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Extinction and reacquisition of a fear-motivated memory require activity of the Src family of tyrosine kinases in the CA1 region of the hippocampus

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Abstract

Evidences indicate that extinction represents a NMDA receptor (NMDAr)-dependent learning rather than erasure of previously stored information. Several members of the Src family of tyrosine kinases are activated by stimulation of the NMDAr and are involved in both induction of hippocampal long-term potentiation and consolidation of hippocampal-dependent, NMDAr-sensitive, memories. Here we analyzed the role of the Src family within the CA1 region of the hippocampus in extinction and reacquisition of the memory for step-down, inhibitory avoidance learning task (IA). Rats trained in IA were submitted to 5 daily extinction sessions during which the avoidance response was elicited in the absence of the unconditioned stimulus. Immediately or 180 min after each extinction session animals received intra-CA1 infusions of either 0.1% DMSO, the Src-family inhibitor PP2 or its inactive analog, PP3. PP2 blocked extinction of the IA response which was otherwise evident in DMSO and PP3-treated animals.

After being submitted to a new training session the animals reacquired the avoidance response; however, they failed to do so if they received intra-CA1 infusions of PP2 immediately following retraining. Our results indicate that, like the original learning, extinction and reacquisition of the IA response require activity of the Src family in the CA1 region of the hippocampus.

Keywords: Memory; Learning; Consolidation; Reconsolidation; Extinction; Reacquisition; Src; Tyrosine kinase; Hippocampus; CA1; PP2; PP3

1. Introduction

Step-down inhibitory avoidance (IA) is a much used animal model for aversive learning in which stepping-down from a platform placed inside an acrylic and wood box with a floor made of an electrifiable grid (contextual conditioned stimulus; CS) is paired with a mild footshock (unconditioned stimulus; US). After just one such training session, the animals learn to refrain from stepping-down to the grid (conditioned response; CR) when placed again on the training box platform. If upon doing so the CS is repeatedly presented without the ensuing US (i.e. the animal is put back on the platform and allowed to step-down from it without receiving a footshock), the learned aversive response gradually extinguishes (Cammarota et al., 2003, 2004).

Although measurable as a decline of the CR, it is thought that extinction does not represent memory erasure or decay (Konorski, 1948; Estes, 1955; Rescorla, 1979). As Pavlov observed (Pavlov, 1927), relearning after extinction occurs typically faster than initial conditioning and, sometimes, the extinguished CR can reappear

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spontaneously. Moreover, reconditioning may result in a stronger CR and it can be obtained through exposure to the US alone (Rescorla, 2001; Anokhin et al., 2002), thus indicating that extinction does not induce unlearning of the original CS–US association but constitutes a secondary learning by which the newly acquired behavior comes to replace the original CR as an animal's first choice. The molecular requirements of extinction have only recently begun to be studied but it is already clear that extinction requires protein synthesis (Vianna et al., 2001; Lin et al., 2003; Izquierdo et al., 2004; Lattal et al., 2004; Santini et al., 2004, but see also Lattal and Abel, 2001and Fischer et al., 2004) and, particularly, NMDAr activation in memory-relevant areas of the brain (Walker and Davis, 2002; Davis, 2002; Richardson et al., 2004), as expected if it was indeed a new learning. For instance, amygdalar NMDAr have been involved in extinction of fear potentiated startle (Falls et al., 1992; Lu et al., 2001) and hippocampal NMDAr are required for extinction of conditioned fear (Szapiro et al., 2003). Blockade of NMDAr in the pigeon neostriatum caudolaterale impairs both learning of a color reversal task and extinction of appetitive instrumental conditioning (Lissek et al., 2002; Lissek and Gunturkun, 2003).

