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## Infantile amnesia reflects a developmental critical period for hippocampal learning

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### Abstract

Episodic memories formed during the first postnatal period are rapidly forgotten, a phenomenon known as *infantile amnesia*. In spite of this memory loss, early experiences influence adult behavior, raising the question of which mechanisms underlie infantile memories and amnesia. Here we show that in rats an experience learned during the infantile amnesia period is stored as a latent memory trace for a long time; indeed, a later reminder reinstates a robust, context-specific and long-lasting memory. The formation and storage of this latent memory requires the hippocampus, follows a sharp temporal boundary, and occurs through mechanisms typical of developmental critical periods, including brain-derived-neurotrophic-factor (BDNF)- and metabotropic-glutamate-receptor-5 (mGluR5)-dependent expression switch of the N-methyl-D-aspartate receptor subunits 2B-2A. BDNF or mGluR5 activation after training rescues the infantile amnesia. Thus, early episodic memories are not lost, but remain stored long-term. These data suggest that the hippocampus undergoes a developmental critical period to become functionally competent.

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#### Author Contributions

A.T., R.B., E.S.S., R.D.B., and C.M.A. designed and developed this study. A.T. and R.B., and E.S.S. carried out the experiments. A.T., E.S.S., R.D.B., and C.M.A. wrote the manuscript.

#### Competing financial interests

The authors declare no competing financial interests.

**A Supplementary Methods Checklist is available.**

#### Data availability.

The data that support the findings of this study are available from the corresponding author upon request.

## Introduction

While salient, episodic memories formed in adulthood can be remembered for years, similar memories formed during early childhood appear to be easily and rapidly forgotten. This rapid forgetting and the inability to recall early life memories in adulthood is found in humans as well as in non human animals, and is known as infantile or childhood amnesia<sup>1-3</sup>. Despite they apparently are rapidly forgotten, experiences during early life have been documented to profoundly affect brain functions and physiology later in life. For example, early threatening experiences predispose to psychopathologies like post-traumatic stress disorder (PTSD), mood and anxiety disorders<sup>4</sup>. This paradox raises the question of how early memories can influence adult life if they cannot actually be remembered.

Several hypotheses have been put forward to explain this paradox. One suggests that the amnesia is due to the immaturity of the infant brain. According to this idea, the neural substrates and cell/circuitry composition of cortical, hippocampal or of other areas processing episodic, or in general hippocampal-dependent memories, being still underdeveloped, lack functional competence<sup>5, 6</sup>. Among the immature functions of the infant brain, recent studies suggested that the rapid forgetting is due to increased rate of hippocampal neurogenesis during development, which would interfere with memory consolidation, the process that mediates long-term memory persistence and storage<sup>7</sup>. In contrast, other authors have proposed that infantile amnesia results from impaired memory retrieval<sup>3, 8, 9</sup>, providing an explanation for how the stored, non-expressed memory traces would affect the behavior later in life. In support of this conclusion, studies in human and animal models reported that reminders of the early experience can reinstate a memory acquired during infancy<sup>10-13</sup>. However, because little is known about the mechanisms underlying acquisition, and possibly storage of memory traces acquired during early life, the paradox is still unsolved.

Using inhibitory avoidance (IA) in rats, here we show that the acquisition and storage of an episodic threatening experience during the infantile amnesia period require hippocampal mechanisms. These mechanisms are typical of developmental critical periods, matures in response to experience, and store hippocampal-dependent traces in a latent form for a long time. These memory traces can influence behavior later in life, as reminders comprising both context and threat reinstate a context-specific long-lasting memory.

## RESULTS

### An early-life memory reinstates with later reminders

In agreement with previous studies done in animal models of contextual threat memories using multiple shocks<sup>14, 15</sup>, we show that a single footshock during IA training in rats at postnatal day 17 (PN17), but not at PN24, recapitulates the infantile amnesia phenomenon. The numeric values with detailed statistical analyses of all experiments of this study are shown in Supplementary Tables. Compared to acquisition, training at PN17 produced significant memory retention immediately after training, which, however, completely decayed 1 day (d) later (Fig. 1a). The latency of PN17 trained rats, although still significantly higher than that at acquisition, was profoundly decreased already 30 minutes

(min) after training (Fig. 1b). This memory, again, was completely lost 1d after training (Fig. 1b). Littermates left undisturbed in their home cage (naïve group), or placed on a shock grid and immediately exposed to a footshock (shock-only group) never showed significant latency above that of acquisition, excluding that the retentions following training were the result of non-specific responses (Fig. 1a-c). In contrast, training at PN24 elicited a strong and long-lasting associative memory at all timepoints tested, including immediately after, 30 min, 1d and 7d after training (Fig. 1d-f); these retentions were comparable to those of adult rats (Supplementary Fig.1).

Furthermore, the first testing (immediately after, or 30 min after training) did not contribute to the amnesia seen 1d after training, as PN17 trained rats tested only 1d after training had no memory compared to their acquisition latency or to latency of naïve rats (Fig. 1c). This amnesia persisted one week after training (Fig. 1c).

The infantile amnesia was not caused by alterations in exploratory behavior, locomotor or nociceptive responses, as latencies to enter the dark compartment at training as well as escape latencies from a hotplate were undistinguishable between PN17 and PN24 rats (Supplementary Table 1 and Supplementary Fig. 2). Moreover naïve, shock-only and trained rats at PN17 or PN24 had comparable weight gain at 1d and 7d following training (Supplementary Fig. 3).

As previous studies suggested that infantile amnesia results from memory retrieval failure, rather than an inability to form long-term contextual memories<sup>8-13</sup>, we tested whether contextual reminders could reinstate memory in PN17 trained rats. Contextual reminder provided by testing at 1 (T1), 7 (T2), 10 (T3) and 16 days after training (T4) produced no memory compared to naïve and shock-only rats (Fig. 1g). However, when the rats were then presented with a reminder footshock (RS) two days after the second test (T2), a robust and long-lasting IA latency emerged (T3 and T4, Fig. 1h). The reinstatement protocol *per se* did not establish any IA latency, as naïve or shock-only control rats exposed to the reinstatement protocol had no latency above acquisition (Fig. 1h). Notably, the reinstated memory was context specific because following reinstatement the rats tested in a different IA box (new context, NC) showed no retention above acquisition (NC, Fig. 1h). Furthermore, an unpaired presentation at PN17 of the context and, one hour later, of the footshock failed to elicit IA memory (Supplementary Fig. 4), indicating that the latent memory trace is associative. Exposure to RS alone (i.e. without any testing) was also not sufficient to reinstate the memory (Fig. 1i). Furthermore, only one test given 7d after training, followed by a RS given 2d thereafter (T+RS) was sufficient to reinstate the memory (T2 and T3, Fig. 1j).

This T+RS effectively reinstated the latent memory for a long time: significant latency was in fact elicited with T+RS given four weeks after training at PN17 (T2 and T3, Fig. 1k).

To determine whether the time interval between T and RS is crucial for IA memory to emerge, the RS was delivered at 4h, 1d or 7d following T. In all these cases T+RS were able to reinstate a long-lasting and context specific memory, suggesting that the time interval between T and RS is not strict (Supplementary Fig. 5).

Thus, a latent and long-term memory trace is acquired at training during the infantile amnesia period; a later reactivation of the memory through exposure to both context and footshock, presented in a temporally unpaired manner (e.g. two days apart), is necessary and sufficient to reinstate a strong, long-lasting and context-specific IA memory.

### **The latent infantile memory trace is hippocampus-dependent**

The hippocampus has a key role in the formation and consolidation of long-term episodic and contextual memories in adulthood<sup>16, 17</sup>, but its role is believed to emerge over time. Several studies in rats reported that hippocampal-dependent learning and memory emerges not earlier than PN21, concluding that the hippocampus before PN21 is unable to support long-term memory formation<sup>5, 15, 18</sup>. Although recently Foster and Burman<sup>19</sup> provided evidence of hippocampal-dependent context pre-exposure learning facilitation in rats at PN17, Robinson-Drummer and Stanton<sup>20</sup> failed to replicate the result.

Here, we asked whether the dorsal hippocampus (dHC) is involved in the acquisition of the IA infantile memory trace. PN24 rats were employed as controls. Compared to vehicle, a bilateral injection of the neural activity blocker GABAA agonist muscimol in the dHC, 30 minutes before training at PN17, prevented memory reinstatement after the T+RS (T2, Fig. 2a), without affecting the latency at training or at tests 1 (T1, Fig. 2a). The muscimol-injected rats learned the IA task when retrained (Tr) upon entering the shock compartment at T2, showing that muscimol had not damaged the hippocampus (T3, Fig. 2a). As expected, compared to vehicle, the bilateral injection of muscimol in the dHC 30 minutes before training at PN24 significantly impaired long-term memory, confirming that, at this age, like in adulthood, hippocampal activity is required to form IA memory (Fig. 2b). Again, muscimol-injected rats were able to acquire the IA task after retraining (T3, Fig. 2b). Furthermore, dHC activity was not required for memory reinstatement. Compared to vehicle, bilateral dHC injection of muscimol 30 min before T, followed by RS 2 days later, did not affect memory reinstatement (T2, Fig. 2c). Likewise, a bilateral injection of muscimol into the dHC 30 min before RS had no effect on memory reinstatement (T2, Fig. 2d).

