaction with stress-specific changes may support the transformation from early into late LTP. The results contribute to the understanding of the rapid consolidation of cellular and possibly systemic memories triggered by stress.

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