HHS Public Access

Author manuscript

Nat Neurosci. Author manuscript; available in PMC 2013 June 01.

Published in final edited form as:

Nat Neurosci. 2012 December ; 15(12): 1707–1714. doi:10.1038/nn.3266.

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

Dillon Y. Chen[2,3], **Dhananjay Bambah–Mukku**[1,2,3], **Gabriella Pollonini**[1], and **Cristina M. Alberini**[1,4]

[1]Center for Neural Science, New York University, New York, NY, 10003

[2]Department of Neuroscience and Friedman Brain Institute, Mount Sinai School of Medicine, New York, NY 10029

Abstract

Emotionally important events are well remembered. Although memories of emotional experiences are known to be mediated and modulated by the stress hormones glucocorticoids, little is known about the underlying molecular mechanisms. Here we show that the hippocampal glucocorticoid receptors critically engaged during the formation of long–term inhibitory avoidance memory in rats are coupled to the activation of CaMKIIα, TrkB, ERK, Akt, PLCγ and CREB, as well as a significant induction of Arc and synaptic GluA1. Most of these changes, which are initiated by a non–genomic effect of glucocorticoid receptors, are also downstream of the activation of brain–derived neurotrophic factor (BDNF). Hippocampal administration of BDNF, but not other neurotrophins, selectively rescues both the amnesia and the molecular impairments produced by glucocorticoid receptor inhibition. Hence, 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/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

[4]Correspondence should be addressed to: Cristina M. Alberini Center for Neural Science, New York University, 4 Washington Square, New York, NY, 10003 ca60@nyu.edu; Phone: 212–998–7721.

[3]Both authors equally contributed to this study

The authors have no conflict of interest to declare

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. and D.Y.C. wrote the manuscript.

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 such as the hippocampus, amygdala and prefrontal cortex, which are enriched in glucocorticoid receptors and play a major role in long–term memory formation[7].

Although several molecular correlates have been described to accompany chronic stress and its negative effects on cognition[8], the molecular mechanisms that are critically recruited by positive, adaptive level of stress/arousal that is 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/1b[10]. Here we employed the inhibitory avoidance learning paradigm in rats to identify the intracellular pathways activated by glucocorticoid receptors in the hippocampus. We show that, to mediate memory consolidation, glucocorticoid receptors recruit the plasticity/survival pathways activated via calcium calmodulin kinase II α (CaMKIIα), brain–derived neurotrophic factor (BDNF) – tropomyosin–related kinase B (TrkB) and cAMP response element binding protein (CREB).

RESULTS

Inhibitory avoidance memory requires hippocampal glucocorticoid receptors

To test the role of hippocampal glucocorticoid receptors in long–term inhibitory avoidance memory formation, groups of rats were bilaterally injected with either the glucocorticoid receptor antagonist RU38486 (RU486)[11] or vehicle into the dorsal hippocampus 15 minutes before or immediately after training elicited by a 0.6 mA footshock. Memory retention was tested 2 (Test 1) and 7 days (Test 2) after training. The numeric values, number of rats used per group (*n*) and statistical analyses of all experiments described in this study are shown in the tables in supporting online material (**Supplementary Tables 1–6**). Compared to vehicle, RU486 completely disrupted memory retention at Test 1 when injected before training and had a partial, but still significant, effect when injected after training (**Fig. 1a,b** and **Supplementary Table 1**). The effect persisted at Test 2. A reminder shock in a different context, one day after Test 2 did not rescue the memory tested one day later (Test 3, **Fig. 1a,b** and **Supplementary Table 1**). The RU486–mediated memory impairment was not due to non–specific locomotor changes (**Supplementary Fig. 1**).

To determine whether a more traumatic memory, elicited by a stronger footshock, is similarly regulated by glucocorticoid receptors, rats underwent the same protocol as described above, except that the training was done with a 0.9 mA footshock (**Fig. 1c,d** and **Supplementary Table 1**). Compared to vehicle, RU486 injected before training significantly decreased retention at both Test 1 and Test 2 (**Fig. 1c** and **Supplementary Table 1**). No re–instatement was seen after a 0.9 mA reminder footshock in a different context one day 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, in line 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 footshock training did not affect short–term memory tested at 1 hour (**Fig. 1e** and **Supplementary Table 1**), confirming that the long–term memory impairment was not due to non–specific effects on task performance.

Thus, hippocampal glucocorticoid receptors rapidly regulate mechanisms essential for the formation of long–term inhibitory avoidance memory without affecting its short–term retention.

Hippocampal molecular pathways recruited by glucocorticoid receptors 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 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 footshock, because a stronger conditioning correlates with a higher degree of molecular changes, which, therefore, can be more easily quantified. Specifically, we tested the effect of RU486 on the phosphorylation and/or expression levels of different classes of proteins 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], the phosphorylation of the synaptic protein Synapsin–1[15], the phosphorylation of the transcription factor cAMP response element binding protein (CREB)[16], the level of the immediate early gene activity regulated cytoskeletal–associated protein (Arc) and of the AMPA receptor subunit GluA1 (also known as GluR1)[17]. Dorsal hippocampal extracts from rats injected with either RU486 or vehicle 15 minutes before training were euthanized either 30 minutes (**Fig. 2a**, **Supplementary Fig. 2a** and **Supplementary Table 2**) or 20 hours after training (**Fig. 2b**, **Supplementary Fig. 2b** and **Supplementary Table 2**) to survey the learning–induced molecular changes over time. Controls consisted of naïve rats injected with vehicle and euthanized at matched time points. Both total cell and synaptoneurosomal extracts were investigated at both time points using quantitative western blot analyses. At 30 minutes after training, compared to naïve conditions, there was a significant increase in CREB phosphorylation at Ser 133 (pCREB) without a change in CREB levels in total cell lysates, a result in agreement with previous studies[18,19]. In the same extracts there was also a significant increase in Arc, and, in the synaptoneurosomal fraction, a significant elevation in CaMKIIα phosphorylation at Thr 286 (pCaMKIIα) and GluA1 levels. All these increases were completely blocked by RU486 (**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 (Ser 603), a downstream presynaptic target of activated CaMKIIα[21], were unaffected by either training or RU486 (**Supplementary Fig. 2a** and **Supplementary Table 2**). All the significant training-induced changes were selective for the paired context–footshock 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 likely due to context exposure[22]), which are both known to not elicit inhibitory

avoidance /fear conditioning[23–25] (**Supplementary Fig. 3**). Training did not change the levels or phosphorylation of hippocampal ERK1/2 (Thr 202/Tyr 204, pERK), Akt (Ser 473, pAkt) and PLCγ (Tyr 783, pPLCγ). However, RU486 significantly decreased pERK, pAkt and pPLCγ, 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 phosphorylation of the BDNF receptor TrkB at Tyr 817 (pTrkB) changed, and found that RU486 significantly decreased pTrkB without affecting TrkB levels (**Fig. 2a** and **Supplementary Table 2**). Hippocampal injections of RU486 in naïve rats also produced a significant decrease in the levels of pCaMKIIα and pAkt, as well as a non–significant trend toward a decrease in the levels of pCREB, pTrkB, pERK and pPLCγ 45 minutes after the injection (same post–injection time point as of 30 min after training), supporting the conclusion that glucocorticoid receptors are coupled to the activation of these factors even in basal (non–trained) conditions (**Supplementary Fig. 4**).