Thus, the hippocampus plays a critical role in encoding and storing a latent IA memory trace at PN17 during the infantile amnesia period, but is not critical for later IA memory reinstatements.

### **Learning at PN17 induces pTrkB and GluN2B-2A subunit switch**

We then compared the hippocampal expression profiles of molecules known to play critical roles in synaptic plasticity of PN17, PN24 and adult (PN80) rats, in either untrained conditions or following IA training. Because of their fundamental roles in plasticity and memory, including IA<sup>17, 21-23</sup>, as well as in development<sup>24</sup>, we examined the levels of brain-derived neurotrophic factor (BDNF), the activation of the receptor TrkB (phosphorylation of TrkB, pTrkB) and the levels of N-methyl-D-aspartate receptor (NMDAR or GluNR) subunits 1 (GluN1 or NR1), 2A (GluN2A or NR2A) and 2B (GluN2B or NR2B). Western blot analyses of dHC extracts revealed that while BDNF levels increased with developmental age (Fig. 3a), pTrkB was significantly higher in PN17 rats compared with adults, with PN24

rats showing intermediate values (Fig. 3a). The level of total TrkB did not change across development (Supplementary Fig. 6). In line with previous data<sup>25, 26</sup>, we found that NMDARs significantly switch their subunit expression levels during postnatal development, going from an elevated expression ratio in favor of GluN2B to predominately expressing GluN2A (Fig. 3a). The level of GluN1 did not change with development (Supplementary Fig. 6). Strong evidence in the visual system and neonatal hippocampal slices support the idea that the GluN2B-2A subunit switch is driven by experience and neuronal activity during a developmental *critical period*, a temporally limited phase during which experience organizes normal functional development and permanently alter performance<sup>27, 28</sup>. Visual experience and deprivation rapidly and reversibly alter the NMDAR subunit composition in the visual cortex, modifying the duration of the critical period<sup>29-31</sup>. Here, we tested whether training at PN17 or PN24 differentially regulates the activation of the BDNF-TrkB pathway and the levels of GluN1, GluN2B and GluN2A over time. Rats were euthanized at 30min, 9h, 24h, and 48h post-training. Controls consisted of naive rats euthanized at matched timepoints (to control for developmental changes independent of training), as well as rats exposed to shock-only or context-only (context exposure without footshock) and euthanized at matched timepoints (to control for changes induced by non-associative experience). Compared to naïve conditions, training at PN17 significantly increased pTrkB at 30min after training. The significant increase persisted and peaked at 24h after training (Fig. 3b). This profile of training-dependent increase in pTrkB was distinct from that found in rats trained at PN24 (Fig. 3c), in which the pTrkB augmentation peaked at 30 min after training and decayed thereafter. No significant changes were found in total TrkB levels at any of the timepoints after training at PN17 or at PN24 (Supplementary Fig. 6). Training at PN17 increased also the level of BDNF but not significantly over that of the developmental increase (Fig. 3b), whereas training at PN24 significantly augmented BDNF levels at 9h and 24h post-training (Fig. 3c).

At PN17, training led to a pronounced and significant induction of GluN2A starting 9h after training, which continued up to 48h after training; GluN2B levels increased more slowly and reached significance only at 24h after training, but not at the other timepoints (Fig. 3b). In contrast, training at PN24 did not change the levels of GluN2A and GluN2B (Fig. 3c). No significant changes were found in GluN1 levels throughout the temporal profiles of PN17 or PN24 rats (Supplementary Fig. 6). Thus, IA training significantly increases the GluN2A/2B ratio in the hippocampus of PN17 but not of PN24 rats (Fig. 3b).

To investigate the functional consequences of training on synaptic NMDAR composition in the hippocampus, we compared EPSCs at the Schaffer collateral-CA1 synapse in acute slices from PN17 and PN24 rats, in either untrained conditions or 24h after IA training. The NR2B component of the NMDAR EPSC was determined by sensitivity to ifenprodil, which selectively blocks receptors that contain NR2B subunits; thus, greater inhibition of the EPSC by ifendopril indicates a higher fraction of NR2B-containing receptors. In agreement with our biochemical findings (Fig. 3c), EPSCs from naive and trained PN24 rats showed essentially identical ifenprodil sensitivities (naive:  $20.1 \pm 4.3\%$  inhibition,  $n=17$  cells; trained:  $20.7 \pm 4.4\%$ ,  $n=7$ ;  $p=.93$ , two-tailed t-test with Welch's correction), and these groups were combined for subsequent analysis (Fig.3d). Ifenprodil had a significantly stronger effect on EPSCs from naive PN17 rats ( $37.2 \pm 6.4\%$ ,  $n=12$  cells;  $p=0.04$ ) compared

to PN24 rats. In contrast, the ifenprodil sensitivity of EPSCs from trained PN17 rats did not differ significantly from P24 rats ( $30.0 \pm 6.1\%$ ,  $n=10$  cells;  $p=0.38$ ), consistent with training-induced conversion of a subset of NMDARs from NR2A- to NR2B-containing in PN17 animals.

Notably, the biochemical changes in NMDA receptor subunit composition found with both development or training strongly correlated with EPSC sensitivity to ifenprodil (Fig. 3e).

All the significant training-induced changes were selective for the associative conditioning and were not found in the shock-only or context-only controls (Supplementary Fig. 7).

No significant changes in pTrkB, GluN2A and GluN2B were found at 30 min or 24h after T +RS, in line with our findings (Fig 2c and 2d) that the hippocampus plays a critical role in encoding the latent IA memory trace at PN17, but not in reinstatement, (Supplementary Figure 8).

We concluded that the latent memory trace acquired at PN17 is accompanied by an activation of TrkB (phosphorylation) and a significant switch in the GluN2A/2B expression level ratio in the hippocampus that persisted at least for 24 hours.

### **BDNF controls early memory formation and the GluN2B-2A switch**

Given the above results, and that BDNF modulates the expression of NMDA receptors<sup>32</sup>, we then tested whether BDNF in the dHC of PN17 rats is required for the formation of the latent IA memory trace and/or the training-induced GluN2B-2A switch.

Hippocampal bilateral injection of either a function-blocking anti-BDNF antibody or its receptor scavenger TrkB-Fc, compared to control IgG, significantly disrupted memory reinstatement (T2, Fig. 4a and 4b) without affecting general locomotor behavior during training or testing (T1, Fig. 4a and 4b). Anti-BDNF- and TrkB-Fc-injected rats learned IA when retrained showing that anti-BDNF and TrkB-Fc had not damaged the hippocampus (T3, Fig. 4a and 4b). Furthermore, anti-BDNF blocked the training-induced increase in pTrkB and the GluN2B/2A switch at 24h after training (Fig.4c), a timepoint when the training-induced molecular changes peaked (Fig. 3b).

Thus, BDNF at training is necessary for the formation of latent infantile memory traces and training-induced NMDA receptor 2B-2A expression switch.

### **mGluR5-dependent GluN2B-2A switch rules early memory formation**

We then asked whether the NMDAR subunits GluN2A and 2B are differentially recruited in the formation of the latent memory at PN17, and whether their contribution changes at PN24 when the system is functionally competent. GluN2A and GluN2B differentially contribute to the NMDAR current kinetics<sup>33</sup>, thus differentially regulating synaptic plasticity<sup>34</sup>. Selective antagonists of GluN2A ([[(1S)-1-(4-Bromophenyl)ethyl]amino](1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxaliny)methyl] phosphonic acid tetrasodium hydrate, or NVP-AAM077 or PEAQX), or 2B ([R-(R\*,S\*)]- $\alpha$ -(4-Hydroxyphenyl)- $\beta$ -methyl-4-(phenylmethyl)-1-piperidinepropanol hydrochloride hydrate, or Ro 25-6981) injected bilaterally into the dHC

30 minutes before training at PN17 revealed that GluN2B but not GluN2A is required for IA memory formation (T2, Fig. 5a), without affecting acquisition or testing (T1, Fig. 5a). All rats acquired the IA task after retraining, indicating that the treatments did not damage the hippocampus (T3, Fig. 5a). In contrast, injection of either GluN2A or GluN2B antagonist 30 min before training at PN24 significantly disrupted memory retention both 1d and 7d after training (Fig. 5b). Notably, the memory loss caused by the GluN2A antagonist at PN24 was complete at both T1 and T2, as in fact the retention of the GluN2A antagonist-injected rats was not significantly different from acquisition (Fig. 5b, Supplementary table 7). These results, in agreement with the relative higher expression levels, suggest that GluN2A plays a major role in NMDAR-mediated memory formation at PN24; in contrast, GluN2A has no effect on the formation of latent memories at PN17.

Because the GluN2B-GluN2A switch has been shown to require the activation of mGluR5 in CA1 pyramidal neurons in acute hippocampal slices<sup>35</sup> and mGluR5 knockout mice confirmed that the developmental GluN2B-GluN2A switch in CA1 is deficient<sup>35</sup>, we tested whether blocking mGluR5 in the dHC affects the training-induced switch of GluN2B-2A and memory formation at PN17 and/or PN24. Western blot analyses of dHC extracts of untrained rats revealed that mGluR5 levels are significantly higher at PN17 compared to adult age, with PN24 showing intermediate values (Fig. 5c).

