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Glucocorticoid receptors recruit the CaMKIIα-BDNF-CREB pathways to mediate memory consolidation

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Emotionally important events are well remembered. Although memories of emotional experiences are known to be mediated and modulated by stress hormones such as glucocorticoids, little is known about the underlying molecular mechanisms. We found that the hippocampal glucocorticoid receptors that are critically engaged during the formation of long-term inhibitory avoidance memory in rats were coupled to the activation of CaMKIIα, TrkB, ERK, Akt, PLCγ and CREB, as well as a to a substantial induction of Arc and synaptic GluA1. Most of these changes, which are initiated by a nongenomic effect of glucocorticoid receptors, were also downstream of the activation of brain-derived neurotrophic factor (BDNF). Hippocampal administration of BDNF, but not of other neurotrophins, selectively rescued both the amnesia and the molecular impairments produced by glucocorticoid receptor inhibition. Thus, glucocorticoid receptors mediate long-term memory formation by recruiting the CaMKIIα-BDNF-CREB–dependent neural plasticity pathways.

Emotionally relevant events, whether positive or negative, are well remembered, and single episodes become long-lasting memories if experienced with a certain level of stress or arousal[1]. Conversely, very high levels of stress or chronic stress lead to amnesia, cognitive impairments and neurodegeneration and contribute to disorders such as depression and anxiety[2–4].

The positive effect of stress and arousal on memory consolidation is likely an adaptive mechanism that has evolved to assure that important information is retained. An acute aversive or traumatic experience induces the activation of several hormonal and neurotransmitter systems, which include the stress hormones glucocorticoids (cortisol in humans and corticosterone in rodents). Glucocorticoids mediate and modulate memory consolidation[5], the process that stabilizes a newly formed memory[6]. Glucocorticoids exert their actions directly on brain regions, including the hippocampus, amygdala and prefrontal cortex, that are enriched in glucocorticoid receptors and are important for long-term memory formation[7].

Although several molecular correlates have been shown to accompany chronic stress and its negative effects on cognition[8], the molecular mechanisms that are critically recruited by positive, adaptive levels of stress and arousal that are critical to transform a learning event into a long-term memory have remained elusive, with the exception that glucocorticoid receptors in the hippocampus enhance contextual fear memory via MAPK-Zif268 activation[9] and the subsequent expression regulation of Synapsin-1a/b[10]. We used the inhibitory avoidance learning task in rats to identify the intracellular pathways activated by glucocorticoid receptors in the hippocampus. We found that, to mediate memory consolidation, glucocorticoid receptors recruit the plasticity and survival pathways activated via calcium calmodulin kinase II α (CaMKIIα), BDNF, tropomyosin-related kinase B (TrkB) and cAMP response element–binding protein (CREB).

RESULTS

RU486 impairs inhibitory avoidance memory

To examine the role of hippocampal glucocorticoid receptors in long-term inhibitory avoidance memory formation, we bilaterally injected groups of rats with either the glucocorticoid receptor antagonist RU38486 (RU486)[11] or vehicle into the dorsal hippocampus 15 min before or immediately after training elicited by a 0.6-mA foot shock. Memory retention was tested 2 d (test 1) and 7 d (test 2) after training (**Supplementary Tables 1–6**). Compared with vehicle, RU486 completely disrupted memory retention at test 1 when injected before training ($P < 0.05$; **Fig. 1a**,**b** and **Supplementary Table 1**). The effect persisted at test 2. A reminder shock in a different context, 1 d after test 2, did not rescue the memory tested 1 d later (test 3; **Fig. 1a**,**b** and **Supplementary Table 1**). The RU486-mediated memory impairment was not a result of nonspecific locomotor changes (**Supplementary Fig. 1**).

To determine whether a more traumatic memory, elicited by a stronger foot shock, is similarly regulated by glucocorticoid receptors, we subjected rats to the same protocol, except that the training was carried out with a 0.9-mA foot shock (**Fig. 1c**,**d** and **Supplementary Table 1**). Compared with vehicle, RU486 injected before training significantly decreased retention at both test 1 and test 2 ($P < 0.01$; **Fig. 1c** and **Supplementary Table 1**). No re-instatement was seen after a 0.9-mA reminder foot shock in a different context 1 d after test 2 (test 3; **Fig. 1c** and **Supplementary Table 1**). However, the same dose of RU486 injected after training had no significant effect on memory retention (**Fig. 1d** and **Supplementary Table 1**), indicating that, consistent with the decreased effect seen with a post-training injection after a 0.6-mA training, RU486 affects long-term memory formation in a very rapid fashion. Furthermore, RU486 injected before 0.9-mA foot shock training did not affect short-term memory tested at 1 h

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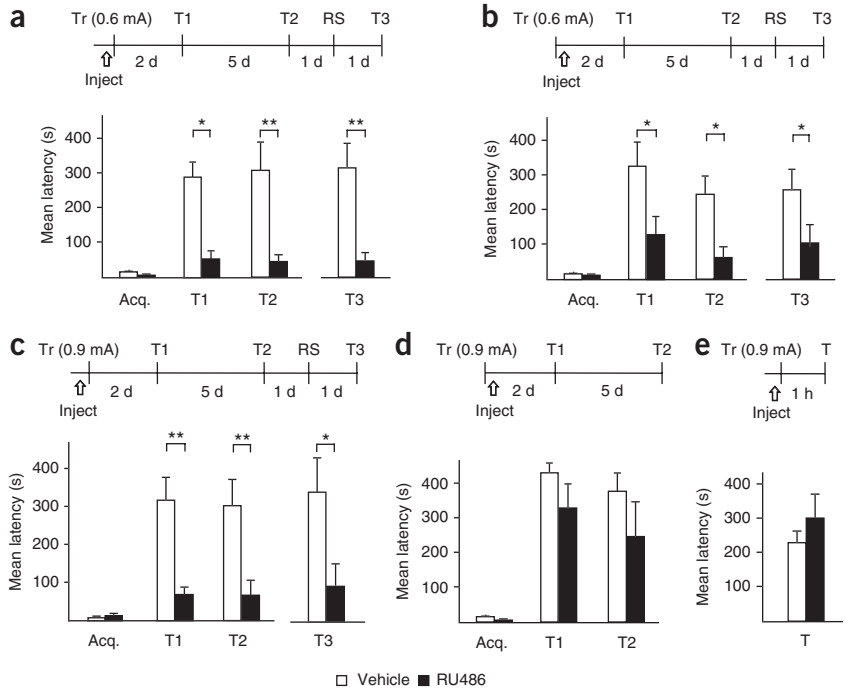

Figure 1 Hippocampal glucocorticoid receptor activation is required for long-term, but not short-term, inhibitory avoidance memory. Data are expressed as mean latency ± s.e.m. Latency scores and *n* can be found in **Supplementary Table 1**. Experimental schedule is shown above each figure. (**a**–**e**) Rats were given hippocampal injections (⇧) of vehicle or RU486 either 15 min before (**a**,**c**,**e**) or immediately after (**b**,**d**) training elicited with a 0.6-mA (**a**,**b**) or 0.9-mA foot shock (**c**–**e**). (**a**,**b**) Two-way ANOVA comparing the effect of treatment (**a**, $F_{1,20} = 24.07$, $P < 0.0001$; **b**, $F_{1,20} = 12.85$, $P = 0.0019$) and time (test 1 (T1) and 2 (T2); **a**, $F_{1,20} = 0.06$, $P = 0.80$; **b**, $F_{1,20} = 0.98$, $P = 0.33$) and time × treatment interaction (**a**, $F_{1,20} = 0.38$, $P = 0.54$; **b**, $F_{1,20} = 0.017$, $P = 0.90$) followed by Bonferroni *post hoc* tests (**a**, $P = 0.004$; **b**, $P = 0.043$). (**c**,**d**) Two-way ANOVA comparing the effect of treatment (**c**, $F_{1,20} = 22.92$, $P < 0.0001$; **d**, $F_{1,22} = 4.20$, $P = 0.053$), time (T1 and T2; **c**, $F_{1,20} = 0.03$, $P = 0.87$; **d**, $F_{1,22} = 1.02$, $P = 0.32$) and time × treatment interaction (**c**, $F_{1,20} = 0.015$, $P = 0.90$; **d**, $F_{1,20} = 0.038$, $P = 0.85$) followed by Bonferroni *post hoc* tests (**c**, $P = 0.049$). (**e**) Student's *t* test, $P = 0.37$. Acq., acquisition; Tr, training; T, test; RS, reminder shock. $^*P < 0.05$, $^{**}P < 0.01$, Student's *t* test.