Compared to naïve, the hippocampi of rats 20 hours after training showed a significant elevation in pCREB, pCaMKIIα and pSynapsin–1 (**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 were 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 hours after training compared to naïve 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 non–genomic regulations. Hence, we tested whether the RNA synthesis inhibitor actinomycin D affected the training–induced molecular changes. Dorsal hippocampal extracts from trained rats injected with either vehicle or an effective dose of actinomycin D[26] 15 minutes before training were euthanized 30 minutes after training and investigated using quantitative western blot analyses. Actinomycin D did not change the training–related inductions of pTrkB, pCaMKIIα, pCREB and GluA1, indicating that these changes were non–genomic. In contrast, actinomycin D decreased the training–dependent Arc induction Arc (**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 (**Fig. 2c, Supplementary Table 2**).

Hence, the training–dependent glucocorticoid receptor activation in the hippocampus is coupled to the phosphorylation of TrkB, ERK1/2, Akt and PLCγ, events 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 post–synaptic 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 in the hippocampus

The overlap between the glucocorticoid receptor–dependent molecular changes found in the hippocampus of trained rats with the known BDNF–coupled molecular pathways led us to test whether BDNF in the hippocampus plays an essential role in 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 anti–BDNF antibody or BDNF–sequestering TrkB–Fc chimera into the dorsal hippocampus 15 minutes before training elicited by either 0.6 or 0.9 mA footshock profoundly and persistently disrupted memory retention at both 2 and 7 days after training compared to IgG (**Fig. 3a, c** and **Supplementary Table 3**), without affecting short–term memory (**Fig. 3b** 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 anti–BDNF on long–term memory retention were not due to non–specific effects on locomotion (**Supplementary Fig. 5**).

Thus, hippocampal BDNF plays a critical role in long–term but not short–term inhibitory avoidance memory formation.

BDNF–TrkB and glucocorticoid receptors recruit overlapping pathways

To confirm that the training–induced BDNF–dependent changes were the same as those we found coupled to glucocorticoid receptor activation, we investigated the effects of blocking BDNF 15 minutes before training with anti–BDNF on the same molecular mechanisms examined following RU486 treatment. Quantitative western blot analyses on total cell and synaptoneurosomal lysates of rat dorsal hippocampi taken at either 30 minutes (**Fig. 4a** and **Supplementary Table 4**) or 20 hours after training (**Fig. 4b**, **Supplementary Fig. 6** and **Supplementary Table 4**) were performed. Control hippocampal extracts were from naïve rats injected with IgG and euthanized at matched time points. At 30 minutes after training, compared to IgG, anti–BDNF blocked the training–related significant increase in pCREB, without affecting CREB levels (**Fig. 4a** and **Supplementary Table 4**) and no effect was found on the training–related significant induction of Arc (**Fig. 4a** and **Supplementary Table 4**). In the synaptoneurosomal fraction, similarly to what was found with RU486, compared to either naïve or trained rats injected with IgG, anti–BDNF significantly lowered the levels of pERK1/2 and pPLCγ without affecting those of ERK1/2 and PLCγ, and resulted in a strong trend toward a decrease, although not significant, of pAkt without changing Akt (**Fig. 4a** and **Supplementary Table 4**). In contrast to RU486, anti–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 anti–BDNF in naïve rats did not change the levels of pCREB, pERK1/2, pAkt or pPLCγ (**Supplementary Fig. 6**), indicating that blocking BDNF in basal (non–trained) conditions has no effect on the molecular changes elicited by training.

At 20 hours after training, similarly to what was found with RU486, compared to IgG, anti–BDNF blocked the learning–dependent increase in phosphorylation of CREB, CaMKIIα and Synapsin–1 (**Fig. 4b** and **Supplementary Table 4**), without changing their total levels

(**Supplementary Table 4**). Furthermore, like RU486, anti–BDNF treatment did not affect pERK1/2 and pAkt, and their respective total protein levels at 20 hours 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 is found at 20 hours after training, when both anti–BDNF and RU486 treatments block 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 minutes before training elicited with a 0.9 mA footshock. The RU486–injected rats were further 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 days after training (Test 1 and 2, respectively). Confirming our previous results, compared to vehicle, RU486 significantly impaired memory retention at both 2 and 7 days after training (**Fig. 5a** and **Supplementary Table 5**). BDNF significantly and persistently rescued this amnesia (**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, compared to vehicle, RU486 significantly impaired long–term memory retention tested 2 and 7 days after training (Test 1 and 2, respectively). BDNF, but not NGF or NT–3 co–administration significantly and persistently rescued memory retention (**Fig. 5b** and **Supplementary Table 5**). Notably, BDNF injections alone immediately after training did not change memory retention (**Fig. 5b** and **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, like corticosterone, is released in response to stress and significantly 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. Similar to glucocorticoid receptor, β–adrenergic receptors are abundantly expressed the hippocampus[28,30].

Here 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 minutes before training done with a 0.9 mA foot shock. Propranolol–injected rats were further 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 days (Test 2) after training. Compared to vehicle, propranolol significantly impaired long–term memory retention at both Test 1 and Test 2, and BDNF failed to rescue the memory impairment caused by propranolol (**Fig. 5c** and **Supplementary Table 5**).

Hence, while 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. Hence, we repeated the experiment of Figure 5a, but extracted total cell and synaptoneurosomal extracts from the dorsal hippocampi of rats either 30 minutes or 20 hours after training. As seen before (**Fig. 2**), at both 30 minutes and 20 hours after training, RU486 significantly reduced the levels of pTrkB, pCREB, and Arc in the total extracts and of pCaMKIIα, pERK1/2, pAkt, pPLCγ and GluA1 in the synaptoneurosomal extract (**Fig. 6a** and **Supplementary Table 6**). BDNF significantly rescued the levels of pTrkB, pERK2, pPLCγ and pCREB (**Supplementary Table 6**). Furthermore, BDNF treatment gave a strong rescuing trend, although not significant, for pERK1, pAkt and GluA1 (**Fig. 6a** and **Supplementary Table 6**) and the only RU486–dependent disruption that remained unaffected by BDNF was that of Arc (**Fig. 6a** and **Supplementary Table 6**).

The rescuing effect of BDNF was also found at 20 hours 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 significantly 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 know for decades, however, the underlying molecular mechanisms have remained elusive. Here we identified several intracellular signaling cascades 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, rescues 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. These results also provide several additional, novel links to the current literature.

First, we speculate that the glucocorticoid receptor's effect on the learning-dependent activation of CaMKIIα may be due to 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 in line with previously reported increased 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 on CaMKIIα, Arc and synaptic GluA1 also extends and link previous evidences of selective Arc expression in CaMKII–positive glutamatergic neurons in both hippocampus and neocortex[37] and Arc critical role in AMPA receptor synaptic trafficking[17].

Furthermore, the pre-training inhibition of glucocorticoid receptor that profoundly reduces the phosphorylation of TrkB, ERK1/2, Akt and PLCγ 30 minutes after training, without affecting the phosphorylation of MSK1, suggests that the functional role of glucocorticoid receptor is upstream of TrkB phosphorylation and the activation of the BDNF–mediated signaling. This intriguing cross–talk between glucocorticoid receptor and TrkB/BDNF signaling is in line with previous findings showing that glucocorticoids administration in the brain or to hippocampal/cortical neuronal cultures leads to TrkB phosphorylation[38]. While the learning–induced glucocorticoid receptor–dependent effect on TrkB phosphorylation is non–genomic, the previous findings indicated 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 increase the evidence for rapid, non–genomic effects of glucocorticoids on the excitability and activation of neurons[39]. Furthermore, in contrast to what was reported in cortical neuronal cultures[40], thus far, we failed to reveal any direct interaction between glucocorticoid receptor and TrkB in our system using immunoprecipitations of either glucocorticoid receptors or TrkB from total cell lysate (data not shown). Although more sensitive techniques, especially *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 increase TrkB surface expression, which, like 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].

Our results showing a coupling of glucocorticoid receptors with ERK1/2 and pSynapsin–1 are 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]. Here, we extended these results by showing that the glucocorticoid receptor–ERK link results from a cross talk between glucocorticoid receptors and the BDNF–dependent pathway, which includes also the activation of Akt and PLCγ.