Hippocampal bilateral injection of the mGluR5 antagonist 3-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]pyridine hydrochloride (MTEP) 30 min before training at PN17 blocked the training-induced GluN2B-GluN2A subunit switch 24h after training (Fig. 5d). Furthermore, compared to vehicle, the same MTEP treatment significantly prevented memory reinstatement without affecting latency at training or at the 7d test (Fig. 5e, Supplementary Table 7). The treatments did not damage the hippocampus as rats acquired IA when retrained (T3, Fig. 5e). Conversely, at PN24, the same MTEP treatment had no effect on memory retention 1d and 7d after training (Fig. 5f).

In addition, hippocampal injections of MTEP 30 min before IA training at PN17 blocked the training-induced increase in TrkB phosphorylation 24h after training, indicating that the mGluR5-TrkB pathways are functionally linked (Fig. 5g).

We concluded that the formation and storage of the latent memory trace during the infantile amnesia period, but not of a normally expressed memory formed a few days later, require GluN2B and the mGluR5-mediated GluN2B-GluN2A subunit switch, and that the activation of mGluR5 is functionally linked to that of TrkB.

### **BDNF or mGluR5 activation close the infantile amnesia period**

Our data thus far indicate that mechanisms underlying the formation of a latent infantile memory trace are similar to those of developmental critical periods of sensory systems, including the experience-dependent switch of GluN2B-2A, requirement of mGluR5 and of BDNF. In the visual cortex, BDNF overexpression is sufficient to close the critical period by promoting structural maturation of cortical circuitry and precocious increase of visual acuity<sup>36, 37</sup>. Here we tested whether, likewise, increasing hippocampal BDNF at the time of training is sufficient to promote functional competence, thus closing the critical period of the

infantile amnesia. BDNF bilaterally injected into the dHC immediately after training at PN17 led to significant and persistent memory expression 1d (T1) and 7d (T2) after training (Fig. 6a). The memory was context specific (NC, Fig. 6a). Furthermore, hippocampal BDNF injection, compared to vehicle, promoted a significant increase the phosphorylation of TrkB (Fig. 6b) and the GluN2B-2A switch (Fig. 6c).

As our previous data showed that mGluR5 is functionally linked and upstream the TrkB activation (Fig. 5g), we tested whether the activation of mGluR5 in the hippocampus at the time of training is, like BDNF, sufficient to close the infantile amnesia period. Dorsal hippocampus bilateral injection of the group I metabotropic glutamate receptor agonist 3,5 dihydroxyphenylglycine (DHPG), compared to vehicle, elicited a significant and persistent memory expression at 1d (T1) and 7d (T2) after training (Fig. 6d).

Thus, like for sensory system functions, an immature hippocampus employs critical period-like mechanisms, which are regulated by experience, to mediate the formation of a latent memory trace during infantile amnesia. BDNF or activation of the upstream mGluR5 at the time of training can close the infantile amnesia period and induce functional competence.

## Discussion

Using a model of episodic contextual fear memory in rats, here we reported that learning occurring during the infantile amnesia period (PN17) produces a persistent, latent retention of that experience; in fact, reminders given later in life reinstate a strong, context-specific and long-lasting memory. These data extend and provide an explanation for previous knowledge that early life experiences, including aversive, appetitive and spatial, affect behavior throughout life<sup>1, 38</sup>. In our paradigm, presentation of both context and footshock (RS) later in life are required to reinstate context specific memories, as exposure to either, alone, is not sufficient. We also found that once context exposure is experienced, the memory trace is accessible for a long time, as in fact it can form an association with a RS given days later. However, as RS given before context is unable to reinstate the memory, we speculate that an associative reactivation is needed in order to reinstate the memory, which is presumably driven by CS-anticipatory responses<sup>39</sup>. Possibly, the reinstatement protocol reflects a facilitated conditioning that builds on the established latent memory trace. Conversely, rats at PN24 were fully competent in learning and expressing IA memory, as their retention after training was similar to that of adult rats. Thus, the infantile amnesia period for explicit memories appears to show a relatively sharp temporal boundary.

Although thus far it has been debated whether the memory learned at PN17 is forgotten<sup>1-3, 7, 8</sup>, and whether an hippocampal-dependent contextual learning at PN17 in rats occurs at all<sup>18-20</sup>, our data show that long-lasting changes through BDNF and mGluR5-dependent mechanisms take place in the dorsal hippocampus with training to store a latent representation. These mechanisms are unique to PN17, and include a prolonged activation of the BDNF pathway, a mGluR5-dependent switch in the ratio of GluN2B/GluN2A expression, and a functional switch toward synaptic GluN2A engagement. These data indicate that the hippocampal system, like sensory systems<sup>34, 35</sup>, matures and develop in response to experience by switching the NMDAR subunit composition. In fact, while the



latent memory formed at PN17 requires mGluR5 and GluN2B but not GluN2A, the strong memory formed by the functionally competent PN24 hippocampus requires GluN2A but not mGluR5. These data are in line with previous reports that a mGluR5-dependent GluN2B-GluN2A switch occurs *in vitro* in the developing hippocampus after acute activity, and parallel the mechanisms described in the developing visual cortex after visual stimuli<sup>28, 35</sup>. The GluN2B-GluN2A switch is in fact classically known as a critical mechanism by which excitatory synapses rapidly mature in response to experience during sensory developmental critical periods and acquire integrative capacity<sup>28-31,40-42</sup>. Our results indicating that similar mechanisms occur in the developing hippocampal memory system lead us to propose that infantile amnesia reflects the existence of a developmental critical period of the hippocampus, and that the functional maturation of the hippocampal system occurs through learning experience. Synaptic strength may also change as a result of IA training. These additional mechanisms as well as circuits important for infantile memories shall be investigated in future studies.

In agreement to what has been reported for the visual or auditory system critical periods<sup>36, 37, 43</sup>, we found that activating mGluR1/5 or increasing BDNF in the dHC accelerates hippocampal functional competence for memory formation and expression and anticipates the end of the critical period. It is possible that, as shown for sensory systems, BDNF regulates the balance of excitatory and inhibitory (E/I) neurotransmission in the hippocampus during the developmental critical period<sup>27</sup>, and further studies shall address this issue. Our data also revealed that mGluR5 and BDNF are functionally linked in promoting hippocampal competence, as in fact the persistent activation of TrkB depends on mGluR5 activation. This link may represent an important node of dysregulation in neurodevelopmental disorders, such as Fragile X syndrome<sup>44</sup>.

In sum, we suggest that experience-driven, mGluR5- and BDNF-mediated GluN2B-2A subunit switch during a developmental critical period is not only typical of sensory systems, but represents a ubiquitous process in the developing brain for acquiring mature, functional competences. This competence, for the hippocampus, includes the ability to form explicit, associative long-term memories. This would also explain how early experiences influence brain development<sup>45</sup> and how experience deprivation in early development impacts learning abilities throughout life<sup>46</sup>. We suggest that alteration of the experience-dependent critical period mechanisms of the medial temporal lobe, thus of related networks, may fundamentally contribute to the etiology of developmental learning disabilities, including autism spectrum disorder, mental retardation and neuropsychiatric disorder in general.

## METHODS

### Animals

Seventeen- and 24-day old male and female rats were obtained from E10/11 pregnant Long Evans female rats (Charles River Laboratories). Pre-weaning rats were housed with their littermates and mother in 30.80 cm × 40.60 cm × 22.23 cm plastic cage, containing ALPHA-dri® bedding, under a 12h light/dark cycle (light on at 07.00 a.m.) with food and water *ad libitum*. All experiments were carried out during the light cycle. The birth date was considered P0 and the litters were culled to 10-12 pups (6 males and 6 females, if

applicable) on PN1 and only one male and/or female per litter was used in any experimental condition. After weaning (P21), rats were group-housed (2-5 per cage). All procedures complied with the US National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the New York University Animals Care Committees.

### **Inhibitory avoidance**

Inhibitory avoidance (IA) was carried out as previously described<sup>17</sup>. The IA chamber (Med Associates Inc., St. Albans, VT) consisted of a rectangular Perspex box divided into a safe compartment and a shock compartment (each 20.3 cm × 15.9 cm × 21.3 cm). The safe compartment was white and illuminated and the shock compartment was black and dark. 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 s, the door separating the compartments was automatically opened, allowing the rat access to the shock compartment. The door closed automatically when the rat entered the shock compartment with all four limbs, and a foot shock (2 s, 1 mA) was administered. Foot shocks were delivered to the grid floor of the shock chamber via a constant current scrambler circuit. Animal remained in the dark compartment for additional 10 s before was returned to its home cages until testing for memory retention at designated timepoints. As controls, we used naïve animals (handled and remained in their home cage) and rat exposed to a footshock without the IA context experience (shock-only). Shock-only consisted in placing the rat onto grid of the shock compartment and, immediately after, delivering a footshock of the same duration and intensity used in IA training. This protocol does not induce any association between the context and the foot shock. Retention tests were done by placing the rat back in the safe compartment and measuring its latency to enter the dark compartment. Foot shocks were not administered during the retention tests (unless otherwise specified), and testing was terminated at 900 s. During retraining session, rats were tested for memory retention and received a footshock upon entering into the dark compartment. Locomotor activity was measured in the inhibitory avoidance chamber by automatically counting the number of times each rat crossed the invisible infrared light photosensor during training and testing. All behavioral tests were carried out blind to training and/or treatment conditions. For biochemical studies, rats were not tested for memory retention. Reminder foot shocks (RS) were administered in a novel, neutral chamber with transparent walls at identical duration and intensity to that of training. Context generalization was tested in a new, modified IA box with a smooth plastic floor and decorated colorful walls, located in a different experimental room.