(**Fig. 1e** and **Supplementary Table 1**), confirming that the long-term memory impairment was not a result of nonspecific effects on task performance. Thus, we can conclude that hippocampal glucocorticoid receptors rapidly regulate mechanisms that are essential for the formation of long-term inhibitory avoidance memory without affecting its short-term retention.

Glucocorticoid receptor signaling following training

To determine which molecular pathways are coupled to the learning-dependent glucocorticoid receptor activation required for long-term memory, we examined the effect of RU486 on numerous molecular changes that were previously established to underlie long-term synaptic plasticity and memory formation. For all biochemical studies, we used inhibitory avoidance training elicited by 0.9-mA foot shock, as stronger conditioning correlates with a higher degree of molecular changes, which can therefore be more easily quantified. Specifically, we tested the effect of RU486 on the phosphorylation and/or expression levels of different classes of proteins that are known to accompany long-term plasticity, including the phosphorylation of the kinases CaMKIIα[12], extracellular signal–regulated kinases (ERK1/2), Akt, phospholipase Cγ (PLCγ)[13], and mitogen and stress-activated kinase 1 (MSK1)[14], phosphorylation of the synaptic protein Synapsin-1 (ref. 15) and the transcription factor CREB[16], the expression of the immediate early gene *Arc* (activity-regulated cytoskeletal-associated protein) and of the AMPA receptor subunit GluA1 (also known as GluR1)[17]. Dorsal hippocampal extracts were taken from rats injected intrahippocampally with either RU486 or vehicle 15 min before training and killed either 30 min (**Fig. 2a**, **Supplementary Fig. 2a** and **Supplementary Table 2**) or 20 h after training (**Fig. 2b**, **Supplementary Fig. 2b** and **Supplementary Table 2**) to survey the learning-induced molecular changes over time. Controls consisted of naive rats injected with vehicle and killed at matched time points. Both total cell and synaptoneurosomal extracts were investigated at both time points using quantitative western blot analyses. At 30 min after training, there was a significant increase in pCREB phosphorylation at Ser133 ($P < 0.01$; pCREB) compared with naive conditions without a change in CREB levels in total cell lysates, consistent with previous findings[18,19]. In the same extracts, there was also a significant increase in Arc ($P < 0.001$) and, in the synaptoneurosomal fraction, a significant elevation in CaMKIIα phosphorylation at Thr286 ($P < 0.01$; pCaMKIIα) and GluA1 levels ($P < 0.01$). All these increases were completely blocked by RU486 (pCREB, $P < 0.05$; Arc, $P < 0.05$; pCaMKIIα, $P < 0.01$; GluA1, $P < 0.05$; **Fig. 2a** and **Supplementary Table 2**). In contrast, the levels of CaMKIIα, pMSK1 (Thr581), a substrate of ERK that can activate the transcription factor CREB[20], and pSynapsin-1 (Ser603), a downstream presynaptic target of activated CaMKIIα[21], were unaffected by either training or RU486 (**Supplementary Fig. 2a** and **Supplementary Table 2**). All of the significant training-induced changes were selective for the paired context–foot shock conditioning and were not found in hippocampi of control rats that were exposed to an immediate shock or an unpaired experience (except for Arc induction, which is likely a result of context exposure[22]), which are both known to not elicit inhibitory avoidance and fear conditioning[23–25] (**Supplementary Fig. 3**). Training did not change the levels or phosphorylation of hippocampal ERK1/2 (Thr202/Tyr204, pERK), Akt (Ser473, pAkt) and PLCγ (Tyr783, pPLCγ). However, RU486 treatment significantly decreased the level of phosphorylation of ERK ($P < 0.05$ for pERK1 and pERK2), Akt ($P < 0.05$) and PLCγ ($P < 0.01$) without changing the respective total protein levels in trained rats (**Fig. 2a** and **Supplementary Table 2**). Given that the phosphorylation of ERK1/2, Akt and PLCγ is known to be activated by BDNF[13], we tested whether the level of phosphorylation of the BDNF receptor TrkB at Tyr817 (pTrkB) was altered, and found that RU486 treatment significantly decreased phosphorylation of TrkB ($P < 0.05$) without affecting TrkB levels (**Fig. 2a** and **Supplementary Table 2**).

Hippocampal injections of RU486 in naive rats also produced a significant decrease in the levels of pCaMKIIα ($P = 0.025$) and pAkt ($P = 0.0064$), as well as a nonsignificant trend toward a decrease in the levels of pCREB ($P = 0.1360$), pTrkB ($P = 0.2723$), pERK ($P = 0.5985$ for pERK1, $P = 0.3435$ for pERK2) and pPLCγ ($P = 0.3019$) 45 min after the injection (same post-injection time point as of 30 min