Although the learning–induced CaMKIIα activation requires glucocorticoid receptors but not BDNF at an early timepoint, these two activations converge on the sustained phosphorylation of CaMKIIα and CREB. Importantly, BDNF but not other neurotrophins rescues both the amnesia and the molecular changes resulting from glucocorticoid receptor inhibition, and the effect does not extend to β–adrenergic receptors, thus indicating selectivity. Our data showing that supplementing BDNF is sufficient to oppose the amnestic effects of glucocorticoid receptor inhibition also have potential clinical applications in conditions in which the glucocorticoid receptor is inactive, saturated or need to be bypassed.

Our results on anti–BDNF treatment on short–term memory disagrees from that of a previous study.[44] on step–down avoidance, perhaps because of different testing time and/or avoidance task used. However, similarly to this study[44], we found that hippocampal injections of BDNF immediately after training do not enhance memory retention. Finally, the failure of BDNF to rescue Arc in the RU486–treated rats, despite it rescueing both memory and the other biochemical changes, suggests that BDNF effects are downstream or independent of Arc.

Hence, we conclude that learning of a traumatic event leads to the activation of glucocorticoid receptors, which rapidly activates CaMKIIα and the BDNF–dependent pathway and control GluA1 receptor trafficking and Arc expression. We speculate that glucocorticoid receptors may regulate all 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 regulates the 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 non–genomic 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. Since BDNF activates a cellular growth/survival response, which involves a CREB–C/EBP–dependent gene expression cascade, we propose that evolution has selected the recruitment of survival/growth responses to stress as conserved, fundamental mechanisms to mediate long–term memory formation.

Given the extensive literature showing the regulation and role of BDNF in chronic stress and mood disorders[3], this glucocorticoid receptor–BDNF–TrkB convergence may be a key

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

Animals

Long Evans adult male rat (Harlan, Indianapolis, IN) weighing between 200–250 grams (approximately 8–9 weeks old) at the beginning of the experiments were used. Rats were housed individually on a 12 hour light–dark cycle with *ad libitum* access to food and water. All experiments were done during the light cycle between 9 AM and 6 PM. All rats were handled for 2–3 minutes per day for 5 days before any behavioral procedure. All protocols complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Mount Sinai School of Medicine and NYU Animal Care Committees.

Inhibitory avoidance

Inhibitory avoidance was carried out as previously described[25]. The inhibitory avoidance chamber (Med Associates. Inc., St. Albans, VT) 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, non–illuminated room. During training sessions, each rat was placed in the safe compartment with its head facing away from the door. After 10 seconds, the door separating the compartments was automatically opened, allowing the rat access to the shock compartment; the rats usually enter the shock (dark) compartment within 10–20 seconds of the door opening. The door closed 1 second after the rat entered the shock compartment, and a 2–second 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 seconds. Locomotor activity was measured in the inhibitory avoidance chamber by the automatic counting of the number of times each rat crosses the invisible infrared light photosensor in 540 seconds. All behavioral tests were done in 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/kg, i.p.) and xylazine (7.5 mg/kg, i.p.), 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 days. 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 were carried out in 1μl 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/minute using an infusion pump. The injection needle was left in place for 2 minutes after the injection to allow complete diffusion of the solution. Rats were randomized to different treatments. To verify proper placement of cannula implants, at the end of the behavioral experiments, rats were euthanized and their brains were fixed with 10% buffered formalin in PBS. Forty–micrometer coronal sections were cut through the hippocampus and examined under a light microscope for cannulae placement. Rats with incorrect placement were discarded from the study. RU486 was purchased from Sigma Aldrich (St. Louis, MO) and was dissolved in 5% DMSO in 1xPBS. All experiments with RU486 were carried out with 10 ng/injection/side. This dosage of RU486 has been successfully used to disrupt inhibitory avoidance long–term memory when injected into the amygdala[48]. The anti–BDNF antibody was purchased from Millipore (Billerica, MA) dissolved in 1xPBS. Anti–BDNF antibody was injected out at 0.5 μg/injection/side. Recombinant human TrkB–Fc chimera was purchased from R&D Systems (Minneapolis, MN) and was dissolved in PBS. TrkB–Fc was injected at 0.5 μg/injection/side. At these dosages, anti–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 1xPBS and injected at 0.5 μg/injection/side. Propranolol was purchased from Sigma Aldrich and was dissolved in 10% DMSO in 1xPBS and it was injected at 5μg/injection/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 (Rocky Hill, NJ.) and dissolved PBS and they were injected at 0.25 μg/injection/side. Actinomycin D was purchased from Sigma Aldrich and dissolved in 10% DMSO in 1×PBS and injected at 4 μg/injection/side. This dose of actinomycin D has been used successfully in the hippocampus to disrupt consolidation of 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 CaCl2, 1 mM MgCl2) followed by homogenization in 10 mM HEPES, 2 mM EDTA, 2 mM EGTA, 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 1000g for 10 minutes. The pellet was resuspended in the homogenization buffer.

Western blot analysis was done 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% glycerol, 2 mM NaF, 2 mM Na4P2O7, 4U/ml aprotonin, 2mM DTT, 1 mM EGTA, 1μM microcystin, 1mM

benzamidine). Protein concentrations were determined using the BioRad protein assay (BioRad Laboratories, Hercules, CA). Equal amounts of total protein (10–20 μg/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 minutes and then washed with 3 changes of water. The membrane was then blocked in 3% milk/TBS or according to manufacturers' instruction for 1 hour at room temperature, then incubated with primary antibody overnight at 4°C in solution per manufacturers' suggestion. A full–length western blot image for each antibody used in this study is shown in **Supplementary Fig. 8**. All antibodies had been previously used and tested for specificity, as specified in the legend of **supplementary Fig.8**. Antibodies: anti–pCREB (1/1000) (Cat # 06-519), anti–GluA1 (1/2000) (Cat # AB-1504), anti–CaMKIIα (1/2000) (Cat # 05-532), anti–PLCγ (1/1000) (Cat # 05-163), anti–Synapsin–1 (1/2000) (Cat # AB-1543P) were purchased from Millipore (Billerica, MA). Anti–CREB (1/1000) (Cat # 9197), anti–pCaMKIIα (1/5000) (Cat # 3361s), anti–ERK1/2 (1/2000) (Cat # 9102), anti–pERK1/2 (1/2000) (Cat # 9101s), anti–Akt (1/1000) (Cat # 4691s), anti–TrkB (1/1000) (Cat # 4603s) and anti–pAkt (1/1000) (Cat # 4060s) and anti–pMSK1 (1/1000) (Cat # 9595P) were purchased from Cell Signaling Technology (Danvers, MA), anti–MSK1 (1/1000) (Cat # AF2518) was purchased from R&D systems, anti–Arc (1/1000) (Cat # 156003) was purchased from Synaptic Systems (Goettingen, Germany), anti–pTrkB (1/1000) (Cat # 2149-1) was purchased from Epitomics (Burlingame, CA), anti–pPLCγ (1/1000) (Cat #700044) was purchased from Invitrogen (Carlsbad, CA), and anti–pSynapsin–1 (Cat #S8192) was purchased from Sigma (St. Louis, MO) anti–Zif268 (egr–1; 1/500) (Cat #Sc-101), and anti–actin–HRP (1/4000) (Cat #Sc-1616) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were washed, treated with secondary HRP–labeled donkey anti–rabbit or –goat anti–mouse antibody (1/4000, GE Healthcare, Waukesha, WI) for 1 hour, washed again and incubated with HRP–streptavidin complex and ECL detection reagents (GE healthcare, Waukesha, WI). Membranes were exposed to Denville Scientific HyBlotCL (Denville Scientific, Metuchen, NJ) and quantitative densitometric analysis was performed using NIH ImageJ. Changes were quantified within gels, and an n=3-4 rats per group in every gel were used. Actin was used as loading control for all markers. All membranes on which the specific markers were investigated were stripped with stripping buffer (1% SDS, 31.25 mM Tris HCl pH 6.8, 0.7% β–mercaptoethanol) and probed with actin. In cases where 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. The experiments were designed as follows: First we investigated the effect of RU486 or anti–BDNF on changes elicited by training. Hence, the expression levels of the markers in trained rats treated either with vehicle, RU486 or anti–BDNF compared to naïve treated with vehicle were determined. Subsequently, having found an effect on trained rats, we determined the effect of RU486 or anti–BDNF on naïve animals. 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 ~ 5 rats is required to have a probability of detecting significant group effects for behavior and biochemistry experiments.