### **Hot-plate test for pain threshold**

The hot-plate (HP) test for pain threshold was performed as previously described<sup>47</sup>. The apparatus consists of a round white ceramic hot-plate surface (20cm diameter) set at 53 ± 1°C and contiguous with and extended area (brown cardboard) at room temperature. The test was performed with naive PN17 and PN24 rats. Pain threshold was measured by the escape latency to withdrawal from the hot plate. The escape latency was averaged from four sessions, with 15-min interval between each session.

## Hippocampal cannula implants and injections

On PN15 or PN22, pups were anesthetized with isoflurane mixed with oxygen. Stainless steel cannulas (26-gauge) were implanted bilaterally in the dorsal hippocampus (for PN15, 3.0 mm anterior, 2.2 mm lateral and 2.3 mm ventral from bregma; for PN22: 3.4 mm anterior, 2.2 mm lateral and 2.5 mm ventral from bregma) through holes drilled in the overlying skull. The cannulas were fixed to the skull with dental cement. After recovery from the surgery, pups were returned to the dam and littermates (for PN15) or their homecage (for PN22) for a 2-day recovery period until experimental manipulations. Hippocampal injections used a 33-gauge needle that extended 1 mm beyond the tip of the guide cannula and connected via polyethylene tubing to a Hamilton syringe. Injections were delivered at a rate of  $0.1 \mu\text{l min}^{-1}$  using an infusion pump on a total volume of  $0.3 \mu\text{l}$  per side over 3min. The injection needle was left in place for 2 min after the injection to allow complete diffusion of the solution. Muscimol (Sigma-Aldrich, St. Louis, MO, cat# M1523) was dissolved in physiological saline (0.9%) and injected at  $0.1 \mu\text{g}$  per side. This dose of muscimol has been successfully used to disrupt long-term contextual fear conditioning when injected into the hippocampus<sup>18</sup>. The antibody anti-BDNF (Millipore, Billerica, MA cat# AB1513P) or control IgG (Sigma-Aldrich, cat# I5131) were dissolved in 1x PBS and injected at  $0.5 \mu\text{g}$  per side. Recombinant human TrkB-Fc chimera (R&D Systems) was dissolved in PBS and injected at  $0.5 \mu\text{g}$  per side. The same dose of anti-BDNF and TrkB-Fc had been shown to disrupt long-term contextual fear memory consolidation when injected into the hippocampus<sup>17</sup>. [R-(R\*,S\*)]- $\alpha$ -(4-Hydroxyphenyl)- $\beta$ -methyl-4-(phenylmethyl)-1-piperidinepropanol hydrochloride hydrate (Ro 25-6981, Sigma-Aldrich, cat# R7150) and [[[1S]-1-(4-Bromophenyl)ethyl]amino](1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxaliny)methyl] phosphonic acid tetrasodium hydrate (PEAQX or NVP-AAM077 tetrasodium hydrate, (Sigma-Aldrich, cat# P1999) were dissolved in 1x PBS and injected at  $0.5 \mu\text{g}$  per side. The same doses of the drugs have been shown to modulate LTP *in vitro* and to impair spatial and contextual memory when injected into hippocampal CA1 and anterior cingulate cortex<sup>33, 48</sup>. 3-(2-Methyl-1,3-thiazol-4-yl)ethynylpyridine hydrochloride (MTEP) (Abcam, Cambridge, MA cat# ab120035) was dissolved in PBS and injected at  $1.0 \mu\text{g}$  per side. This dose has been used *in vitro* to control the activity-dependent switch in NMDAR subunit<sup>35</sup> and *in vivo* to attenuated the expression morphine-associated contextual memory when injected into the nucleus accumbens<sup>49</sup>. Recombinant BDNF (PeproTech, Rocky Hill, NJ, cat# 450-02) was dissolved in 1x PBS and injected at  $0.25 \mu\text{g}$  per side. This dose was shown to rescue memory impairment caused by inhibition of hippocampal protein synthesis and glucocorticoid receptor<sup>17</sup>. (S)-3,5 dihydroxyphenylglycine (DHPG) (Sigma-Aldrich, cat# D3689) was dissolved in PBS and injected at 2 ng per side. This dose was shown to enhance fear memory when injected into the basolateral amygdala<sup>50</sup>. To verify proper placement of the cannula implants, rats were euthanized at the end of the behavioral experiments and their brain frozen in isopentane, sliced in  $40\mu\text{m}$  coronal sections in a  $-20^\circ\text{C}$  cryostat and examined under a light microscope for cannula placement. Rats with incorrect placement (6%) were discarded from the study.

## Western blot analysis

Western blot analysis was carried out as previously reported<sup>17</sup>. Rats were euthanized and their brains were rapidly removed and frozen in isopentane. Dorsal hippocampus punches were obtained with a neuro punch (19 gauge; Fine Science Tools, Foster City, CA) from frozen brains mounted on a cryostat. Samples were homogenized in ice cold RIPA buffer (50mM Tris base, 150mM NaCl, 0.1% SDS, 0.5% Na-Deoxycholate, 1% NP-40) with protease and phosphatase inhibitors [0.5mM PMSF, 2mM DTT, 1mM EGTA, 2mM NaF, 1μM Microcystine, 1mM Benzamidine, 1mM Sodium Orthovanadate and commercial protease and phosphatase inhibitor cocktails (Sigma Aldrich)]. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of total protein (20 μg per lane) were resolved on denaturing SDS-PAGE gels and transferred to Immobilon-FL Transfer membrane (Millipore) by electroblotting. Membranes were dried, reactivated in methanol and washed with water, before they were blocked in 5% (wt) milk and TBS for 1 h at room temperature. Membranes were then incubated with primary antibody overnight at 4 °C in solution according manufacturer's suggestion. Antibodies to TrkB (1:1000, cat# 4603s) and NMDAR2B (1:1000, cat# 5580) were purchased from Cell Signaling Technology (Danvers, MA), mGluR5 (1:1000, Abcam, cat# 76316), pTrkB (1:10,000, Abcam cat # 2149-1 or 1:1000, Millipore, cat# 1381), NMDAR2A (1:1000, Millipore, cat# 07-632), anti-BDNF (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, cat# sc-546.). The membranes were then washed TBS with 0.2% Tween20 (TBST) and incubated with a species-appropriate fluorescently conjugated secondary antibody [goat anti-mouse IRCye 680LT (1:10,000) or goat anti-rabbit IRDye 800CW (1:10,000) from LI-COR Bioscience (Lincoln, NE)] for 1h at room temperature. Membranes were again washed in TBST and scanned using the Odyssey Infrared Imaging system (Li-Cor Bioscience). Data were quantified using pixel intensities with the Odyssey software according to the protocols of the manufacturer (Li-Cor). Actin (1:20,000, Santa Cruz Biotechnology, cat# sc-47778) was used to co-stain all membranes and used as loading control for all markers.

## Whole-cell patch electrophysiology

Rats were trained in IA and euthanized 24 hours later. 200-μm-thick slices of dorsal hippocampus were prepared in ice-cold sucrose cutting solution (in mM: 1.25 Na<sub>2</sub>HPO<sub>4</sub>, 3 KCl, 25 NaHCO<sub>3</sub>, 254 sucrose, 10 dextrose, 2 MgSO<sub>4</sub>, 2 CaCl<sub>2</sub>) on a vibratome (Leica VT1000). The slices recovered while submerged at 30 °C for 30 minutes in bubbled ACSF (in mM: 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 3.5 KCl, 24 NaHCO<sub>3</sub>, 118 NaCl, 1.3 MgSO<sub>4</sub>, 2.5 CaCl<sub>2</sub>, and 11 dextrose; pH=7.4), followed by at least 30 minutes at room temperature. Recordings were obtained in submersion chambers superfused with room temperature ACSF, using a Multiclamp 700B and Digidata 1440A (Molecular Devices). Area CA3 was dissected away immediately prior to recording to eliminate recurrent feedback contamination. Cells were visualized on an Olympus BX51W1 microscope with DIC-IR optics, and whole-cell recordings made from pyramidal cells in the CA1 region. Signals were filtered using a 2 kHz lowpass filter and digitized at 20 kHz, and no adjustment was made for pipette junction potential. All recordings were made using pipettes filled with (in mM): 130 Cs-methanesulfonate, 10 HEPES, 0.5 EGTA, 8 NaCl, 5 TEA-Cl, 4 Mg-ATP, 0.4 Na-GTP, 10 Na-phosphocreatine, 1 QX-314; 290 – 300 mOsm, pH=7.3. All NMDA mediated currents were recorded at V<sub>m</sub> = +40 mV in the presence of picrotoxin (50 μM) and NBQX (10 μM).