The Src family of non-receptor tyrosine kinases is named after its prototypic member, pp60c-src (Src) and includes nine highly homologous enzymes, five of which– Src, Fyn, Lyn, Lck and Yes–are abundantly expressed in the mammalian brain and localize to the postsynaptic density (Suzuki and Okumura-Noji, 1995; Salter, 1998; Kalia and Salter, 2003; Huang et al., 2001), an electron-dense structure attached to the cytoplasmic surface of the postsynaptic membrane at glutamatergic synapses that gathers receptors and signaling proteins (Husi and Grant, 2001; Walikonis et al., 2000). Although initially studied because of their role in differentiation and development, over the last decade it has became clear that some members of the Src family might play an important role in the regulation of neuronal plasticity and memory formation acting through modulation of the NMDAr functionality (for recent reviews see Ali and Salter, 2001; Purcell and Carew, 2003; Kalia et al., 2004). Src phosphorylates the NR2A and NR2B subunits of the NMDAr thus enhancing the receptor-mediated currents (Wang and Salter, 1994; Kohr and Seeburg, 1996). In fact, induction of NMDAr-dependent long-term potentiation (LTP) in the hippocampus, a process that many consider a cellular model for learning and memory (Martin et al., 2000; Brun et al., 2001; Gerlai, 2002; Daoudal and Debanne, 2003; Lynch, 2004; Mehta, 2004), is blocked by inhibition of Src and accompanied by both the Src-mediated upregulation of the NMDAr conductance and the increased phosphorylation of NR2B on tyrosine residues (Lu et al., 1998; Rostas et al., 1996). It is known that Src activity is required in the rat hippocampus for the normal formation of one-trial avoidance memory (Bevilaqua et al., 2003a) and that the activity and expression of Src are increased in the hippocampal formation after spatial learning (Zhao et al., 2000). Moreover, Fyn knock-out mice show impaired acquisition of some types of spatial memories (Grant et al., 1992).

Using the specific inhibitor of the Src family, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-D] pyrimidine (PP2; Hanke et al., 1996), here we analyzed the hypothesis that activity of these kinases is necessary in the CA1 region of the dorsal hippocampus for extinction and reacquisition of the memory for IA.

2. Materials and methods

2.1. Surgery and intrahippocampal infusions

Three-months-old male Wistar rats (220–250 g) were used. The animals were raised in our own facilities, had ad libitum access to food and water, were housed 3–5 to a cage, and were kept at 22 °C in a 12 h light/dark cycle (lights on at 7:00 A.M.). To implant the cannulas, rats were deeply anesthetized with thiopental (30–50 mg/kg, i.p.), and 27 gauge cannulas were stereotaxically aimed 1.0 mm above the stratum pyramidale of the dorsal CA1 region of the hippocampus [coordinates: anterior, −4.3; lateral, ±4.3; ventral, 2.6, in accordance with the description by Paxinos and Watson (1986)]. To deliver the drugs, we used a 30 gauge infusion cannula connected by a polyethylene tube to a microsyringe. Infusions (0.8 μl/side) were performed over 60 s, first on the left side and then on the right side; the infusion cannula was kept in place for an additional 1 min to minimize backflow of the injectant. Placement of infusion cannulas was verified postmortem by standard histological procedures (Bonini et al., 2003), and was correct (i.e., within the pyramidal cell layer of CA1) in 95% of the implanted animals. Only data from animals with correct cannula implants were analyzed. Animals were allowed to recover from surgery for 4 days before submitting them to behavioral tests.

2.2. Behavioral procedures

Rats were subjected to one-trial, step-down inhibitory avoidance training as described previously (Bevilaqua et al., 2003b; Cammarota et al., 2003, 2004). The training apparatus was a $50 \times 25 \times 25$ cm white acrylic box, the floor of which was a series of 1-mm-caliber bronze bars spaced 1 cm apart. The left end of the floor was covered by a 7-cm wide, 5-cm-high wood platform. Animals were gently placed on the platform facing the left rear corner. When they stepped down onto the grid and had placed their four paws on it, they received a mild 2 s, 0.5 mA scrambled shock to the foot, and were then immediately removed from the training box and placed in their home cages. The long-term memory associated with the learning

of this task persists for at least 31 days (Izquierdo et al., 2000). Rats were tested for retention several times after training, with an interval of 24 h between sessions. A ceiling of 180 s was imposed on retention test measures. In the test sessions the footshock was omitted and the animals were left to freely explore the apparatus for 30 s after they had stepped down. During this period, they stepped up onto the platform and down again several times. All experiments and surgical procedures were conducted according to National Institutes of Health of the United States of America guidelines for animal care.