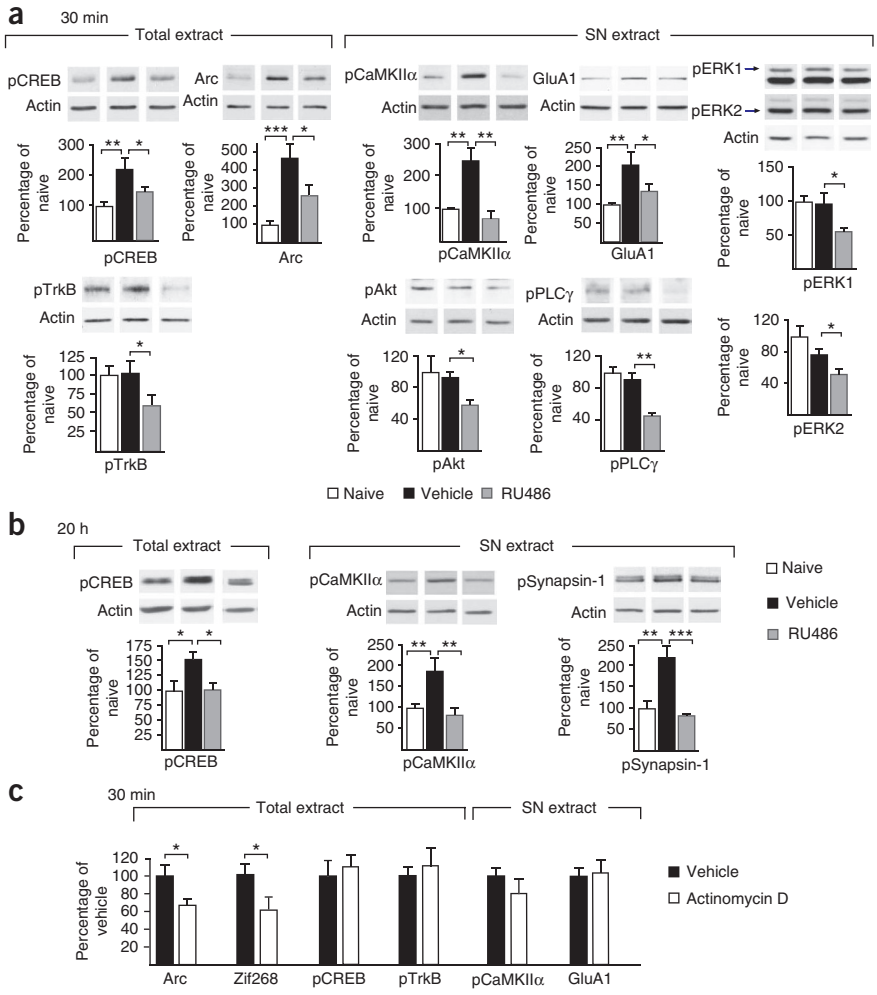

Figure 2 Molecular pathways coupled to hippocampal glucocorticoid receptors following inhibitory avoidance training. Mean percentage, *n* and ANOVA *F* values can be found in **Supplementary Table 2**. (**a**) Example (full-length blots images shown in **Supplementary Fig. 8**) and densitometric quantitative western blot analyses of hippocampal total extracts and synaptoneurosomal (SN) extracts from naive or trained rats injected with either vehicle or RU486 15 min before training and killed 30 min after training (normalized to actin). Data are expressed as mean percentage ± s.e.m. of naive rats injected with vehicle (one-way ANOVA followed by Newman-Keuls *post hoc* test for all markers and Student's *t* test for pTrkB and pAkt; pCREB, $F_{2,25} = 6.23$, $P = 0.0069$; pTrkB, $F_{2,26} = 3.564$, $P = 0.044$; Arc, $F_{2,22} = 10.89$, $P = 0.0006$; pCaMKIIα, $F_{2,25} = 14.48$, $P < 0.0001$; GluA1, $F_{2,24} = 5.464$, $P = 0.0118$; pERK1, $F_{2,23} = 4.813$, $P = 0.019$; pERK2, $F_{2,27} = 9.34$, $P = 0.0009$; pAkt, $F_{2,16} = 3.75$, $P = 0.049$; pPLCγ, $F_{2,22} = 9.63$, $P = 0.0012$). (**b**) Examples and densitometric quantitative western blot analyses of hippocampal total extracts and SN extracts from naive or trained rats injected with vehicle or RU486 15 min before training and killed 20 h after training (normalized to actin). Data are expressed as mean percentage ± s.e.m. of naive rats injected with vehicle (one-way ANOVA followed by Newman-Keuls *post hoc* test; pCREB, $F_{2,23} = 4.57$, $P = 0.0225$; pCaMKIIα, $F_{2,21} = 8.05$, $P = 0.0029$; pSynapsin-1, $F_{2,21} = 11.2$, $P = 0.0006$). (**c**) Densitometric quantitative western blot analyses of hippocampal total extracts and SN extracts from trained rats injected with vehicle or actinomycin D 15 min before training and killed 30 min after training (normalized to actin). Data are expressed as mean percentage ± s.e.m. of trained rats injected with vehicle (Student's *t* test; Arc, $P = 0.0309$; Zif268, $P = 0.0445$; pCREB, $P = 0.6230$; pTrkB, $P = 0.5590$; pCaMKIIα, $P = 0.3607$; GluA1, $P = 0.8214$). Full-length western blots are shown in **Supplementary Figures 8–11** and **13–16**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

after training), indicating that glucocorticoid receptors are coupled to the activation of these factors even in basal (nontrained) conditions (**Supplementary Fig. 4**).

Compared with naive rats, the hippocampi of rats 20 h after training showed a significant elevation in the expression of pCREB ($P < 0.05$), pCaMKIIα ($P < 0.01$) and pSynapsin-1 ($P < 0.01$; **Fig. 2b** and **Supplementary Table 2**) and no change in their respective total levels (**Supplementary Table 2**). The increase in pCREB, pCaMKIIα and pSynapsin-1 expression was completely blocked by RU486 (**Fig. 2b** and **Supplementary Table 2**). In contrast, neither training nor RU486 treatment changed the levels of pTrkB, pERK1/2, pAkt, their respective total protein levels or GluA1 at 20 h after training compared to naive conditions (**Supplementary Fig. 2b** and **Supplementary Table 2**).

Given the relatively rapid effect of the glucocorticoid receptor–dependent molecular changes, we asked whether these changes are dependent on genomic or nongenomic regulation. We tested whether the RNA synthesis inhibitor actinomycin D affected the training-induced molecular changes. Dorsal hippocampal extracts from trained rats injected intra-hippocampally with either vehicle or an effective dose of actinomycin D[26] 15 min before training were killed 30 min after training and investigated using quantitative western blot analyses. Actinomycin D did not alter the training-related induction of pTrkB, pCaMKIIα, pCREB and GluA1, indicating that these changes were nongenomic. In contrast, actinomycin D decreased the training-dependent Arc induction ($P = 0.0309$, **Fig. 2c** and **Supplementary Table 2**), indicating that this change required transcription. The effectiveness of the actinomycin D treatment was confirmed by the significant inhibition of the training dependent induction of another immediate early gene, *Zif268* (also known as *Egr1*, $P = 0.0445$; **Fig. 2c** and **Supplementary Table 2**).

Thus, the training-dependent glucocorticoid receptor activation in the hippocampus is coupled to the phosphorylation of TrkB, ERK1/2, Akt and PLCγ, events that are known to constitute the cellular response to BDNF[13]. Furthermore, glucocorticoid receptor activation is coupled to a sustained phosphorylation of CaMKIIα and CREB. Finally, glucocorticoid receptors also regulate the learning-dependent increase of Arc, synaptic GluA1 and pSynapsin-1, indicating that they affect both pre- and postsynaptic mechanisms. In summary, to mediate memory consolidation, glucocorticoid receptors recruit the CaMKIIα-BDNF-CREB–mediated synaptic plasticity pathways.

Inhibitory avoidance memory requires BDNF-TrkB signaling

The overlap between the glucocorticoid receptor–dependent molecular changes found in the hippocampus of trained rats with the known

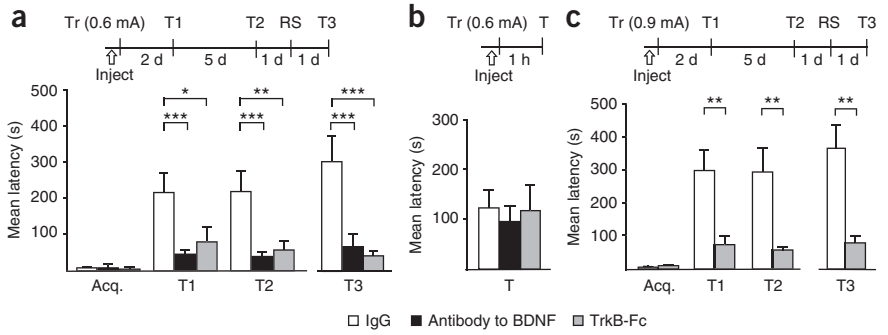

Figure 3 Hippocampal BDNF is required for long-term, but not short-term, inhibitory avoidance memory. Data are expressed as mean latency ± s.e.m. Latency scores and *n* can be found in **Supplementary Table 3**. Experimental schedule is shown above each figure. (**a**–**c**) Rats were given hippocampal injections (⇧) of either IgG, antibody to BDNF or TrkB-Fc 15 min before training elicited with a 0.6-mA (**a**,**b**) or 0.9-mA foot shock (**c**). (**a**) Two-way ANOVA comparing the effect of treatment ($F_{2,84} = 15.66$, $P < 0.0001$) and time (T1 and T2; $F_{1,84} = 0.105$, $P = 0.747$), treatment × time interaction ($F_{2,84} = 0.073$, $P = 0.929$) followed by Bonferroni *post hoc* tests; one-way ANOVA comparing the effect of treatment on T3 ($F_{2,44} = 9.842$, $P = 0.003$) followed by Newman-Keuls *post hoc* test. (**b**) One-way ANOVA ($F_{2,26} = 0.1348$, $P = 0.8745$). (**c**) Two-way ANOVA comparing the effect of treatment ($F_{1,22} = 21.21$, $P < 0.0001$), and time (T1 and T2; $F_{1,22} = 0.041$, $P = 0.8413$) and treatment × time interaction ($F_{1,22} = 0.0143$, $P = 0.9058$) followed by Bonferroni *post hoc* tests; Student's *t* test for T3. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

BDNF-coupled molecular pathways led us to test whether BDNF in the hippocampus is essential for inhibitory avoidance long-term memory formation. BDNF was previously shown to be required for memory formation in different learning tasks[27].