For biochemical studies, using data from **Fig. 2a**, power calculation of one–way ANOVA comparing the 3 treatment groups analyzed by G*Power software indicated a sample size of 4–5 rats/group was necessary to achieve power of 0.8 and a error probability of 0.05. For behavioral experiments, similar power analysis using data from **Fig. 1a** calculated the requirement of a sample size of 6 for two–way ANOVA to achieve power of 0.8 and an error probability of 0.05. Statistical tests are designed based on 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 days after inhibitory avoidance training and injection follow a normal distribution. For multi–group comparison, one– or two–way analysis of variance (ANOVA) followed by the post hoc tests: Newman–Keuls or Student's t–test where indicated for one–way ANOVA, or Bonferroni post hoc tests for two–way ANOVA were used. Two–tailed Student's *t*–test was used for pair–wise comparisons. P value < 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by grants R01–MH065635, NARSAD, Philoctetes Foundation awarded to C.M.A., F31–MH816213 to D.Y.C. We thank S. Stern, S. Sheng, S. Taubenfeld and S. Katz for technical assistance. We thank A. Suzuki and Xiaojing Ye for comments on the manuscript, Roberto Testi (Tor Vergata, Rome) for valuable discussions.

REFERENCES

1. McGaugh JL. Memory and Emotion: The Making of Lasting Memory. Weidenfeld and Nicolson. 2003
2. De Kloet ER. Hormones and the stressed brain. Ann. N. Y. Acad. Sci. 2004; 1018:1–15. [PubMed: 15240347]
3. McEwen BS. Physiology and neurobiology of stress and adaptation: central role of the brain. Physiol. Rev. 2007; 87:873–904. [PubMed: 17615391]
4. Sapolsky RM. Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch. Gen. Psychiatry. 2000; 57:925–935. [PubMed: 11015810]
5. Roozendaal B. 1999 Curt P. Richter award. Glucocorticoids and the regulation of memory consolidation. Psychoneuroendocrinology. 2000; 25:213–238. [PubMed: 10737694]
6. Dudai Y. Molecular bases of long–term memories: a question of persistence. Curr. Opin. Neurobiol. 2002; 12:211–216. [PubMed: 12015239]
7. Morimoto M, Morita N, Ozawa H, Yokoyama K, Kawata M. Distribution of glucocorticoid receptor immunoreactivity and mRNA in the rat brain: an immunohistochemical and in situ hybridization study. Neurosci. Res. 1996; 26:235–269. [PubMed: 9121734]
8. McEwen BS, Eiland L, Hunter RG, Miller MM. Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. Neuropharmacology. 2012; 62:3–12. [PubMed: 21807003]
9. Revest JM, et al. The MAPK pathway and Egr–1 mediate stress–related behavioral effects of glucocorticoids. Nat. Neurosci. 2005; 8:664–672. [PubMed: 15834420]
10. Revest JM, et al. The enhancement of stress–related memory by glucocorticoids depends on synapsin–Ia/Ib. Mol. Psychiatry. 2010; 15:1125, 1140–1151. [PubMed: 20368707]

11. Brogden RN, Goa KL, Faulds D. Mifepristone. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential. Drugs. 1993; 45:384–409. [PubMed: 7682909]
12. Lisman J, Schulman H, Cline H. The molecular basis of CaMKII function in synaptic and behavioural memory. Nat. Rev. Neurosci. 2002; 3:175–190.
13. Minichiello L. TrkB signalling pathways in LTP and learning. Nat. Rev. Neurosci. 2009; 10:850–860. [PubMed: 19927149]
14. Sindreu CB, Scheiner ZS, Storm DR. Ca2+ –stimulated adenylyl cyclases regulate ERK–dependent activation of MSK1 during fear conditioning. Neuron. 2007; 53:79–89. [PubMed: 17196532]
15. Hosaka M, Hammer RE, Sudhof TC. A phospho–switch controls the dynamic association of synapsins with synaptic vesicles. Neuron. 1999; 24:377–387. [PubMed: 10571231]
16. Alberini CM. Transcription factors in long–term memory and synaptic plasticity. Physiol. Rev. 2009; 89:121–145. [PubMed: 19126756]
17. Bramham CR, Worley PF, Moore MJ, Guzowski JF. The immediate early gene arc/arg3.1: regulation, mechanisms, and function. J. Neurosci. 2008; 28:11760–11767. [PubMed: 19005037]
18. Taubenfeld SM, Wiig KA, Bear MF, Alberini CM. A molecular correlate of memory and amnesia in the hippocampus. Nat. Neurosci. 1999; 2:309–310. [PubMed: 10204535]
19. Impey S, et al. Stimulation of cAMP response element (CRE)–mediated transcription during contextual learning. Nat. Neurosci. 1998; 1:595–601. [PubMed: 10196567]
20. Arthur JS, et al. Mitogen– and stress–activated protein kinase 1 mediates cAMP response element–binding protein phosphorylation and activation by neurotrophins. J. Neurosci. 2004; 24:4324–4332. [PubMed: 15128846]
21. McGuinness TL, et al. Phosphorylation–dependent inhibition by synapsin I of organelle movement in squid axoplasm. J. Neurosci. 1989; 9:4138–4149. [PubMed: 2512374]
22. Pevzner A, Miyashita T, Schiffman AJ, Guzowski JF. Temporal dynamics of Arc gene induction in hippocampus: Relationship to context memory formation. Neurobiol. Learn. Mem. 2012; 97:313–320. [PubMed: 22390855]
23. Fanselow MS, DeCola JP, Young SL. Mechanisms responsible for reduced contextual conditioning with massed unsignaled unconditional stimuli. J. Exp. Psychol. Anim. Behav. Process. 1993; 19:121–137. [PubMed: 8505593]
24. Garcia–Osta A, et al. MuSK expressed in the brain mediates cholinergic responses, synaptic plasticity, and memory formation. J. Neurosci. 2006; 26:7919–7932. [PubMed: 16870737]
25. Chen DY, et al. A critical role for IGF–II in memory consolidation and enhancement. Nature. 2011; 469:491–497. [PubMed: 21270887]
26. Lee JL, Everitt BJ, Thomas KL. Independent cellular processes for hippocampal memory consolidation and reconsolidation. Science. 2004; 304:839–843. [PubMed: 15073322]
27. Bekinschtein P, Cammarota M, Izquierdo I, Medina JH. BDNF and memory formation and storage. Neuroscientist. 2008; 14:147–156. [PubMed: 17911219]
28. Cahill L, Prins B, Weber M, McGaugh JL. Beta–adrenergic activation and memory for emotional events. Nature. 1994; 371:702–704. [PubMed: 7935815]
29. Hu H, et al. Emotion enhances learning via norepinephrine regulation of AMPA–receptor trafficking. Cell. 2007; 131:160–173. [PubMed: 17923095]
30. Duncan GE, et al. Beta–adrenergic receptor distribution in human and rat hippocampal formation: marked species differences. Brain Res. 1991; 561:84–92. [PubMed: 1665753]
31. Black JW, Crowther AF, Shanks RG, Smith LH, Dornhorst AC. A New Adrenergic Betareceptor Antagonist. Lancet. 1964; 1:1080–1081. [PubMed: 14132613]
32. Chameau P, Qin Y, Spijker S, Smit G, Joels M. Glucocorticoids specifically enhance L–type calcium current amplitude and affect calcium channel subunit expression in the mouse hippocampus. J. Neurophysiol. 2007; 97:5–14. [PubMed: 17021021]
33. Takahashi T, et al. Corticosterone acutely prolonged N–methyl–d–aspartate receptor–mediated Ca2+ elevation in cultured rat hippocampal neurons. J. Neurochem. 2002; 83:1441–1451. [PubMed: 12472898]