2.3. Drugs

4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-D] pyrimidine (PP2) and 4-amino-7-phenylpyrazol [3,4-D] pyrimidine (PP3) were purchased from Calbiochem (San Diego, CA), dissolved in DMSO and brought to final concentration (20 μmol/l) with saline. The dose of PP2 utilized was determined based on pilot studies and on previous results showing the effect of this drug on hippocampal Src signaling and memory formation (Sanna et al., 2000; Grosshans and Browning, 2001; Derkinderen et al., 2001; Kim et al., 2002; Bevilaqua et al., 2003a,b).

2.4. Statistics

Since the variable being analyzed (step-down latency) does not follow a normal distribution and its variance does not fulfill the assumption of homoscedasticity, data were expressed as median (interquartile ranges) and analyzed by Mann–Whitney or Kruskal–Wallis non-parametric tests followed by Dunn's post hoc comparisons versus Vehicle or PP3 = – treated experimental groups when appropriate.

3. Results

To extinguish the long-term memory for the IA task, IA trained animals were submitted to daily non-reinforced retention test sessions starting 24 h after training. As previously shown (Cammarota et al., 2003) five such sessions (TT1 to TT5) resulted in significant extinction of the IA response in control animals (Fig. 1; VEH); i.e., the latency to step-down from the elevated platform to the grid decreased over consecutive sessions. Extinction was not seen in animals receiving intra-CA1 PP2 (0.8 μl; 20 μmol/l) immediately after the first four extinction sessions (Fig. 1A; PP2; $p < 0.05$ in Dunn's post hoc comparison versus VEH and PP3 groups at TT4 and TT5) but it proceeded normally when PP2 was infused into CA1 180 min after every non-reinforced test (Fig. 1B; PP2). The intra-CA1 infusion of PP3, a PP2 inactive analog, had no consequence whatsoever on IA extinction (Fig. 1A and B; PP3). The effect of 4 daily intra-CA1 infusions of PP2 on extinction was totally reversible and the animals normally extinguished the IA response upon deferment of PP2 treatment (Fig. 2A). When PP2 was given into CA1 immediately after the first two non-reinforced test sessions (i.e. after TT1 and TT2, see Fig. 2B), extinction was delayed but it progressed upon suspension of PP2 infusion. When PP2 was infused into CA1 just immediately after TT3 and TT4 it had no effect on memory extinction or retention (Fig. 2C).

To analyze whether activity of the Src family within the hippocampus is required to reacquire the extinguished IA response, trained animals were submitted to five daily extinction sessions as explained above. At TT5, instead of allowing them to freely explore the training box upon stepping down from the platform, the animals received a footshock identical to the one they had received during the original training. Immediately or 180 min after TT5 the