Injection of either a function-blocking antibody to BDNF or BDNF-sequestering TrkB-Fc chimera into the dorsal hippocampus 15 min before training elicited by either 0.6- or 0.9-mA foot shock markedly and persistently disrupted memory retention at both 2 and 7 d after training compared with IgG injection, and had no effect on short-term memory (**Fig. 3** and **Supplementary Table 3**). Furthermore, memory was not re-instated by a reminder foot shock given a day later (test 3; **Fig. 3a,c** and **Supplementary Table 3**). The effects of antibody to BDNF on long-term memory retention were not a result of nonspecific effects on locomotion (**Supplementary Fig. 5**). Thus, hippocampal BDNF is critical for long-term, but not short-term, inhibitory avoidance memory formation.

BDNF-TrkB and glucocorticoid receptor pathways overlap

To confirm that the training-induced BDNF-dependent changes were the same as those that we found coupled to glucocorticoid receptor activation, we investigated the effects of blocking BDNF 15 min before training with an antibody to BDNF on the same molecular mechanisms examined following RU486 treatment. Quantitative western blot analyses of total cell and synaptoneurosomal lysates of rat dorsal hippocampi taken at either 30 min (**Fig. 4a** and **Supplementary Table 4**) or 20 h after training (**Fig. 4b**, **Supplementary Fig. 6** and **Supplementary Table 4**) were performed. Control hippocampal extracts were from naive rats injected with IgG and killed at matched time points. At 30 min after training, compared to IgG, antibody to BDNF blocked the training-related significant increase in pCREB ($P < 0.05$) without affecting CREB levels (**Fig. 4a** and **Supplementary Table 4**), and no effect was found on the training-related significant induction of Arc ($P < 0.01$; **Fig. 4a** and **Supplementary Table 4**). In the synaptoneurosomal fraction, similarly to what was found with RU486, antibody to BDNF significantly lowered the levels of pERK1/2 ($P < 0.05$) and PLCγ ($P < 0.05$) compared with either naive or trained rats injected with IgG and resulted in a strong trend toward a decrease, although not significant, of pAkt ($P > 0.05$) without changing Akt (**Fig. 4a** and **Supplementary Table 4**). In contrast to RU486, antibody to BDNF had no effect on the training-related increase in pCaMKIIα, synaptic GluA1 and total level of CaMKIIα (**Fig. 4a** and **Supplementary Table 4**).

Bilateral hippocampal injection of antibody to BDNF in naive rats did not change the levels of pCREB, pERK1/2, pAkt or pPLCγ (**Supplementary Fig. 6**), indicating that blocking BDNF in basal (nontrained) conditions had no effect on the molecular changes elicited by training. At 20 h after training, similarly to what was found with RU486, antibody to BDNF blocked the learning-dependent increase in phosphorylation of CREB, CaMKIIα and Synapsin-1 compared with IgG treatment (**Fig. 4b** and **Supplementary Table 4**), without changing their total levels (**Supplementary Table 4**). Furthermore, similarly to RU486 treatment, antibody to BDNF did not affect pERK1/2 and pAkt, or their respective total protein levels, at 20 h after training (**Supplementary Fig. 7** and **Supplementary Table 4**).

Thus, long-term inhibitory avoidance memory formation critically recruits the BDNF-dependent pathways, which largely overlap with those downstream of glucocorticoid receptors. Moreover, more convergence of the two pathways was found at 20 h after training, when both antibody to BDNF and RU486 treatment blocked the training-dependent long-lasting increase in pCREB, pCaMKIIα and pSynapsin-1.

BDNF selectively rescues the amnesia caused by RU486

Given the overlap between the intracellular activation pathways coupled to glucocorticoid receptors and the BDNF-TrkB signaling pathway in the hippocampus during memory formation, we investigated whether BDNF rescues the memory impairment produced by glucocorticoid receptor inhibition. Rats were bilaterally injected with either RU486 or vehicle into the hippocampus 15 min before training elicited with a 0.9-mA foot shock. The RU486-injected rats were injected immediately after training with either recombinant BDNF or vehicle. The rats that received vehicle injections before training received another vehicle injection immediately after training. All rats were tested 2 and 7 d after training (tests 1 and 2, respectively). Confirming our previous results, RU486 significantly impaired memory retention at both 2 and 7 d after training compared with vehicle ($P < 0.05$; **Fig. 5a** and **Supplementary Table 5**). BDNF significantly and persistently rescued the amnesia ($P < 0.01$; **Fig. 5a** and **Supplementary Table 5**).

To confirm these data and target memory consolidation independently from the learning phase, as well as to test the specificity of the BDNF effect, we co-injected the same concentration of RU486 with BDNF, nerve-growth factor (NGF), Neurotrophin-3 (NT-3) or vehicle immediately after training. Confirming the previous results, RU486 significantly impaired long-term memory retention tested 2 and 7 d after training ($P < 0.001$; tests 1 and 2, respectively) compared with vehicle. BDNF, but not NGF or NT-3 co-administration, significantly and persistently rescued memory retention ($P < 0.05$; **Fig. 5b** and **Supplementary Table 5**). Notably, BDNF injections alone immediately after training did not change memory retention (**Fig. 5b** and