Ifenprodil (3  $\mu$ M) was washed in for 20 min prior obtaining recordings to measure ifenprodil sensitivity. NMDA EPSCs were evoked by a 100- $\mu$ s monophasic stimuli delivered by a bipolar electrode at  $V_m = +40$  mV, and EPSC amplitude was limited to 200 pA. Recordings at each age were taken from naive and trained animals in pairs to control for maternal rearing. All drugs were obtained from Tocris Bioscience.

### Statistical analysis

Data were analyzed with the Prism 5 (GraphPad Software Inc.). No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. No randomization was used to collect all the data. Statistical analyses were designed using the assumption of normal distribution and similar variance among groups, but this was not formally tested. The data were analyzed by one- or two-way analysis of variance (ANOVA) followed by post hoc tests. One-way ANOVA followed by Newman–Keuls post hoc tests was performed when comparing groups where a pairwise post hoc analysis of each group was required. One-way ANOVA followed by Dunnett’s post hoc tests was used when each group was compared with a single control group. One-way ANOVA with Tukey’s multiple comparisons was used for electrophysiology experiments. Two-way ANOVAs followed by Bonferroni post hoc tests were used when two factors were compared (e.g., treatment and testing). For paired comparisons, Student’s t-test was used. All analyses are two-tailed. The significance of the results was accepted at  $p < 0.05$ .

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

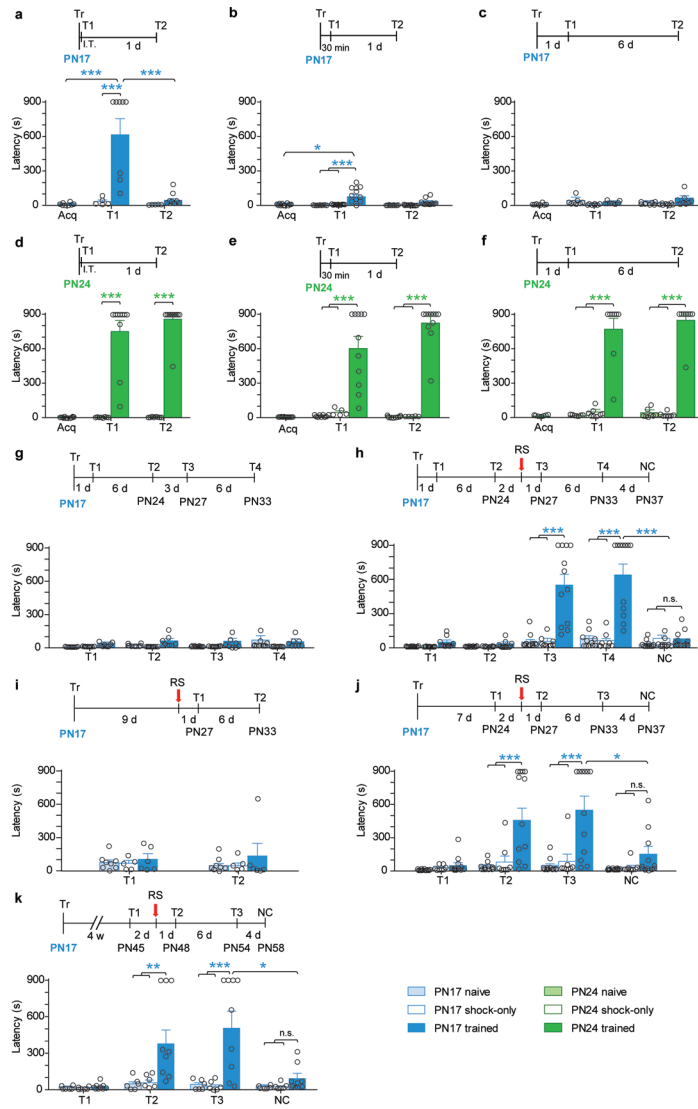
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**Figure 1. Latent infantile memories are rapidly forgotten but reinstate later in life with reminders**

Experimental schedule is shown above each panel. Acquisition (Acq.) and memory retention are expressed as mean latency ± s.e.m (in seconds, s).

(a-c) Mean latency ± s.e.m. of naïve, shock-only and rats trained at PN17 and tested (T): (a) immediately (immediate test, I.T.) [n= 5, 8; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(1,22)=11.53$ ,  $P=0.0026$ , Testing  $F(1,22)=10.71$ ,  $P=0.0035$ , Interaction  $F(1,22)=9.209$ ,  $P=0.0061$ ; 3 independent experiments]; (b) 30min [n= 9, 11, 11; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,56)=14.60$ ,  $P<0.001$ , Testing  $F(1,56)=5.48$ ,  $P=0.023$ , Interaction  $F(2,56)=2.73$ ,  $P=0.074$ ; 3 independent experiments]; (a-c) 1d; and (c) 7d after training [n=8, 8, 8; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,42)=2.437$ ,  $P=0.0997$ , Testing  $F(1,42)=0.4311$ ,  $P=0.515$ , Interaction  $F(2,42)=0.9929$ ,  $P=0.379$ ; 3 independent experiments].



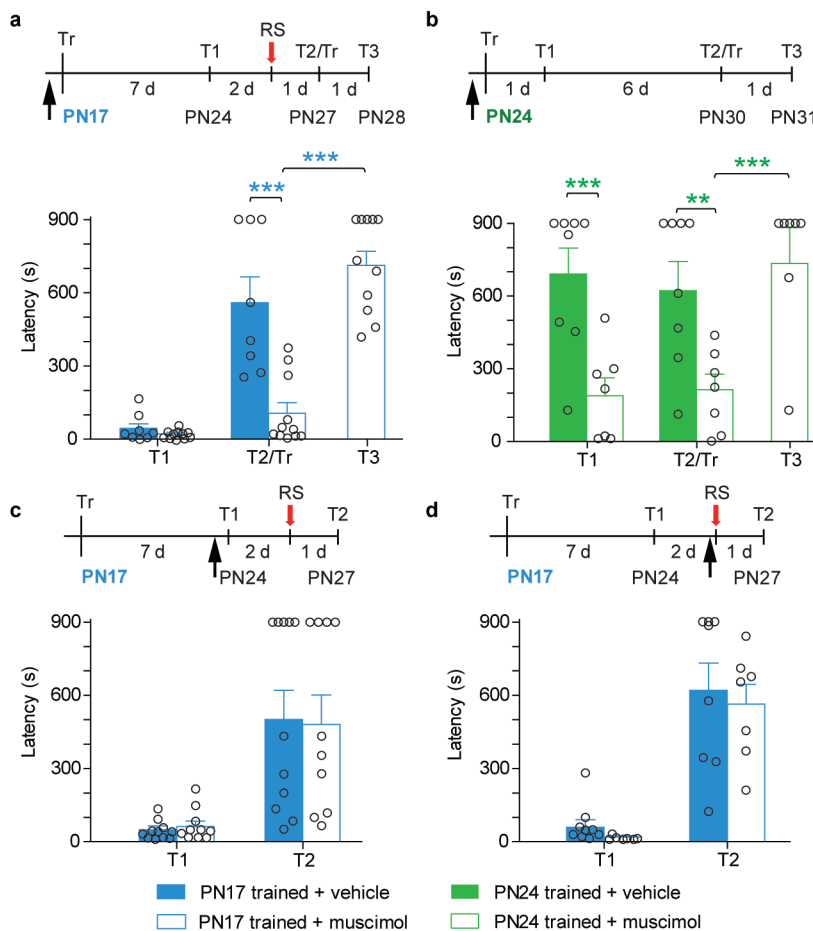
(**d-f**) Mean latency  $\pm$  s.e.m. of naïve, shock-only and rats trained at PN24 and tested (T): (**d**) immediately [n=7, 10; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(1,30)=153.6$ ,  $P<0.0001$ , Testing  $F(1,30)=0.7410$ ,  $P=0.3962$ , Interaction  $F(1,30)=0.6629$ ,  $P=0.4220$ ; 3 independent experiments]; (**e**) 30min [n= 9, 5, 11; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,44)=55.51$ ,  $P<0.001$ , Testing  $F(1,44)=0.97$ ,  $P=0.33$ , Interaction  $F(2,44)=1.72$ ,  $P=0.19$ ; 3 independent experiments]; (**d-f**) 1d; and (**f**) 7d after training [n= 8, 8, 8; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,42)=183.8$ ,  $P<0.0001$ , Testing  $F(1,42)=0.48$ ,  $P=0.489$ , Interaction  $F(2,44)=0.5949$ ,  $P=0.5562$ , 3 independent experiments].

(**g**) Mean latency  $\pm$  s.e.m. of naïve, shock-only and rats trained at PN17 and tested (T) 1d, 7d, 10d and 16d after training (n=10, 9, 8; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,96)=5.542$ ,  $P=0.0053$ , Testing  $F(3,96)=0.9441$ ,  $P=0.4226$ , Interaction  $F(6,96)=1.056$ ,  $P=0.3945$ ; 3 independent experiments].

(**h**) Mean latency  $\pm$  s.e.m. of naïve, shock-only and rats trained at PN17 and tested (T) 1d and 7d after training, and after a reminder shock (RS) given 2d thereafter in a different context [n=11, 10, 12; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,138)=43.48$ ,  $P<0.0001$ , Testing  $F(4,138)=21.81$ ,  $P<0.0001$ , Interaction  $F(8,138)=12.27$ ,  $P<0.0001$ , 3 independent experiments].