34. McReynolds JR, et al. Memory–enhancing corticosterone treatment increases amygdala norepinephrine and Arc protein expression in hippocampal synaptic fractions. Neurobiol. Learn. Mem. 2010; 93:312–321. [PubMed: 19932757]
35. Molteni R, et al. Depression–prone mice with reduced glucocorticoid receptor expression display an altered stress–dependent regulation of brain–derived neurotrophic factor and activity–regulated cytoskeleton–associated protein. J. Psychopharmacol. 2010; 24:595–603. [PubMed: 19074532]
36. Liu W, Yuen EY, Yan Z. The stress hormone corticosterone increases synaptic alpha–amino–3–hydroxy–5–methyl–4–isoxazolepropionic acid (AMPA) receptors via serum– and glucocorticoid–inducible kinase (SGK) regulation of the GDI– Rab4 complex. J. Biol. Chem. 2010; 285:6101–6108. [PubMed: 20051515]
37. Vazdarjanova A, et al. Spatial exploration induces ARC, a plasticity–related immediate–early gene, only in calcium/calmodulin–dependent protein kinase II–positive principal excitatory and inhibitory neurons of the rat forebrain. J. Comp. Neurol. 2006; 498:317–329. [PubMed: 16871537]
38. Jeanneteau F, Garabedian MJ, Chao MV. Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:4862–4867. [PubMed: 18347336]
39. Groeneweg FL, Karst H, de Kloet ER, Joels M. Rapid non–genomic effects of corticosteroids and their role in the central stress response. J. Endocrinol. 2011; 209:153–167. [PubMed: 21357682]
40. Numakawa T, et al. Glucocorticoid receptor interaction with TrkB promotes BDNF–triggered PLC–gamma signaling for glutamate release via a glutamate transporter. Proc. Natl. Acad. Sci. U. S. A. 2009; 106:647–652. [PubMed: 19126684]
41. Lu B. BDNF and activity–dependent synaptic modulation. Learn. Mem. 2003; 10:86–98. [PubMed: 12663747]
42. Meyer–Franke A, et al. Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron. 1998; 21:681–693. [PubMed: 9808456]
43. Ji Y, Pang PT, Feng L, Lu B. Cyclic AMP controls BDNF–induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. Nat. Neurosci. 2005; 8:164–172. [PubMed: 15665879]
44. Alonso M, et al. BDNF–triggered events in the rat hippocampus are required for both short– and long–term memory formation. Hippocampus. 2002; 12:551–560. [PubMed: 12201640]
45. Allaman I, Pellerin L, Magistretti PJ. Glucocorticoids modulate neurotransmitter–induced glycogen metabolism in cultured cortical astrocytes. J. Neurochem. 2004; 88:900–908. [PubMed: 14756811]
46. Sandi C. Glucocorticoids act on glutamatergic pathways to affect memory processes. Trends Neurosci. 2011; 34:165–176. [PubMed: 21377221]
47. Bekinschtein P, et al. Persistence of long–term memory storage requires a late protein synthesis– and BDNF– dependent phase in the hippocampus. Neuron. 2007; 53:261–277. [PubMed: 17224407]
48. Roozendaal B, McGaugh JL. Basolateral amygdala lesions block the memory–enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats. Eur. J. Neurosci. 1997; 9:76–83. [PubMed: 9042571]
49. Ou LC, Yeh SH, Gean PW. Late expression of brain–derived neurotrophic factor in the amygdala is required for persistence of fear memory. Neurobiol. Learn. Mem. 2010; 93:372–382. [PubMed: 19995614]
50. Ji JZ, Wang XM, Li BM. Deficit in long–term contextual fear memory induced by blockade of beta–adrenoceptors in hippocampal CA1 region. Eur. J. Neurosci. 2003; 17:1947–1952. [PubMed: 12752794]

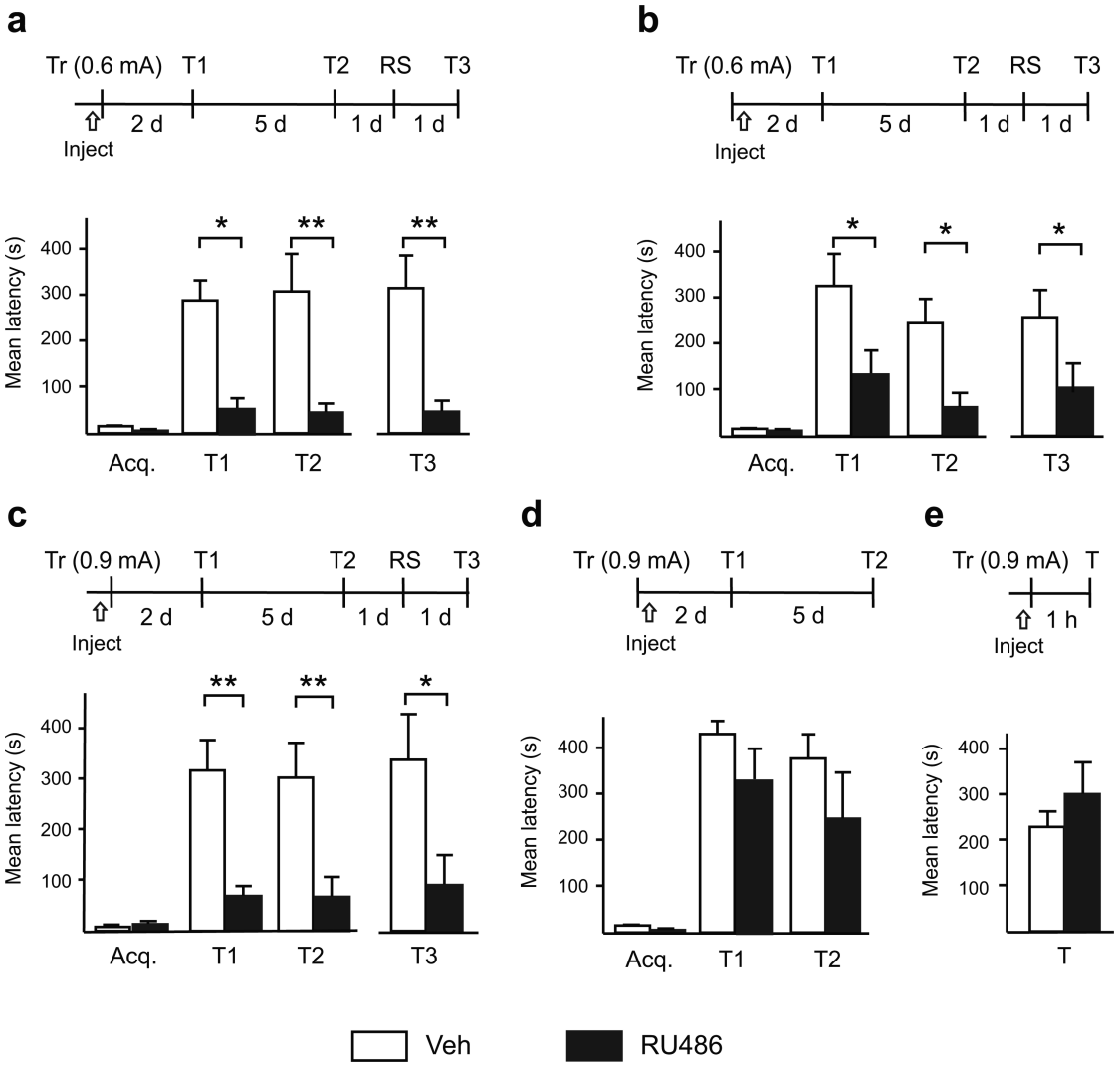

Figure 1.