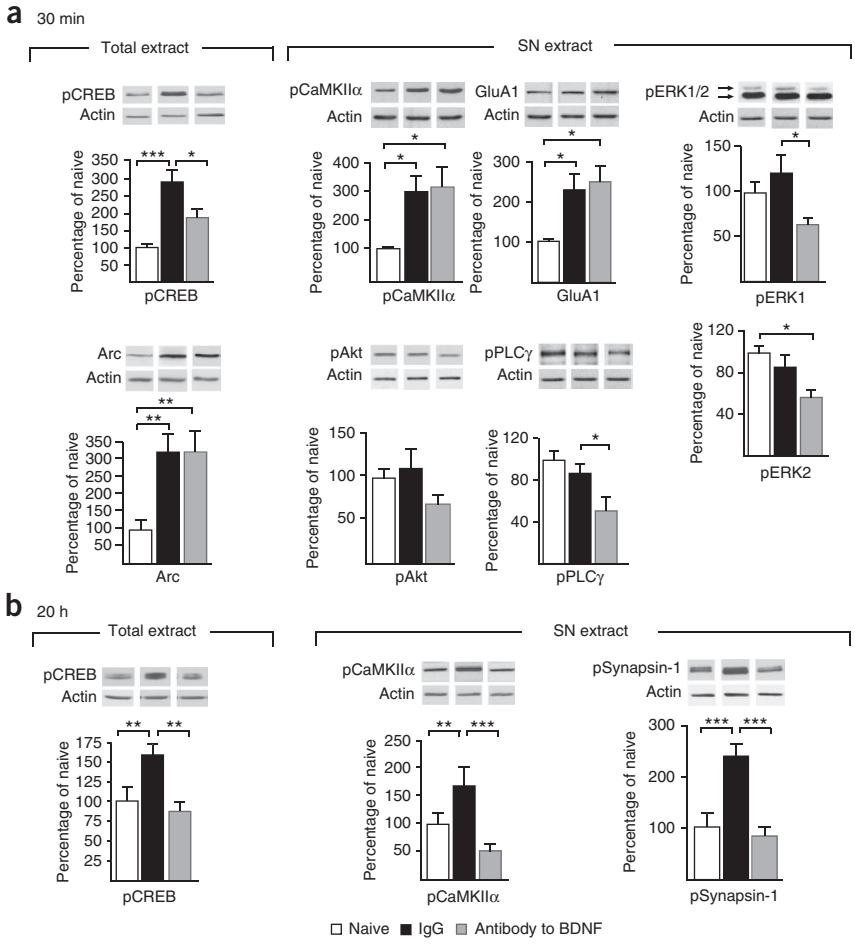

Figure 4 Molecular pathways coupled to BDNF following inhibitory avoidance training. Mean percentage, *n* and ANOVA *F* values can be found in **Supplementary Table 4**. (**a**) Examples and densitometric quantitative western blot analyses of hippocampal total extracts and synaptoneurosomal (SN) extracts from naive and trained rats injected with either IgG or antibody to BDNF 15 min before training and killed 30 min after training (normalized to actin). Data are expressed as mean percentage ± s.e.m. of naive rats injected with IgG (one-way ANOVA followed by Newman-Keuls *post hoc* tests; pCREB, $F_{2,14} = 15.61$, $P = 0.0005$; Arc, $F_{2,19} = 9.093$, $P = 0.0021$; pCaMKIIα, $F_{2,22} = 4.975$, $P = 0.0176$; GluA1, $F_{2,17} = 4.444$, $P = 0.0305$; pERK1, $F_{2,25} = 4.28$, $P = 0.0275$; pERK2, $F_{2,25} = 5.22$, $P = 0.0135$; pPLCγ, $F_{2,14} = 8.422$, $P = 0.0052$; pAkt, $F_{2,16} = 2.352$, $P = 0.1316$). (**b**) Examples and densitometric quantitative western blot analyses of hippocampal total extracts and SN extracts from naive and trained rats given hippocampal injections of IgG or antibody to BDNF 15 min before training and killed 20 h after training (normalized to actin). Data are expressed as mean percentage ± s.e.m. of naive rats injected with IgG (one-way ANOVA followed by Newman-Keuls *post hoc* test; pCREB, $F_{2,23} = 7.809$, $P = 0.0029$; pCaMKIIα, $F_{2,25} = 10.17$, $P = 0.0007$; pSynapsin-1, $F_{2,20} = 15.67$, $P = 0.0001$). Full-length western blots for are shown in **Supplementary Figures 9–12, 14** and **15**. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Supplementary Table 5). We conclude that BDNF selectively rescues the amnesia caused by hippocampal glucocorticoid receptor inhibition.

BDNF does not rescue propranolol-induced amnesia

Noradrenaline, similarly to corticosterone, is released in response to stress and substantially modulates memory consolidation[28,29]. Administration of noradrenaline, both peripherally and intracerebrally, enhances memory retention and, conversely, antagonists to its β-adrenergic receptors block memory consolidation. Similarly to glucocorticoid receptor, β-adrenergic receptors are abundantly expressed in the hippocampus[28,30].

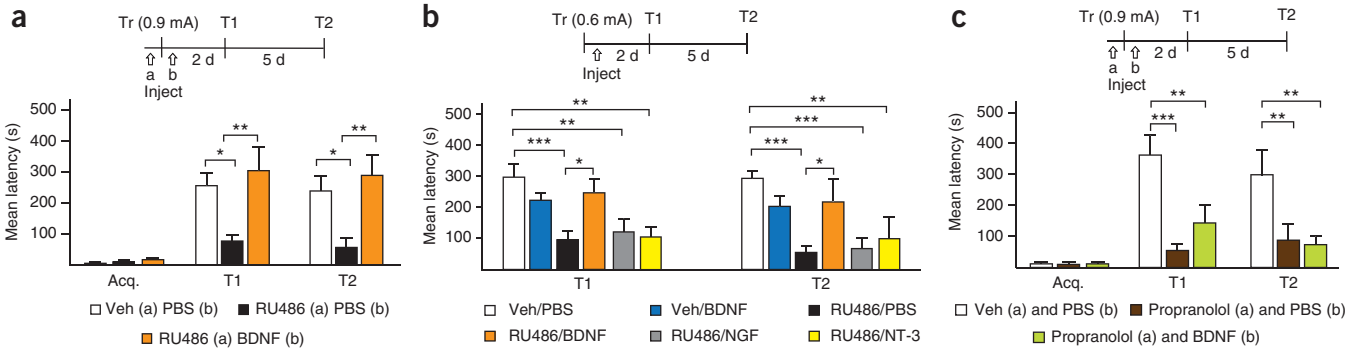

Figure 5 BDNF selectively rescues the RU486-mediated amnesia. Data are expressed as mean latency ± s.e.m. Latency scores and *n* can be found in **Supplementary Table 3**. Experimental schedule is shown above each figure. (**a**) Rats were given hippocampal injections (⇑) of either vehicle or RU486, 15 min before training elicited with a 0.9-mA foot shock and then either phosphate-buffered saline (PBS) or BDNF immediately after training (two-way ANOVA comparing the effect of treatment ($F_{2,44} = 13.44$, $P < 0.0001$), time ($F_{1,44} = 0.2185$, $P = 0.6425$) and treatment × time interaction ($F_{2,44} = 0.001$, $P = 0.999$) followed by Bonferroni *post hoc* tests). (**b**) Rats were given hippocampal injections (⇑) of vehicle (Veh) and PBS, Veh and BDNF, RU486 and PBS, RU486 and BDNF, RU486 and NGF, or RU486 and NT-3 immediately after training elicited with a 0.6-mA foot shock (two-way ANOVA comparing the effect of treatment ($F_{5,112} = 9.414$, $P < 0.0001$), time ($F_{1,112} = 1.163$, $P = 0.2832$) and treatment × time interaction ($F_{5,112} = 0.1749$, $P = 0.9715$) followed by Bonferroni *post hoc* tests). (**c**) Rats were given hippocampal injections (⇑) of either vehicle of propranolol 15 min before training elicited with a 0.9-mA foot shock and then PBS or BDNF immediately after training (two-way ANOVA comparing the effect of treatment ($F_{2,36} = 17.44$, $P < 0.0001$), time ($F_{1,36} = 0.3516$, $P = 0.5569$) and treatment × time interaction ($F_{2,36} = 0.5369$, $P = 0.5892$) followed by Bonferroni *post hoc* tests). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