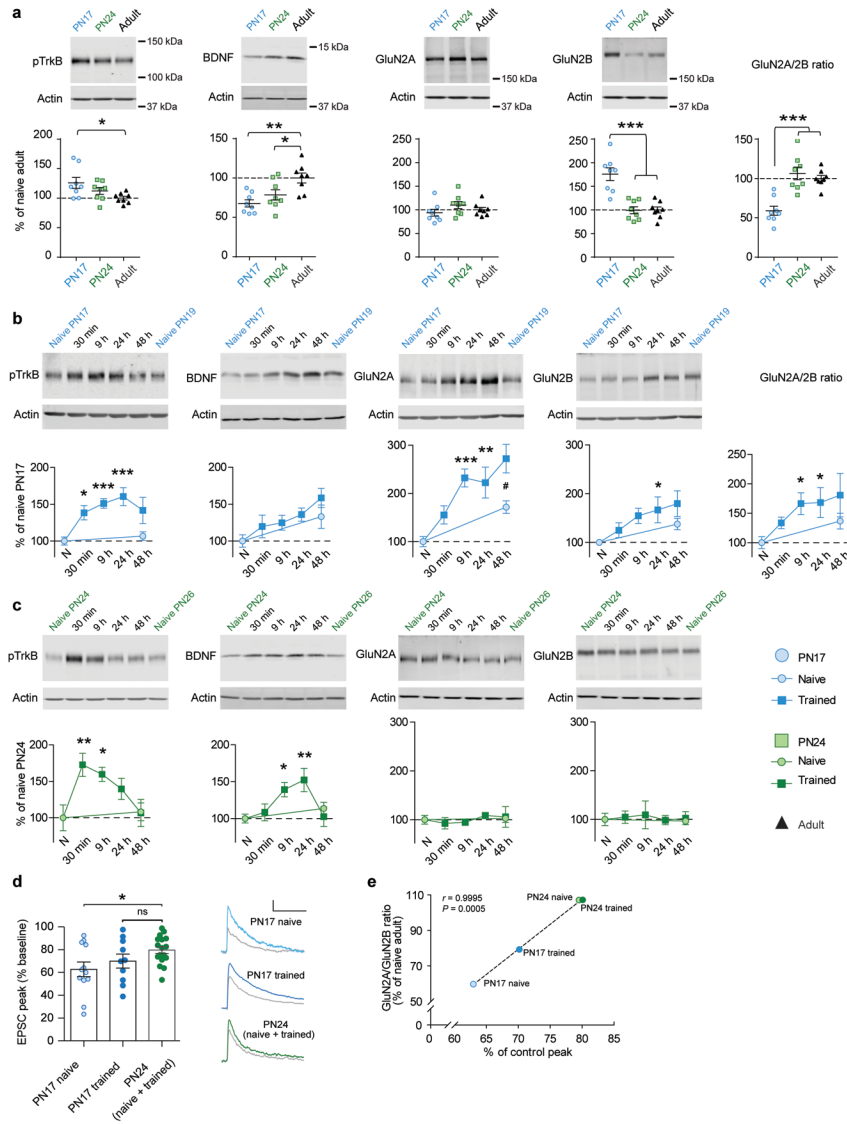
(**i**) Mean latency  $\pm$  s.e.m. of naïve, shock-only and rats trained at PN17 and given a RS 9d after training and tested (T) 1d (T1) and again 6d later (T2) [n= 8, 5, 6; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,32)=0.8669$ ,  $P=0.4299$ , Testing  $F(1,32)=0.0259$ ,  $P=0.8731$ , Interaction  $F(1,32)=0.1791$ ,  $P=0.8368$ ; 3 independent experiments].

(**j-k**) Mean latency  $\pm$  s.e.m. of naïve, shock-only and rats trained at PN17 and tested (T): (**j**) 7d [n=12, 8, 12; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,116)=24.0$ ,  $P<0.0001$ , Testing,  $F(3,116)=6.733$ ,  $P=0.0003$ , Interaction  $F(6,116)=4.137$ ,  $P=0.0008$ ; 3 independent experiments] or (**k**) 4 weeks after training (T1) [n= 6, 6, 9; Two-way ANOVA followed by Bonferroni post hoc, Condition  $F(2,71)=13.18$ ,  $P<0.0001$ , Testing  $F(3,71)=3.98$ ,  $P<0.011$ , Interaction  $F(6,71)=3.54$ ,  $P=0.004$ ; 3 independent experiments]. A RS was given 2d later and the rats were tested 1d (T2) and again 6d later (T3). Four days after T3 the rats were tested in a novel context (NC). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . Latency scores are reported in Supplementary Table 1.



### Figure 2. The latent infantile memory trace is hippocampus-dependent

Experimental schedule is shown above each panel. Memory retention are expressed as mean latency  $\pm$  s.e.m. (in seconds, s). **(a-b)** Mean latency  $\pm$  s.e.m. of rats injected ( $\uparrow$ ) in the dorsal hippocampus with vehicle or muscimol 30min before training (Tr) at **(a)** PN17 [n= 8, 10; Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,48)=17.74$ ,  $P=0.0001$ , Testing  $F(2,48)=32.02$ ,  $P<0.0001$ , Interaction  $F(2,48)=17.43$ ,  $P<0.0001$ ; 3 independent experiments] or **(b)** PN24 [n= 8, 7; Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,39)=22.57$ ,  $P<0.0001$ , Testing  $F(2,39)=16.65$ ,  $P<0.0001$ , Interaction  $F(2,39)=5.108$ ,  $P=0.0107$ ; 3 independent experiments] and tested (T) at the indicated times. At T2, upon entering the shock compartment rats were trained again (Tr) and tested 1d later. **(c-d)** Mean latency  $\pm$  s.e.m. of rats trained at PN17 and injected ( $\uparrow$ ) in the dorsal hippocampus with vehicle or muscimol 30min before **(c)** test 1 (T1) which was given 7d after training [n= 11, 10; Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,57)=0.0013$ ,  $P=0.9719$ , Testing  $F(2,57)=27.68$ ,  $P<0.0001$ , Interaction  $F(2,57)=0.03027$ ,  $P=0.9702$ ; 3 independent experiments] or **(d)** a reminder shock (RS) given 2d after T1 [n=8, 7; Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,39)=0.4162$ ,  $P<0.5226$ , Testing  $F(2,39)=60.59$ ,  $P<0.0001$ , Interaction  $F(2,39)=0.1302$ ,  $P=0.8783$ ; 3 independent experiments]. Rats were tested again 1d after RS (T2). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . Latency scores are reported in Supplementary Table 2.