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 Veh or RU486 either 15 minutes before (**a**, **c** and **e**) or immediately after (**b** and **d**) training elicited with a 0.6 mA footshock (**a** and **b**) or 0.9 mA footshock (**c**, **d** and **e**). [**a** and **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 (T1 and 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 *$P <$ 0.05, ** $P < 0.01$, Student's *t*–test for T3, **a**: $P = 0.004$; **b**: $P = 0.043$]; [**c** and **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 *$P < 0.05$, ** $P < 0.01$, Student's *t*–test for T3, **c**: $P = 0.049$]. **e**:

Student's *t*–test $P = 0.37$. Acq. = Acquisition; Tr = Training; T = Test; RS = Reminder Shock.

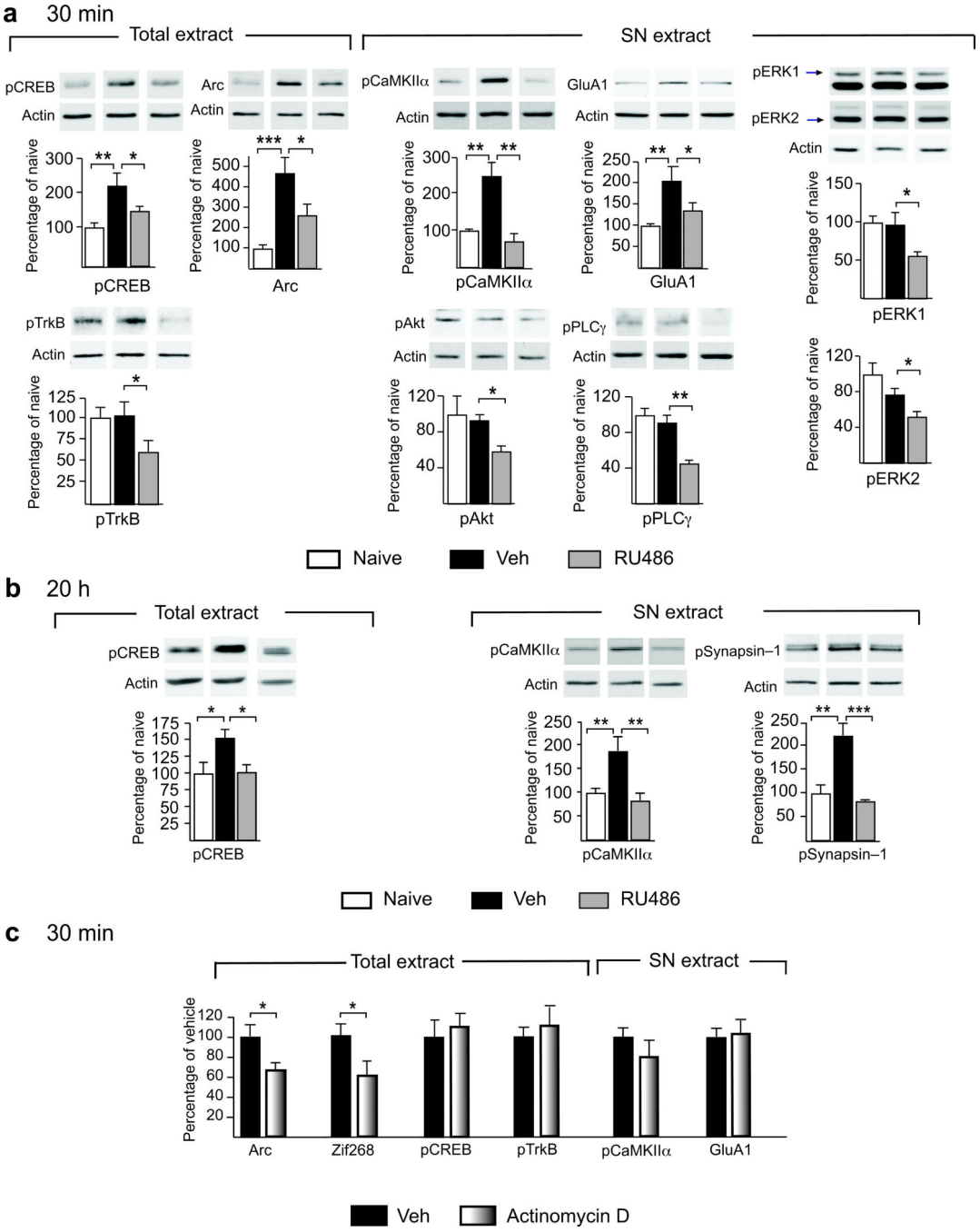

Figure 2.

Molecular pathways coupled to hippocampal glucocorticoid receptors following inhibitory avoidance training. Mean %, n and ANOVA *F* values can be found in **Supplementary Table 2**.

(a) Examples (full–length blots images shown in Supplementary Fig. 8) and densitometric quantitative western blot analyses of hippocampal total extracts and synaptoneurosomal (SN) extracts from naïve or trained rats injected with either vehicle or RU486 15 minutes before training and euthanized 30 minutes after training (normalized against actin). Data are

expressed as mean percentage ± s.e.m. of naïve 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 $F_{2,22} = 10.89$, $P = 0.0006$; pCaMKII⟨ $F_{2,25} = 14.48$, $P < 0.0001$; 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 $F_{2,16} = 3.75$, $P = 0.049$; pPLC© $F_{2,22} = 9.63$, $P = 0.0012$,*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(**b**) Examples and densitometric quantitative western blot analyses of of hippocampal total extracts and SN extracts from naïve or trained rats injected with vehicle or RU486 15 minutes before training and euthanized 20 hours after training (normalized against actin). Data are expressed as mean percentage ± s.e.m. of naïve 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 $F_{2,21} = 11.2$, $P = 0.0006$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(**c**) Densitometric quantitative western blot analyses of hippocampal total extracts and SN extracts from trained rats injected with vehicle or Actinomycin D 15 minutes before training and euthanized 30 minutes after training (normalized against 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 $P = 0.8214$; *$P < 0.05$). Full-length western blots for this figure are shown in **supplementary figures 8, 9, 10, 11, 13, 14 and 16**.