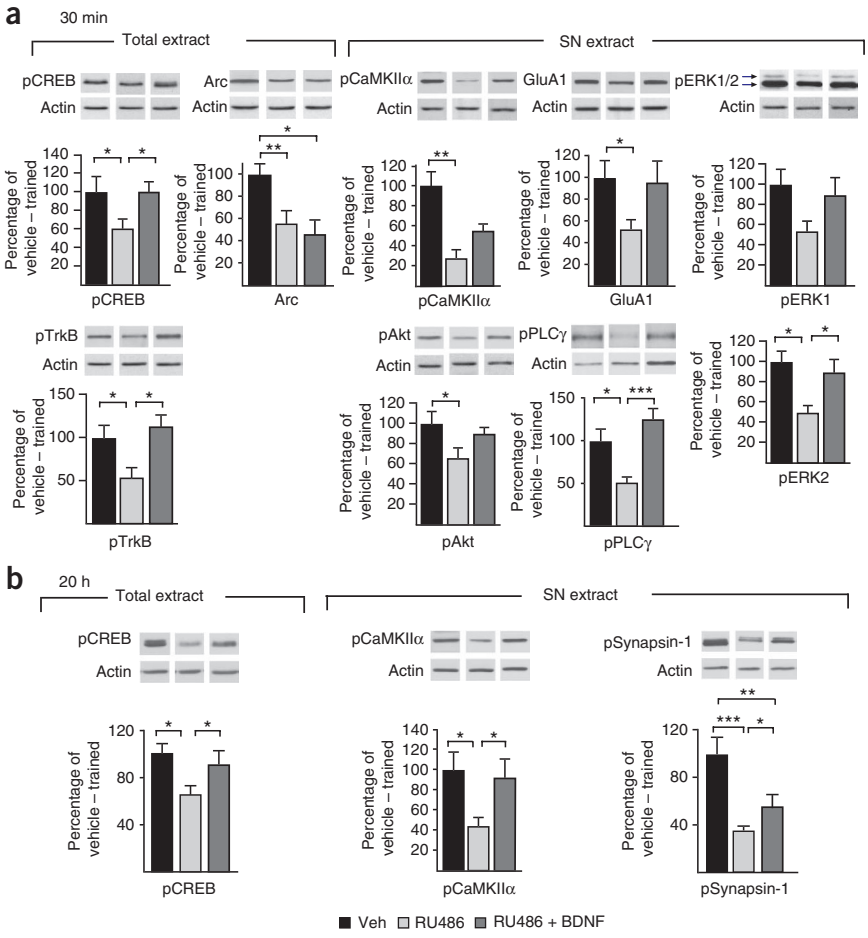

Figure 6 BDNF rescues the RU486-mediated molecular disruption. Mean percentage, *n* and ANOVA *F* values can be found in **Supplementary Table 6**. (**a**) Examples and densitometric quantitative western blot analyses of hippocampal total extracts and synaptoneurosomal (SN) extracts from trained rats injected with vehicle, RU486 or RU486 and BDNF 15 min before training and killed 30 min after training (normalized to actin). Data are expressed as mean percentage ± s.e.m. of trained rats injected with vehicle (one-way ANOVA followed by Newman-Keuls *post hoc* tests for all markers and Student's *t* test for GluA1; pCREB, $F_{2,23} = 5.543$, $P = 0.0117$; pTrkB, $F_{2,21} = 4.916$, $P = 0.019$; Arc, $F_{2,19} = 7.006$, $P = 0.006$; pCaMKIIα, $F_{2,25} = 10.44$, $P = 0.0006$; GluA1, $F_{2,25} = 3.455$, $P = 0.0488$; pERK1, $F_{2,29} = 2.347$, $P = 0.1148$; pERK2, $F_{2,29} = 4.267$, $P = 0.0245$; pAkt, $F_{2,14} = 4.064$, $P = 0.0449$; pPLCγ, $F_{2,26} = 14.63$, $P < 0.0001$). (**b**) Examples and densitometric quantitative western blot analyses of hippocampal total extracts and SN extracts from rats given hippocampal injections of vehicle, RU486, or RU486 and BDNF 15 min before training and killed 20 h after training (normalized to actin). Data are expressed as mean percentage ± s.e.m. of trained rats injected with vehicle (one-way ANOVA followed by Newman-Keuls *post hoc* tests; pCREB, $F_{2,23} = 3.606$, $P = 0.0451$; pCaMKIIα, $F_{2,22} = 5.522$, $P = 0.0123$; pSynapsin-1, $F_{2,20} = 12.81$, $P = 0.0003$). Student's *t* test was used to compare pSynapsin-1 RU486 and BDNF to RU486. Full-length western blots for this figure are shown in **Supplementary Figures 11** and **17**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

We examined whether BDNF rescues the memory impairment caused by β-adrenergic receptor blockers. Rats received a bilateral injection into the hippocampus of either vehicle or the β-adrenergic receptor antagonist propranolol[31] 15 min before training with a 0.9-mA foot shock. Propranolol-injected rats were injected immediately after training with either BDNF or vehicle. The vehicle-injected rats received another vehicle injection after training and served as controls. All rats were tested for memory retention 2 (test 1) and 7 d (test 2) after training. Compared to vehicle, propanolol significantly impaired long-term memory retention at both test 1 ($P < 0.001$) and test 2 ($P < 0.01$), and BDNF failed to rescue the memory impairment caused by propranolol (**Fig. 5c** and **Supplementary Table 5**). Thus, although both glucocorticoid receptor and β-adrenergic receptor–mediated stress responses are required in the hippocampus for long-term memory formation, BDNF selectively rescues the amnesia caused by glucocorticoid receptor inhibition.

BDNF rescues the molecular impairments caused by RU486

We asked whether BDNF also rescues the molecular impairments produced by RU486, in addition to rescuing memory retention. We repeated the bilateral injection experiment described above (**Fig. 5a**) but extracted total cell and synaptoneurosomal extracts from the dorsal hippocampi of rats either 30 min or 20 h after training. As described above (**Fig. 2**), at both 30 min and 20 h after training, RU486 significantly reduced the levels of pTrkB ($P < 0.05$), pCREB ($P < 0.05$) and Arc ($P < 0.01$) in the total extracts and pCaMKIIα ($P < 0.01$), pERK1/2 ($P < 0.05$), pAkt ($P < 0.05$), pPLCγ ($P < 0.05$) and GluA1 ($P < 0.05$) in the synaptoneurosomal extract (**Fig. 6a** and **Supplementary Table 6**). BDNF significantly rescued the levels of pTrkB ($P < 0.05$), pERK2 ($P < 0.05$), pPLCγ ($P < 0.001$) and pCREB ($P < 0.05$) (**Supplementary Table 6**). Furthermore, BDNF treatment resulted in a strong rescuing trend, although not significant ($P > 0.05$) for pERK2, pAkt and GluA1 expression (**Fig. 6a** and **Supplementary Table 6**). The only RU486-dependent disruption that remained unaffected by BDNF treatment was that of Arc (**Fig. 6a** and **Supplementary Table 6**).

The rescuing effect of BDNF was also found at 20 h after training for pCREB, pCaMKIIα and pSynapsin-1 (**Fig. 6b** and **Supplementary Table 6**). The levels of total TrkB, PLCγ, ERK, Akt, CREB, CaMKIIα and Synapsin-1 were unaffected by BDNF administration (**Supplementary Table 6**). Thus, BDNF is not only sufficient for recovering the memory loss but also substantially rescues most molecular disruptions caused by RU486, confirming that there is an early convergence on the intracellular mechanisms activated by glucocorticoid receptor and BDNF during long-term memory formation.

DISCUSSION

The strengthening effects of adaptive stress and relative concentrations of glucocorticoids on memory consolidation have been known for decades, but the underlying molecular mechanisms have remained elusive. We identified several intracellular signaling cascades that are coupled to glucocorticoid receptors following a single traumatic experience that becomes a long-lasting memory: activation of CaMKIIα, increase in Arc and synaptic GluA1, and activation of the

BDNF-dependent pathways. BDNF, but not other neurotrophins, rescued both the amnesia and molecular disruptions caused by glucocorticoid receptor inhibition at training. We conclude that glucocorticoid receptors recruit the CaMKIIα-BDNF-CREB–dependent pathways to mediate long-term memory formation.

First, we speculate that the glucocorticoid receptor's effect on the learning-dependent activation of CaMKIIα may be a result of an increase in corticosterone-dependent Ca^{2+} influx during training, as suggested by *in vitro* data on glucocorticoid receptor activation enhancing L-type calcium current amplitude and affecting calcium channel subunit expression[32] and/or Ca^{2+} elevation via NMDA receptor activation[33]. Second, the glucocorticoid receptor's control on the learning-dependent hippocampal increase in Arc and synaptic GluA1 is consistent with previously reported increases in hippocampal Arc expression following memory-enhancing doses of systemically administered corticosterone[34], impairment of stress-dependent modulation of Arc in the hippocampus of glucocorticoid receptor (+/−) mice[35] and corticosterone-dependent synaptic GluA1 recycling[36]. The glucocorticoid receptor's control of CaMKIIα, Arc and synaptic GluA1 suggests the existence of functional links between these molecules, and this result extends previous evidence showing selective Arc expression in CaMKII-positive glutamatergic neurons in both hippocampus and neocortex[37] and a critical role of Arc in AMPA receptor synaptic trafficking[17].