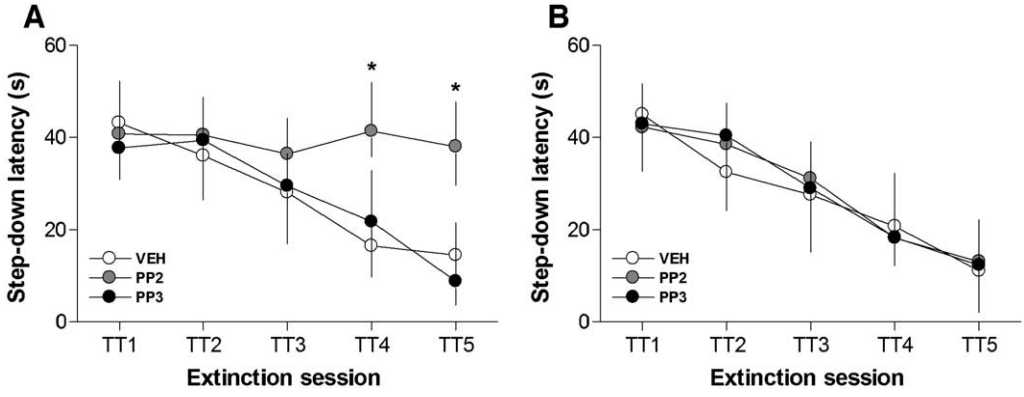

Fig. 1. (A and B) Rats bilaterally implanted with guide cannulas aimed to the CA1 region of the dorsal hippocampus were randomly assigned to different experimental groups and trained into a one-trial, step-down inhibitory avoidance task. After that the animals were tested for 5 consecutive days (TT1–TT5; first test 24 h after training). Immediately (A) or 180 min (B) after the first 4 sessions (i.e., TT1–TT4), each experimental group received bilateral, 0.8 μl intra-CA1 infusions of either 0.1% DMSO in saline (VEH), PP2 (20 μmol/l) or PP3 (20 μmol/l). During test sessions animals were allowed to freely explore the training box for 30 s after they stepped down from the platform. Data ($n = 9–12$) are presented as median ± interquartile range of the step-down latency (i.e., the time animals spend on the platform before stepping down to the grid). *$p < 0.05$ versus VEH or PP3 groups in Dunn's post hoc comparison after Kruskal–Wallis test.

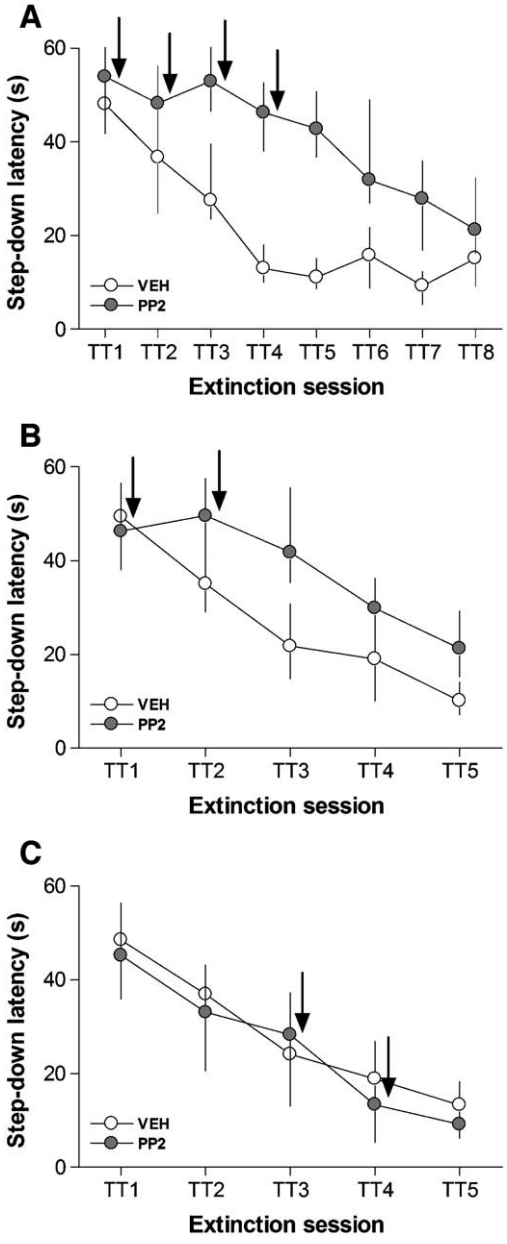

Fig. 2. (A) Rats bilaterally implanted with guide cannulas aimed to the CA1 region of the dorsal hippocampus were randomly assigned to different experimental groups and trained into a one-trial, step-down inhibitory avoidance task. After that the animals were tested for 8 consecutive days (TT1–TT8; first test 24 h after training). Immediately after the first 4 sessions (i.e., TT1–TT4) each experimental group received bilateral, 0.8 μl intra-CA1 infusions of either 0.1% DMSO in saline (VEH) or PP2 (20 μmol/l). (B and C) Rats were treated as in A except that 0.1% DMSO in saline (VEH) or PP2 (20 μmol/l) were infused into CA1 immediately after TT1 and TT2 (B) or TT3 and TT4 (C). The black arrows indicate the time of infusion. Data ($n=12-15$) are depicted as median ± interquartile range of the step-down latency (i.e., the time animals spend on the platform before stepping down to the grid).