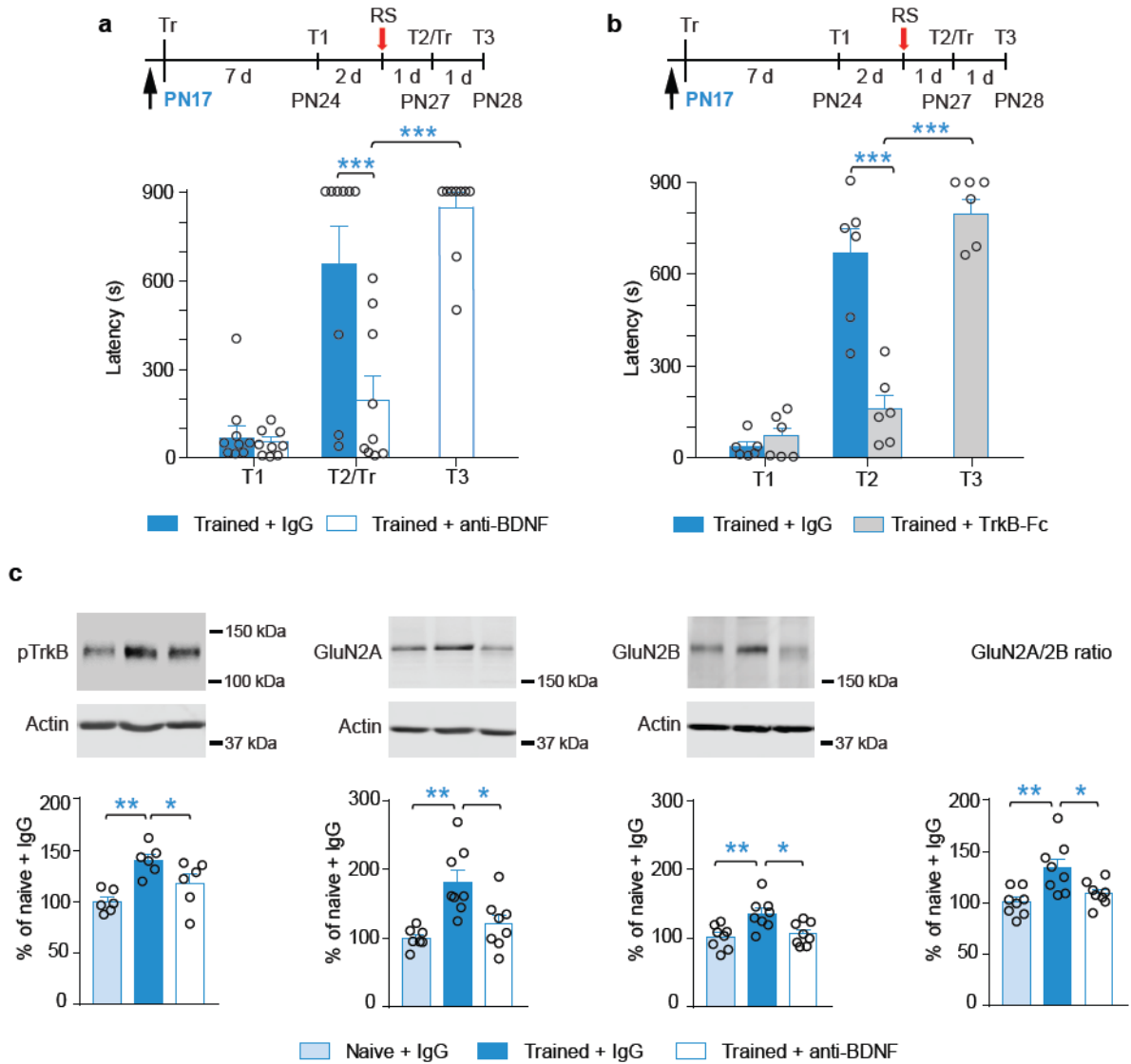


**Figure 3. Training at PN17 increases pTrkB and switches the ratio of GluN2B/GluN2A levels in the dorsal hippocampus**

(a) Examples and densitometric western blot analyses of dHC total extracts from naïve rats euthanized at PN17, PN24 or PN80 (adult) (n=8, 8, 8). Data are expressed as mean percentage ± s.e.m. of adult naïve rats [One-way ANOVA followed by Newman-Keuls Multiple Comparison Test, pTrkB F(2,21)=3.342, P=0.0549; BDNF F(2,21)=7.125, P=0.0043; GluN2A F(2,21)=1.524, P=0.2410; GluN2B F(2,21)=12.41, P=0.0003; GluN2A/GluN2B ratio F(2,21)= 17.68, p< 0.0001; 3 independent experiments]. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001.

(b-c) Examples and densitometric western blot analyses of dHC total extracts from rats trained in IA at (b) PN17 or (c) PN24, and euthanized 30min, 9h, 24h, 48h after training (n=6-10/group). To take into account developmental differences, two groups of naïve were used [(b) PN17 and PN19 or (c) PN24 and PN26]. Data are expressed as mean percentage ± s.e.m. of (b) PN17 naïve rats [n=8, 6, 10, 7, 6, 6, One-way ANOVA followed by Dunnett’s

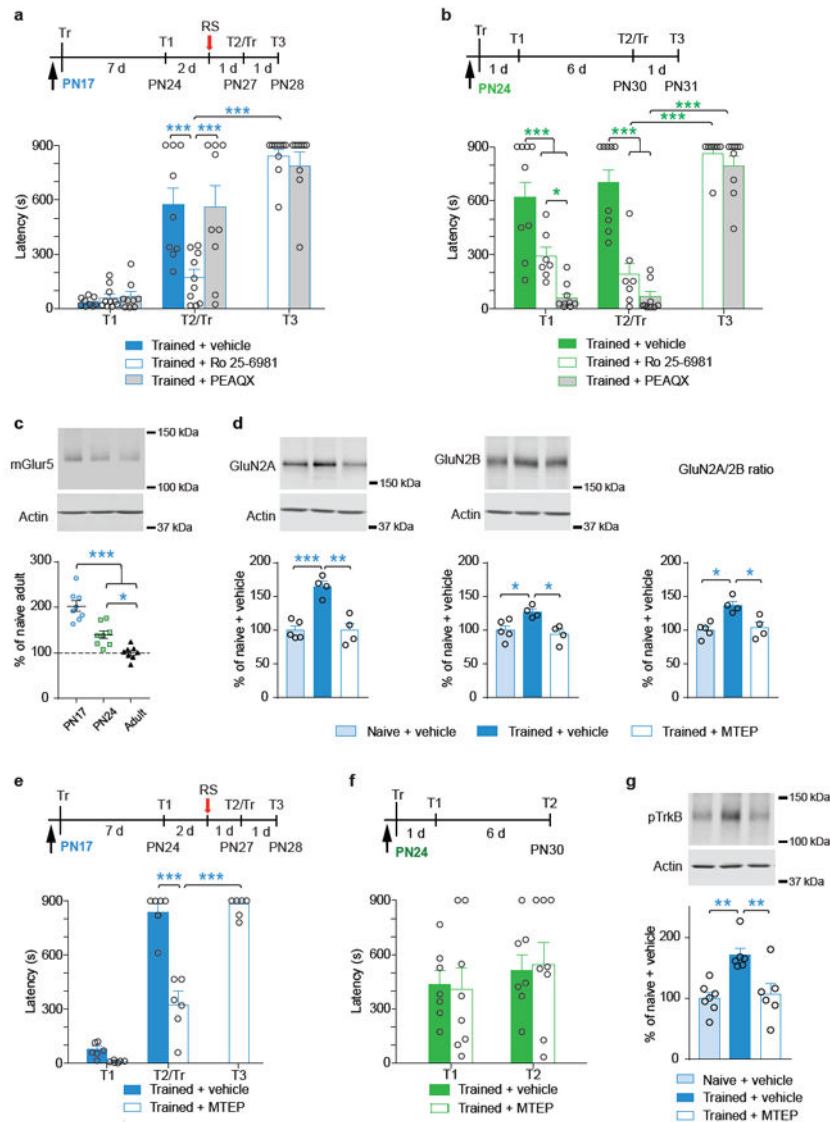
Multiple Comparison Test, pTrkB  $F(3,27)=10.29$ ,  $P=0.0001$ ; BDNF  $F(3,27)=1.998$ ,  $P=0.1381$ ; GluN2A  $F(3,27)=8.580$ ,  $P=0.0004$ ; GluN2B  $F(3,27)=2.923$ ,  $P=0.0527$ ; GluN2A/2B  $F(3,27)=3.243$ ,  $P=0.0389$ ; 3 independent experiments] or (c) PN24 naïve rats [n=8, 6, 6, 7, 7, 8; One-way ANOVA followed by Dunnett's Multiple Comparison Test, pTrkB  $F(3,23)=4.489$ ,  $P=0.0128$ ; BDNF  $F(3,23)=5.256$ ,  $P=0.0066$ ; GluN2A  $F(3,23)=0.7538$ ,  $P=0.5314$ ; GluN2B  $F(3,23)=0.08686$ ,  $P=0.9665$ ; 3 independent experiments]. \* Indicates significance compared to (b) PN17 naïve or (c) PN24 naïve rats \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . # indicates significance levels comparing PN19 naïve to 48h trained groups (GluN2A, Unpaired two-tailed Student's t-test,  $t=3.113$  df=10, # $P = 0.0110$ ). (d) Ifenprodil (3  $\mu\text{M}$ ) depressed the amplitude of NMDA EPSCs recorded at  $V_m = +40$  mV in PN17 naïve rats (n= 6 rats, 12 cells) when compared to P24 animals (n=10,17;  $p < 0.05$ ), but not in PN17 trained animals (n=6,10) [One-way ANOVA followed by Bonferroni's Multiple Comparison's Test,  $F(2,36) = 3.298$ ,  $P = 0.0484$ ]. Representative sample traces from before (color) and 20 min after ifenprodil (grey) are shown on the right. Error bars, SEM. Scale bars, X-axis = 200 ms, Y-axis = 10 pA (top), 40 pA (middle), and 25 pA (bottom). (e) Correlation of western blot (GluN2A/GluN2B ratio, % of Naïve Adult) and electrophysiology data (% of Control Peak).  $r$  the Pearson correlation. The numeric values are reported in Supplementary Table 3. Full-length blots/gels are presented in Supplementary Figure 9.



**Figure 4. BDNF is required for the formation of the latent infantile memory and for the GluN2B-2A switch**

Experimental schedule is shown above each panel. **(a-b)** Mean latency  $\pm$  s.e.m. (expressed in seconds, s) of rats injected ( $\uparrow$ ) in the dorsal hippocampus with **(a)** IgG or anti-BDNF [ $n=9, 9$ , Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,32)=9.021$ ,  $P=0.0051$ , Testing  $F(1,32)=21.72$ ,  $p<0.0001$ , Interaction  $F(1,32)=8.234$ ,  $P=0.0072$ ; 3 independent experiments] or **(b)** IgG or TrkB-Fc [ $n=6, 6$ ; Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,20)=25.48$ ,  $p<0.0001$ , Testing  $F(1,20)=59.34$ ,  $P<0.0001$ , Interaction  $F(1,20)=33.81$ ,  $p<0.0001$ ; 2 independent experiments] 30min before training (Tr) at PN17. Rats were tested 7d after training (T1) and two days later received a reminder shock (RS), and were tested again 1d later (T2). At T2, upon entering the shock compartment rats were trained again (Tr) and tested 1d later (T3).