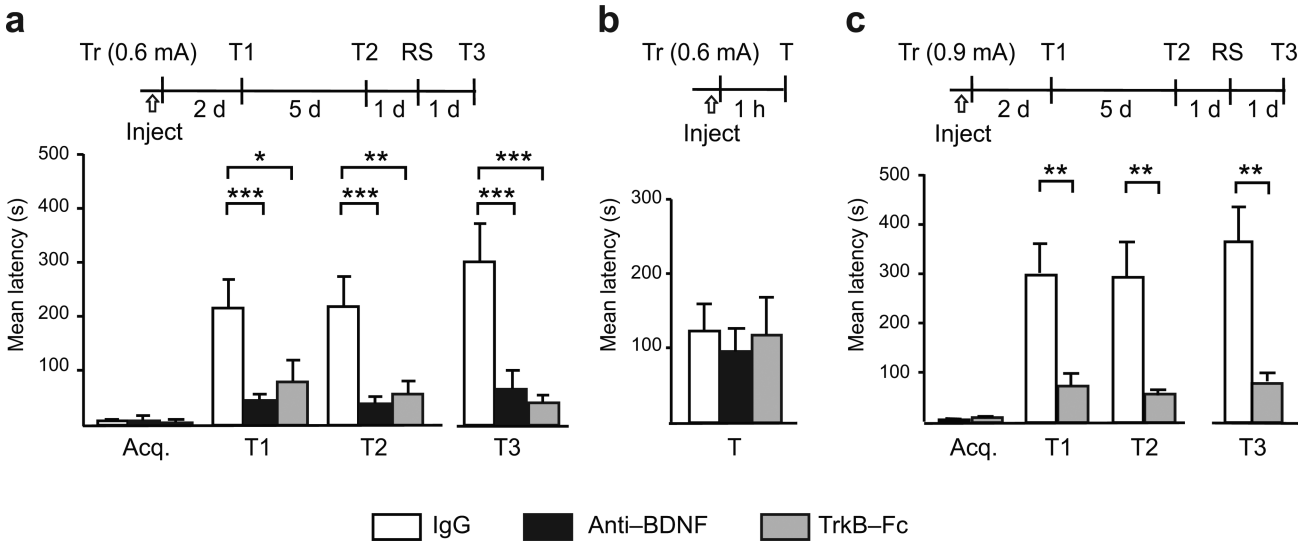

Figure 3.

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, anti–BDNF antibodies, or TrkB–Fc 15 minutes before training elicited with a 0.6 mA footshock (**a** and **b**) or 0.9 mA (**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 *P < 0.05, **P < 0.01, *** P < 0.001; 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 ***P < 0.001); **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), treatment × time interaction ($F_{1,22}$ = 0.0143, P = 0.9058) followed by Bonferroni post hoc tests **P < 0.01); Student's t–test for T3, **P < 0.01]. Acq. = Acquisition; Tr = Training; T = Test; RS = Reminder Shock.

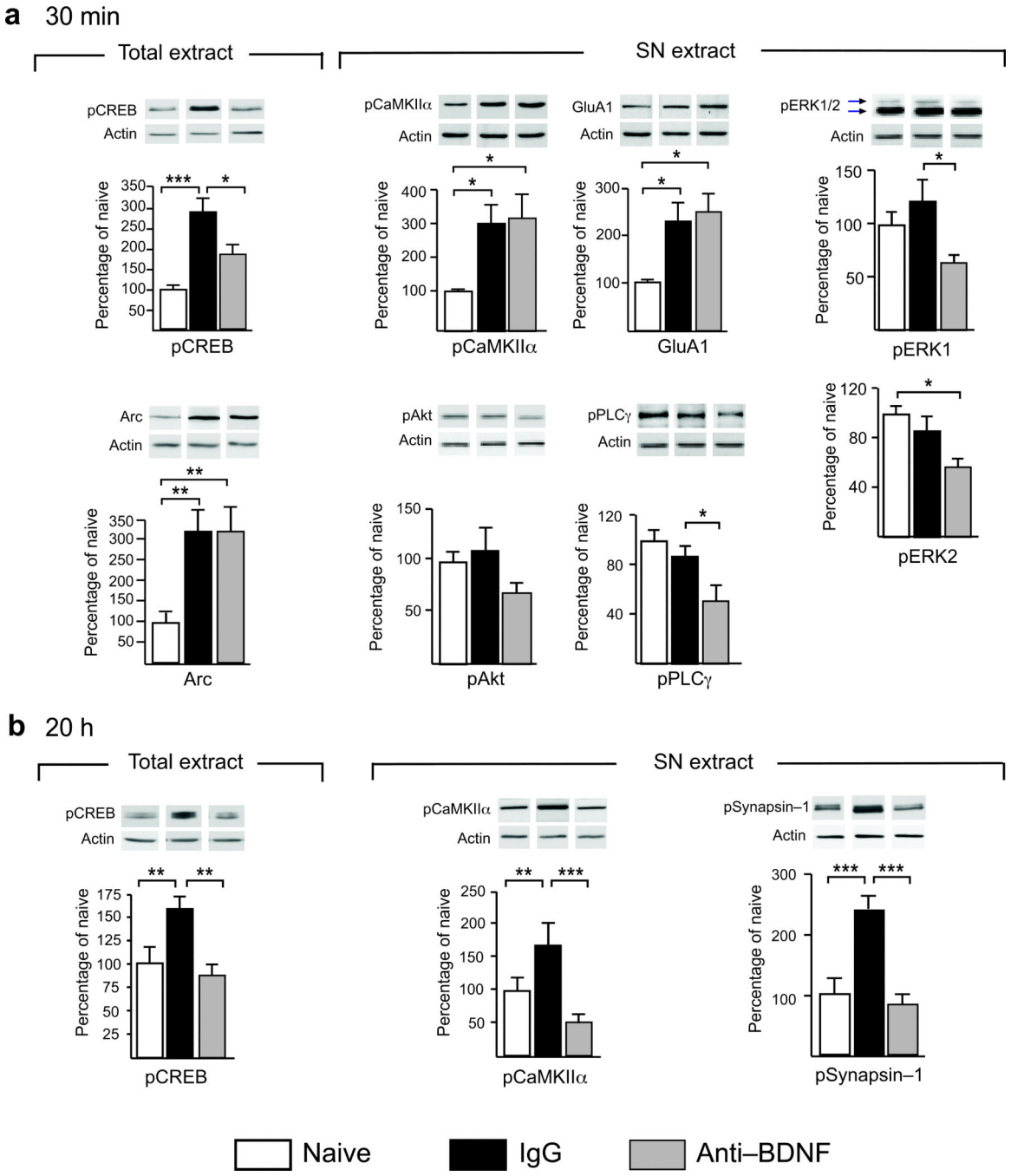

Figure 4.

Molecular pathways coupled to BDNF following inhibitory avoidance training. Mean %, 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 naïve and trained rats injected with either IgG or anti–BDNF antibodies 15 minutes before training and euthanized 30 minutes after training (normalized against 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; pCaMKII⟨ $F_{2,22}$ = 4.975, P = 0.0176; GluA1 $F_{2,17}$ = 4.444, P = 0.0305; pERK 1 $F_{2,25}$ = 4.28, P = 0.0275; pERK2 $F_{2,25}$ = 5.22 P = 0.0135; pPLC© $F_{2,14}$ = 8.422, P = 0.0052, *P < 0.05, **P < 0.01, ***P < 0.001). (**b**) Examples and densitometric quantitative western blot analyses of hippocampal total extracts and SN extracts from naïve and trained rats given hippocampal injections of IgG or anti–BDNF antibodies 15 minutes before training and euthanized 20 hours after training (normalized against actin). Data are expressed as mean percentage ± s.e.m. of naïve 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, **P < 0.01, ***P < 0.001). Full-length western blots for this figure are shown in **supplementary figures 9, 10, 11, 12, 14 and 15**.