animals received bilateral intra-CA1 infusions of 0.1% DMSO in saline (VEH), PP2 (20 μmol/l) or PP3 (20 μmol/l). Animals were tested again (TT6) one day after this retraining session. PP2 blocked memory reacquisition when given immediately (Fig. 3A; $p<0.01$ in Dunn's post hoc comparison versus VEH and PP3 groups at TT6) but not 180 min after retraining (Fig. 3B). In animals infused with VEH or PP3, retraining caused retention levels to return to TT1 values notwithstanding the moment of the infusion (Fig. 3).

4. Discussion

The experiments presented above show that activity of the Src family of non-receptor tyrosine kinases is necessary in the CA1 region of the dorsal hippocampus for extinction and reacquisition of IA long term memory. The observed results can clearly be interpreted as due to an effect of PP2 on the Src family, inasmuch as infusion of its inactive analogue PP3 had no consequence on extinction or reacquisition and the dosage of PP2 used here has been demonstrated to specifically block the activity of this family of kinases and to effectively impede the consolidation of fear-motivated memory without affecting locomotor activity or anxiety state (Sanna et al., 2000; Grosshans and Browning, 2001; Derkinderen et al., 2001; Kim et al., 2002; Bevilaqua et al., 2003a). Moreover, the fact that PP2 reversibly blocked extinction and hindered reacquisition only when infused immediately but not 180 min after each non-reinforced retrieval session or retraining clearly indicates that this drug produces a bonafide amnesic effect which is in no way due to any permanent insult on hippocampal functionality.

It has been shown that extinction of IA memory requires functional NMDAr and activation of the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) in the CA1 region of the hippocampus at the time of the first CS–no US presentation (Szapiro et al., 2003) and there is abundant evidence of cross-talk between those proteins and members of the Src family (Shanley et al., 2001; Derkinderen et al., 2003; Wu et al., 2004; Choi et al., 2004). In fact, it is known that in neurons inhibition of the Src family blocks both glutamate signaling to ERK1/2 (Crossthwaite et al., 2004) and the NMDAr- and ERK1/2-dependent phosphorylation/activation of the cAMP-responsive element binding protein (CREB) (Kawasaki et al., 2004). CREB activation is a molecular marker for learning and memory throughout the animal kingdom (Cammarota et al., 2000a; Ribeiro et al., 2003; Lin et al., 2003; Perazzona et al., 2004; Josselyn et al., 2004) and it has been shown that IA learning induces CREB upregulation through a mechanism that requires the early activation of hippocampal NMDAr (Cammarota et al., 2000b). It is therefore quite possible that the effects of PP2 shown here are at least partially due to an indirect influence on the activation state of the NMDAr–ERK1/2–CREB pathway. Notwithstanding that, it is important to mention that our results apparently are at odds with those of Szapiro et al. using NMDAr and ERK1/2 blockers. Szapiro and coworkers reported that inhibition of hippocampal NMDAr and