Furthermore, pre-training inhibition of glucocorticoid receptor markedly reduced the phosphorylation of TrkB, ERK1/2, Akt and PLCγ 30 min after training, without affecting the phosphorylation of MSK1, which suggests that the functional role of glucocorticoid receptor is upstream of TrkB phosphorylation and the activation of the BDNF-mediated signaling. This cross-talk between glucocorticoid receptor and TrkB/BDNF signaling is consistent with previous findings showing that glucocorticoid administration in the brain or to hippocampal/cortical neuronal cultures leads to TrkB phosphorylation[38]. Although the learning-induced glucocorticoid receptor–dependent effect on TrkB phosphorylation was nongenomic, previous findings have suggested a genomic effect. This difference may be attributed to differences between *in vivo* learning-induced effects on hippocampal TrkB and *in vitro* treatments of PC12 cells on TrkA[38]. Our findings therefore point to rapid, nongenomic effects of glucocorticoids on the excitability and activation of neurons[39]. Furthermore, in contrast to what was reported in cortical neuronal cultures[40], we failed to find any direct interaction between glucocorticoid receptor and TrkB in our system using immunoprecipitation of either glucocorticoid receptors or TrkB from total cell lysate (data not shown). Although more sensitive techniques, particularly *in vivo*, may be required to fully address the question of how glucocorticoid receptors activate the BDNF pathway, we speculate that glucocorticoid receptors may control TrkB phosphorylation via other types of mechanisms; for example, they may regulate BDNF release and/or TrkB membrane trafficking. The latter would agree with previous findings showing that depolarization rapidly increases TrkB surface expression, which, similarly to the activity-dependent insertion of AMPA receptors, requires Ca^{2+} influx through NMDA receptors or voltage-gated Ca^{2+} channels and activation of CaMKIIα[41,42]. Finally, glucocorticoid receptors, as suggested by their control of pCREB, may regulate cAMP activation, which modulates signaling and trafficking of TrkB[43].

We found an interaction between glucocorticoid receptors with ERK1/2 and pSynapsin-1, which is consistent with previous findings[9,10] reporting that, in the hippocampus of mice after stress, as well as in cell lines, activation of glucocorticoid receptors increases both the expression and activation of MAPK signaling and the expression of Egr-1 (Zif268)[9], and subsequent regulation of Synapsin-1a/1b[10]. We found that the glucocorticoid receptor–ERK link results from cross-talk between glucocorticoid receptors and the BDNF-dependent pathway, which includes also the activation of Akt and PLCγ.

Although learning-induced CaMKIIα activation requires glucocorticoid receptors, but not BDNF, at an early time point, these two activations converge on the sustained phosphorylation of CaMKIIα and CREB. Notably, BDNF, but not other neurotrophins, rescued both the amnesia and the molecular changes resulting from glucocorticoid receptor inhibition, and the effect did not extend to β-adrenergic receptors, indicating selectivity. Our finding that supplementing BDNF was sufficient to oppose the amnesic effects of glucocorticoid receptor inhibition has potential clinical applications in conditions in which the glucocorticoid receptor is inactive, saturated or needs to be bypassed.

The results from our antibody to BDNF treatments on short-term memory disagree with those of a previous study[44] on step-down avoidance, perhaps because of different testing time and/or the avoidance task that we used. However, similarly to results of that study[44], we found that hippocampal injections of BDNF immediately after training did not enhance memory retention. Finally, the failure of BDNF to rescue Arc in the RU486-treated rats, despite it rescuing both memory and the other biochemical changes, suggests that BDNF effects are downstream or independent of Arc.

We conclude that learning of a traumatic event leads to the activation of glucocorticoid receptors, which rapidly activate CaMKIIα and the BDNF-dependent pathway and control GluA1 receptor trafficking and Arc expression. We speculate that glucocorticoid receptors may regulate all of these cellular activations by controlling an upstream, perhaps general, mechanism, such as BDNF release, receptor membrane trafficking (including that of TrkB), TrkB phosphorylation, activation of mechanisms that control TrkB activation of trafficking[43], or metabolic mechanisms[45]. Glucocorticoid receptor activation also regulated learning-dependent Arc induction, which, together with GluA1 AMPA receptor trafficking, accompanies synaptic strengthening. Given the numerous rapid functional effects of glucocorticoids, alternative, parallel and/or sequential regulations mediated by these hormones may include the increase of extracellular glutamate levels and activation of NMDA receptors[46], as well as other nongenomic effects mediated by different types of glucocorticoid receptors[39]. We also suggest that BDNF release and subsequent genomic effects, which include sustained BDNF expression increase[47], support the persistent activation of CaMKIIα, CREB and Synapsin-1. Given that BDNF activates a cellular growth and survival response, which involves a CREB-C/EBP–dependent gene expression cascade, we propose that evolution has selected the recruitment of survival and growth responses to stress as conserved, fundamental mechanisms for mediating long-term memory formation.

Given the extensive literature regarding the regulation and role of BDNF in chronic stress and mood disorders[3], this convergence between glucocorticoid receptor BDNF and TrkB may be an important contributor to the inverted U effect of stress-mediated responses, which include performance, growth, memory and cognitive functions in general. Thus, it likely represents a critical biological node of dysfunction in affective disorders.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.M.A., D.Y.C. and D.B.-M. designed and developed the study. D.Y.C. carried out the behavioral studies. D.Y.C., D.B.-M. and G.P. carried out the biochemical studies and analyses. C.M.A., D.Y.C. and D.B.-M. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Long Evans adult male rat (Harlan) weighing between 200–250 g (approximately 8–9 weeks old) at the beginning of the experiments were used. Rats were housed individually on a 12-h light-dark cycle with *ad libitum* access to food and water. All experiments were carried out during the light cycle between 9 a.m. and 6 p.m. All rats were handled for 2–3 min per d for 5 d before any behavioral procedure. All protocols complied with the US National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Mount Sinai School of Medicine and New York University Animal Care Committees.

Inhibitory avoidance. Inhibitory avoidance was carried out as previously described[25]. The inhibitory avoidance chamber (Med Associates) consisted of a rectangular Perspex box divided into a safe compartment and a shock compartment. The safe compartment was white and illuminated and the shock compartment was black and dark. Foot shocks were delivered to the grid floor of the shock chamber via a constant current scrambler circuit. The apparatus was located in a sound-attenuated, nonilluminated room. During training sessions, each rat was placed in the safe compartment with its head facing away from the door. After 10 s, the door separating the compartments was automatically opened, allowing the rat access to the shock compartment; the rats usually entered the shock (dark) compartment within 10–20 s of the door opening. The door closed 1 s after the rat entered the shock compartment, and a 2-s foot shock (0.6 mA or 0.9 mA as specified in each behavioral experiment) was administered. For biochemical studies, training was done with 0.9 mA. Latency to enter the shock compartment was taken in seconds as acquisition. The rat was then returned to its home cage and tested for memory retention at the designated time point(s). Retention tests were done by placing the rat back in the safe compartment and measuring its latency to enter the shock compartment. Foot shocks were not administered on the retention tests, and testing was terminated at 540 s. Locomotor activity was measured in the inhibitory avoidance chamber by automatically counting the number of times each rat crossed the invisible infrared light photosensor in 540 s. All behavioral tests were carried out blind. For biochemical studies, rats were not tested for memory retention.