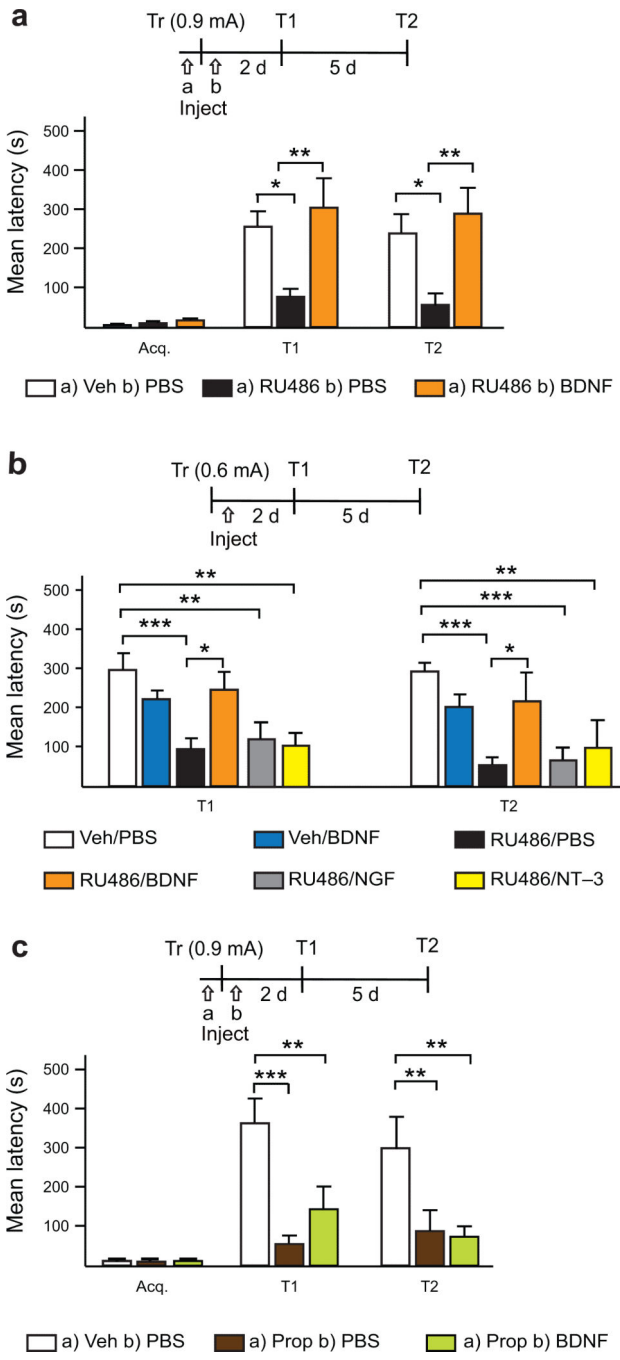

Figure 5.

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 minutes before training elicited with a 0.9 mA footshock and then either 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 *$P < 0.05$, **$P < 0.01$]. (**b)** Rats were given hippocampal injections (⇑) of Veh/PBS, Veh/BDNF, RU486/PBS, RU486/BDNF, RU486/NGF, RU486/NT–3 immediately after training elicited with a 0.6 mA footshock [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 *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$]. (**c**) Rats were given hippocampal injections (⇑) of either vehicle of propranolol 15 minutes before training elicited with a 0.9 mA footshock 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 ($F_{1,36} = 0.3516$, $P = 0.5569$), treatment × time interaction ($F_{2,36} = 0.5369$, $P = 0.5892$) followed by Bonferroni post hoc tests **$P < 0.01$, *** $P < 0.001$]. Acq. = Acquisition; Tr = Training; T = Test.

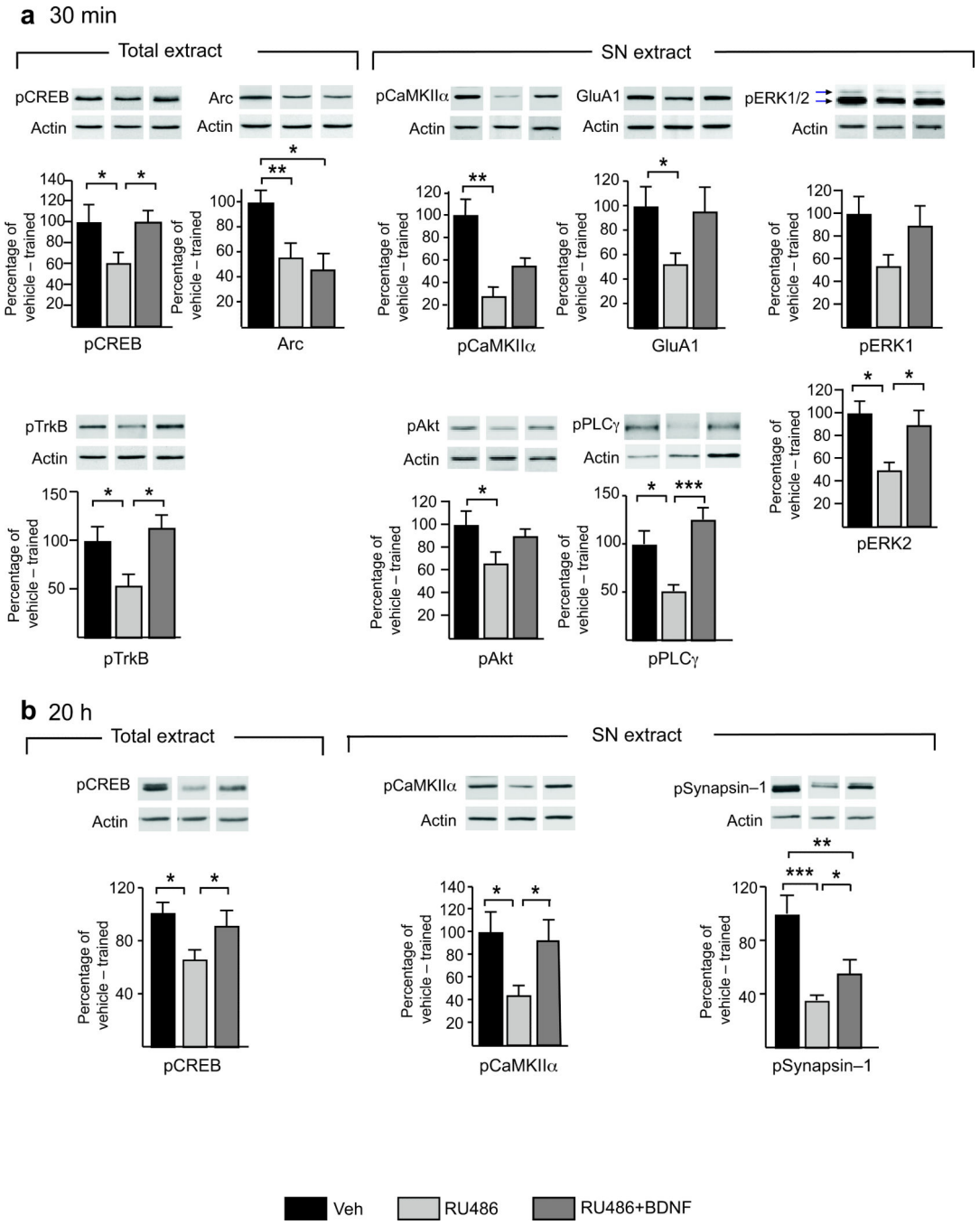

Figure 6.

BDNF rescues the RU486–mediated molecular disruption. Mean %, 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+BDNF 15 minutes before training and euthanized 30 minutes after training (normalized against 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; pCaMKII⟨ $F_{2,25}$ = 10.44, P = 0.0006; GluA1 $F_{2,25}$ = 3.455, P = 0.0488; pERK2 $F_{2,29}$ = 4.267, P = 0.0245; pAkt $F_{2,14}$ = 4.064, P = 0.0449; pPLC© $F_{2,26}$ = 14.63, P < 0.0001,*P < 0.05, **P < 0.01, ***P < 0.001). (**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+BDNF 15 minutes before training and euthanized 20 hours after training (normalized against 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 were used for 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+BDNF to RU486; *P < 0.05, ***P < 0.001;). Full-length western blots for this figure are shown in **supplementary figures 11 and 17**.