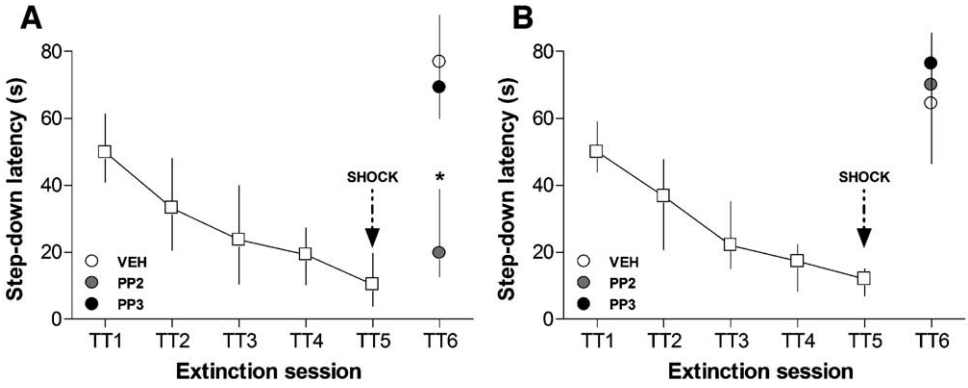

Fig. 3. Rats bilaterally implanted with cannulas aimed to the CA1 region of the dorsal hippocampus were trained in a one-trial, step-down IA task and tested for 4 consecutive days (TT1–TT4; first test 24 h after training). After that, the animals were randomly assigned to different experimental groups. Immediately (A) or 180 min (B) after the fifth test session (TT5), each experimental group received bilateral intra-CA1 infusions of either 0.1% DMSO in saline (VEH), PP2 (20 μmol/l) or PP3 (20 μmol/l). During this session, instead of being allowed to freely explore the training box, rats received a scrambled electric footshock equal to that received in the training session (0.5 mA, 2 s) immediately after they stepped down to the grid. Memory retention was measured in a subsequent test session performed 24 h later (TT6). Data ($n = 11–12$) are depicted as median ± interquartile range of the step-down latency *$p < 0.01$ versus VEH or PP3 groups at TT6 in Dunn's post hoc comparison after Kruskal–Wallis test.

ERK1/2 around the moment of the first extinction session actually cancelled extinction of IA memory (i.e. infusion of the drugs immediately before or immediately after the first non-reinforced retrieval test prevented extinction in a long-lasting manner; Szapiro et al., 2003) but we found that the effect of PP2 on this process was readily reversible and could not be observed when the drug was infused just after TT1 and TT2 or TT3 and TT4 (Fig. 2). The reasons for this discrepancy are not clear but they are perhaps hidden behind the fact that although the IA training protocol utilized was almost identical, the two studies employed different extinction procedures. While Szapiro et al. removed the animals to their home cages immediately after they stepped-down to the grid on test sessions (Szapiro et al., 2003, see also Vianna et al., 2001), we left them to freely explore the training box for 30 s. During this 30 s period, the rats stepped up onto the platform and down again several times resulting in deeper extinction after which there is no spontaneous recovery, the original memory cannot be reestablished by treatments known to facilitate retrieval and gene expression and protein synthesis in the hippocampus are required for reacquisition, as if the task had to be learned completely anew (Cammarota et al., 2003). In this respect, it was reported that when a weak extinction protocol is utilized and the onset of extinction is prevented on the first test session, extinction usually becomes detectable only many trials later. Since under such circumstances expression of the original memory prevails, the acquisition of extinction learning turns out to be much more difficult (Konorski, 1948; Izquierdo et al., 1965). At any rate, our findings showing that the Src family is required for the formation of the memory for extinction immediately after each non-reinforced test session but not 180 min thereafter, just like consolidation of the original IA memory (Bevilaqua et al., 2003a,b), strongly endorse the view that extinction is indeed a very active form of learning (Berman and Dudai, 2001; Rescorla, 2001; Santini et al., 2001, 2004; Myers and Davis, 2002; Izquierdo et al., 2004) involving a finely tuned, time-dependent and tightly knitted assortment of molecular processes.

Those results showing that reacquisition of the IA CR requires activity of the Src family in the CA1 region of the dorsal hippocampus as if, after extinguished, the memory had to be consolidated again, add force to the notion that strengthening the unconditioned aspect of the CS–no US association during extinction trials can produce the behavioral uninstallment of the mnemonic trace. However, taking into account that there may be remnants of the original task that had only became momentarily inaccessible due to the extinction procedure, it is not possible to conclude whether, despite appearances, extinction as described here effectively reflects erasure of the original learning. Indeed, there is copious evidence indicating that extinction does not result in forgetting (Rescorla, 1979, 2001, 2004; Davis et al., 2003). Further experiments on the molecular and behavioral properties of memory reacquisition after extinction are needed to unravel this dilemma.

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