Cannulae implants and hippocampal injections. Hippocampal injections were given as previously described[25]. Rats were anesthetized with ketamine (65 mg per kg of body weight, intraperitoneal) and xylazine (7.5 mg per kg, intraperitoneal), and stainless-steel guide cannulae (22 gauge) were stereotactically implanted to bilaterally target the dorsal hippocampus (4.0 mm posterior to the bregma, 2.6 mm lateral from midline and 2.0 mm ventral). The rats were returned to their home cages and allowed to recover from surgery for 7 d. At the indicated time points before or after training, rats received bilateral injections of compounds as specified. All injections are indicated by arrow in the experimental schedule. All hippocampal injections consisted of 1 µl per side. Hippocampal injections used a 28-gauge needle that extended 1.5 mm beyond the tip of the guide cannula and connected via polyethylene tubing to a Hamilton syringe. The infusions were delivered at a rate of 0.33 µl min^{-1} using an infusion pump. The injection needle was left in place for 2 min after the injection to allow complete diffusion of the solution. Rats were randomized to different treatments. To verify proper placement of cannula implants, rats were killed at the end of the behavioral experiments and their brains were fixed with 10% (vol) buffered formalin in PBS. We cut 40-µm coronal sections through the hippocampus and examined them under a light microscope for cannulae placement. Rats with incorrect placement were discarded from the study. RU486 was purchased from Sigma-Aldrich and was dissolved in 5% (vol) DMSO in 1× PBS. All experiments with RU486 were carried out with 10 ng per injection per side. This dosage of RU486 has been successfully used to disrupt inhibitory avoidance long-term memory when injected into the amygdala[48]. The antibody to BDNF was purchased from Millipore and dissolved in 1× PBS. Antibody to BDNF was injected at 0.5 µg per injection per side. Recombinant human TrkB-Fc chimera was purchased from R&D Systems and was dissolved in PBS. TrkB-Fc was injected at 0.5 µg per injection per side. At these dosages, antibody to BDNF and TrkB-Fc have been used to disrupt long-term memory consolidation when injected into either the hippocampus or amygdala[44,49]. Control sheep IgG was purchased from Sigma-Aldrich and dissolved in 1× PBS and injected at 0.5 µg per injection per side. Propranolol was purchased from Sigma-Aldrich and was dissolved in 10% DMSO in 1× PBS and injected at 5 µg per injection per side, a dose that has been successfully used in the hippocampus to disrupt long-term contextual fear conditioning[50]. Recombinant BDNF, NGF and NT-3 were purchased from PeproTech and dissolved in PBS and they were injected at 0.25 µg per injection per side. Actinomycin D was purchased from Sigma-Aldrich and dissolved in 10% DMSO in 1× PBS and injected at 4 µg per injection per side. This dose of actinomycin D has been used successfully in the hippocampus to disrupt consolidation in a shock-motivated brightness discrimination task and reconsolidation of contextual fear conditioning[26].

Synaptoneurosomal preparation and western blot analysis. Synaptoneurosomal preparation was carried out as previously described[25]. Briefly, dorsal hippocampi were rapidly dissected in cold dissection buffer (2.6 mM KCl, 1.23 mM sodium phosphate monobasic, 26 mM sodium bicarbonate, 5 mM kynurenic acid, 212 mM sucrose, 10 mM dextrose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$) followed by homogenization in 10 mM HEPES, 2 mM EDTA, 2 mM EGTA and 0.5 mM DTT, with phosphatase and protease inhibitor cocktails (Sigma-Aldrich) using glass-teflon homogenizer. Homogenates were filtered through 100-µm nylon mesh filter and 5-µm nitrocellulose filters sequentially. Synaptoneurosomes were obtained by centrifugating the filtrate at 1,000*g* for 10 min. The pellet was resuspended in the homogenization buffer.

Western blot analysis was carried out as previously reported[25]. Specifically, hippocampal total extracts from rat were obtained by polytron homogenization in cold lysis buffer with protease and phosphatase inhibitors (0.2 M NaCl, 0.1 M HEPES, 10% (vol) glycerol, 2 mM NaF, 2 mM $Na_4P_2O_7$, 4 U ml^{-1} aprotonin, 2 mM DTT, 1 mM EGTA, 1 µM microcystin and 1 mM benzamidine). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Equal amounts of total protein (10–20 µg per lane) were resolved on denaturing SDS-PAGE gels and transferred to Hybond-P membranes (Millipore) by electroblotting. Membranes were dried and then reactivated in methanol for 5 min and then washed with three changes of water. The membrane was then blocked in 3% (wt) milk and TBS or according to manufacturer's instruction for 1 h at room temperature, then incubated with primary antibody overnight at 4–8 °C in solution per manufacturer's suggestion. Full-length western blot images for each antibody used are shown in **Supplementary Figure 8**. All antibodies had been previously used and tested for specificity (**Supplementary Fig. 8**). Antibodies to pCREB (1:1,000, cat # 06-519), GluA1 (1:2,000, cat # AB-1504), CaMKIIα (1:2,000, cat # 05-532), PLCγ (1:1,000, cat # 05-163) and Synapsin-1 (1:2,000, cat # AB-1543P) were purchased from Millipore. Antibodies to CREB (1:1,000, cat # 9197), pCaMKIIα (1:5,000, cat # 3361s), ERK1/2 (1:2,000, cat # 9102), pERK1/2 (1:2,000, cat # 9101s), Akt (1:1,000, cat # 4691s), TrkB (1:1,000, cat # 4603s), pAkt (1:1,000, cat # 4060s) and pMSK1 (1:1,000, cat # 9595P) were purchased from Cell Signaling Technology, MSK1 (1:1,000, cat # AF2518) from R&D systems, Arc (1:1,000, cat # 156003) from Synaptic Systems, pTrkB (1:1,000, cat # 2149-1) from Epitomics, pPLCγ (1:1,000, cat # 700044) from Invitrogen, and pSynapsin–1 (1:1,000 cat # S8192) from Sigma, Zif268 (1:500, cat # Sc-101) and actin–horseradish peroxidase (HRP) (1:4,000, cat # Sc-1616) from Santa Cruz Biotechnology. The membranes were washed, treated with secondary HRP-labeled donkey antibody to rabbit (1:4,000 cat # NA934V GE Healthcare) or sheep antibody to mouse (1:4,000 cat# NA931V GE Healthcare) for 1 h, washed again and incubated with HRP-streptavidin complex and ECL detection reagents (GE Healthcare). Membranes were exposed to Denville Scientific HyBlotCL and quantitative densitometric analysis was performed using ImageJ (US National Institutes of Health). Changes were quantified within gels, and 3–4 rats per group were used in every gel. Actin was used as loading control for all markers. All membranes on which the specific markers were investigated were stripped with stripping buffer (1% (wt) SDS, 31.25 mM Tris HCl pH 6.8, 0.7% (vol) β-mercaptoethanol) and probed with actin. In cases in which phospho-markers were probed, the same membranes, whenever possible, were stripped again with stripping buffer after actin levels have been assessed and probed for their respective total levels. First, we investigated the effect of RU486 or antibody to BDNF on changes elicited by training. The expression levels of the markers in trained rats treated either with vehicle, RU486 or antibody to BDNF compared with naive rats treated with vehicle were determined. Subsequently, having found an effect on trained rats, we determined the effect of RU486 or antibody to BDNF on naive rats. As these experiments were run separately, they are shown separately in the relative figures. Examples of full-length blots are shown in **Supplementary Figures 8–17**.

Statistical analysis. A minimum final group size of about five rats is required to have a probability of detecting significant group effects for behavior and biochemistry experiments. For biochemical studies (**Fig. 2a**), power calculation of one-way ANOVA comparing the three treatment groups analyzed by G*Power software indicated a sample size of 4–5 rats per group was necessary to achieve power of 0.8 and an error probability of 0.05. For behavioral experiments, similar power analysis (**Fig. 1a**) calculated the requirement of a sample size of six for two-way ANOVA to achieve power of 0.8 and an error probability of 0.05. Statistical tests were designed using the assumption of normal distribution and variance for control versus treatment groups. D'Agostino-Pearson omnibus test carried out in groups with sufficient *n* confirmed that memory latency 2 d after inhibitory avoidance training and injection followed a normal distribution. For multi-group comparison, one- or two-way ANOVA followed by the *post hoc* tests were used (Newman-Keuls or Student's *t* test where indicated for one-way ANOVA, or Bonferroni *post hoc* tests for two-way ANOVA). Two-tailed Student's *t* test was used for pair-wise comparisons. $P < 0.05$ was considered significant.

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