(c) Representative examples and densitometric western blot analyses of dorsal hippocampal extracts obtained from naive and trained rats given hippocampal injections of IgG or anti-BDNF 30min before training (Tr) at PN17 and euthanized 24h after training. Data are expressed as mean percentage  $\pm$  s.e.m. of naive rats injected with IgG and euthanized at the matched time point (i.e. PN18) [n= 8,8,8; One-way ANOVA followed by Newman-Keuls Multiple Comparison Test, pTrkB F(2,15)=8.858, P=0.0029; GluN2A F(2,21)= 6.864, P=0.0051; GluN2B F(2,21)=6.731, P=0.0055; GluN2A/2B F(2,21)=7.632, P=0.0032, 3 independent experiments]. \*p < 0.05, \*\*p < 0.01). The numeric values are reported in Supplementary Table 4 and 5. Full-length blots/gels are presented in Supplementary Figure 10.

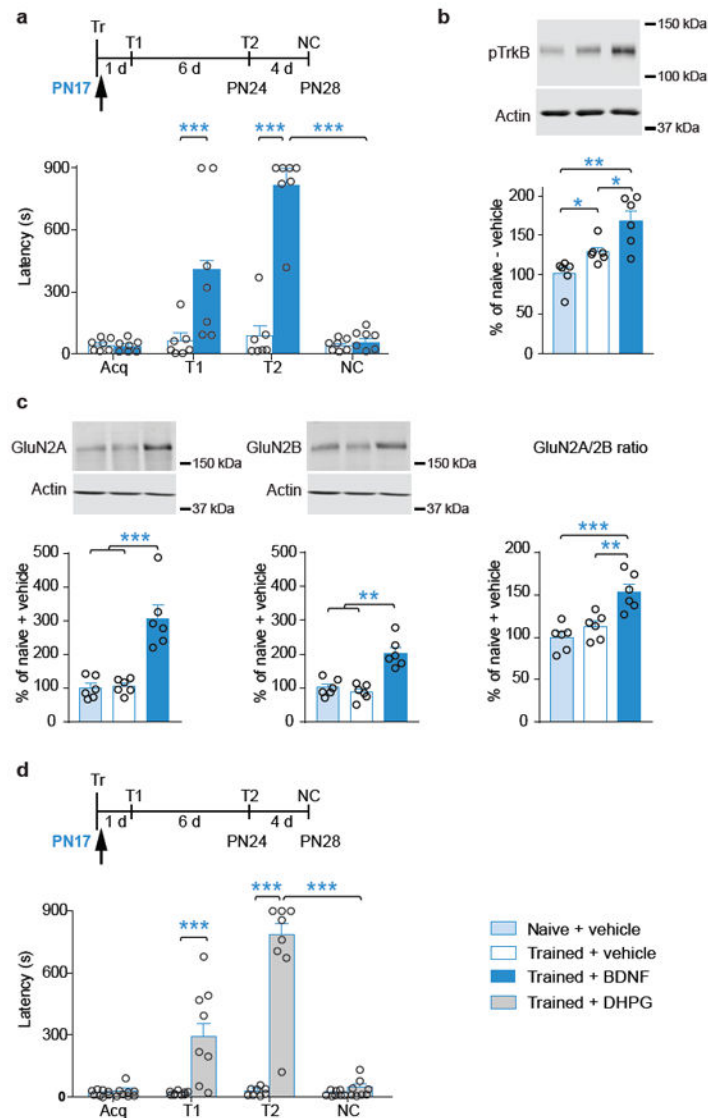


### Figure 5. GluN2B and mGluR5-dependent switch of GluN2B-2A is required to form the latent infantile memory

Experimental schedule is shown above each panel. **(a-b)** Mean latency  $\pm$  s.e.m. (expressed in seconds, s) of rat injected ( $\uparrow$ ) in the dorsal hippocampus with vehicle, Ro 25-6981 or PEAQX 30min before training at **(a)** PN17 [n=9, 10, 9, Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(2,75)=6.639$ ,  $P=0.0022$ , Testing  $F(2,75)=58.35$ ,  $P<0.0001$ , Interaction  $F(4,75)=6.496$ ,  $P=0.0022$ ; 3 independent experiments] or **(b)** PN24 [n=9, 7, 9, Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(2,66)=43.55$ ,  $P<0.0001$ , Testing  $F(2,66)=32.29$ ,  $P<0.0001$ , Interaction  $F(4,66)=12.81$ ,  $P<0.0001$ ; 3 independent experiments]. T1, first test. RS, reminder shock. T2, second test. At T2, upon entering the shock compartment rats were trained again (Tr) and tested 1d later (T3). **(c-d)** Representative examples and densitometric western blot analyses of dorsal hippocampal total extracts obtained from **(c)** naive rats euthanized at PN17, PN24 or PN80 (adult) (n=8/group, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test  $F(2,21)=$

33.89,  $p < 0.0001$ ; 3 independent experiments); **(d)** naive and trained rats injected into the dorsal hippocampus with either vehicle or MTEP 30min before training (Tr) at PN17 and euthanized 24h after training [n= 5, 4, 4, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test, GluN2A  $F(2,12) = 18.64$ ,  $P = 0.0004$ ; GluN2B  $F(2,12) = 6.314$ ,  $P = 0.0169$ ; GluN2A/2B  $F(2,12) = 4.481$ ,  $P = 0.0408$ ; 2 independent experiments]. Data are expressed as mean percentage  $\pm$  s.e.m. of **(c)** adult naïve rats or **(d)** naive rats injected with vehicle and euthanized at the matched time point (i.e. PN18). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). **(e-f)** Mean latency  $\pm$  s.e.m. (in seconds, s) of rat injected ( $\uparrow$ ) in the dorsal hippocampus with either vehicle or MTEP 30min before training at **(e)** PN17 [n= 6, 6, Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,30) = 41.18$ ,  $P < 0.0001$ , Testing  $F(2,30) = 134.9$ ,  $P < 0.0001$ , Interaction  $F(2,30) = 25.02$   $p < 0.0001$ ; 2 independent experiment] or **(f)** PN24 [n= 7, 8, Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,39) = 0.003504$ ,  $P < 0.9531$ , Testing  $F(2,39) = 19.68$ ,  $P < 0.0001$ , Interaction  $F(2,39) = 0.05990$ ,  $P = 0.9419$ ; 3 independent experiments]. T1, first test. RS, reminder shock. T2, second test. At T2, upon entering the shock compartment rats were trained again (Tr) and tested 1d later (T3). **(g)** Representative examples and densitometric western blot analyses of dorsal hippocampal total extracts obtained from naive and trained rats given hippocampal injections of vehicle or MTEP 30min before training (Tr) at PN17 and euthanized 24h later [n= 7, 6, 6, One-way ANOVA followed by Newman-Keuls Multiple Comparison Test, pTrkB  $F(2,18) = 9.162$ ,  $P = 0.0022$ ; 3 independent experiments]. Data are expressed as mean percentage  $\pm$  s.e.m. of naive rats injected with vehicle and euthanized at the matched time point (i.e. PN18). \* $p < 0.05$  and \*\*\* $p < 0.001$ . Latency scores are reported in Supplementary Table 6 and 7. Full-length blots/gels are presented in Supplementary Figure 10.





### Figure 6. BDNF closes the infantile amnesia period

Experimental schedule is shown above each panel. Acquisition (Acq) and memory retention are expressed as mean latency  $\pm$  SEM (in seconds, s). **(a)** Mean latency  $\pm$  s.e.m. (expressed in seconds, s) of rats that received hippocampal injections ( $\uparrow$ ) of vehicle or BDNF immediately after IA training at PN17. Memory retention was tested 1d (T1) and 7d (T2) after training and, 4d after T2, in a new context (NC) [n=7, 7, Two-way ANOVA followed by Bonferroni post hoc, Treatment  $F(1,36)=18.49$ ,  $P<0.0001$ , Testing  $F(2,36)=22.57$ ,  $P<0.0001$ , Interaction  $F(2,36)=14.36$ ,  $P<0.0001$ ; 3 independent experiments]. **(b-c)** Representative examples and densitometric western blot analyses of dorsal hippocampal total extracts obtained from naive or trained rats given hippocampal injections of either vehicle or BDNF immediately after IA training (Tr) at PN17 and euthanized 2 hours later [n= 6, 6, 6; One-way ANOVA followed by Newman-Keuls Multiple Comparison Test, pTrkB  $F(2,17)=10.35$ ,  $P=0.0015$ ; GluN2A  $F(2,17)=14.66$ ,  $P=0.0003$ ; GluN2B

F(2,17)=19.17,  $P<0.0001$ ; GluN2A/2B F(2,17)= 11.82  $P=0.0008$ ; 3 independent experiments]. Data are expressed as mean percentage  $\pm$  s.e.m. of naive rats injected with vehicle and euthanized at the matched time point. **(d)** Mean latency  $\pm$  s.e.m. (expressed in seconds, s) of rats that received a bilateral hippocampal injection ( $\uparrow$ ) of either vehicle or DHPG immediately after IA training at PN17. Memory retention was tested at 1d (T1) and 7 d (T2) after training and, 4d after T2, in a new context (NC) [n= 8, 8, Two-way ANOVA followed by Bonferroni post hoc, Treatment F(1,42)=157.2,  $P<0.0001$ , Testing F(1,42)=61.70,  $P<0.0001$ , Interaction F(2,42)=57.4,  $P<0.0001$ ]. \*\*\* $P < 0.001$ . Latency score in Supplementary Table 8 and 9. Full-length blots/gels are presented in Supplementary Figure 10.

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