Nonreceptor tyrosine protein kinase pp60c-src in spatial learning: Synapse-specific changes in its gene expression, tyrosine phosphorylation, and protein–protein interactions

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c-src is a nonreceptor tyrosine protein kinase that is highly concentrated in synaptic regions, including synaptic vesicles and growth cones. Here, we report that the mRNA signal of pp60c-src is widely distributed in the rat brain with particularly high concentrations in the hippocampus. After spatial maze learning, up-regulation of c-*src* mRNA was observed in the CA3 region of the hippocampus, which was accompanied by increases in pp60c-src protein in hippocampal synaptosomal preparations. Training also triggered an increase in c-src protein tyrosine kinase activity that was correlated with its tyrosine dephosphorylation in the synaptic membrane fraction. After training, pp60c-src from hippocampus showed enhanced interactions with synaptic proteins such as synapsin I, synaptophysin, and the type 2 *N*-methyl-D-aspartate receptor, as well as the cytoskeletal protein actin. The association of pp60c-src with insulin receptor in the synaptic membrane fraction, however, was temporally decreased after training. Furthermore, *in vitro* results showed that Ca^{2+} and protein kinase C might be involved in the regulation of protein–protein interactions of pp60c-src. These results suggest, therefore, that pp60c-src participates in the regulation of hippocampal synaptic activity during learning and memory.

Memory formation after a learning experience has been shown to be correlated with changes in efficacy of synaptic communication, synaptogenesis, and other morphological alterations. These processes require functions of diverse signaling molecules localized at both pre- and postsynaptic sites, among which are protein kinases and various substrate synaptic proteins. c-src protein has been a subject of growing interest because of its specific distribution in synaptic regions, where it interacts with other functional proteins.

The highly conserved protooncogene c-*src* is a cellular homologue of the viral src oncogene (v-src) of the transforming Rous sarcoma virus. Its protein product, pp60c-src, a nonreceptor protein tyrosine kinase (PTK), is widely expressed in many cell types with particularly high abundance in neurons (1–3). In neural cells, pp60c-src is enriched in nerve terminals and accounts for the majority of presynaptic vesicle tyrosine kinase activity (4–8). High concentrations of pp60c-src are also distributed in nerve growth cones (9, 10), where it is thought to play a role in guiding neurites toward their synaptic targets by modulating cytoskeletal dynamics (11, 12). Furthermore, association of pp60c-src with the postsynaptic density also has been reported (13).

The kinase activity of pp60c-src is regulated by its autophosphorylation at particular tyrosine residues. Phosphorylation at Tyr-416 has a positive effect on c-src PTK activity, whereas at Tyr-517 it regulates negatively the c-src PTK activity (14, 15). In synaptic vesicles, pp60c-src interacts with proline-rich domains of different synaptic proteins such as dynamin and synapsin I through its SH3 domain (16), resulting in elevation of c-src PTK activity (17). c-src also phosphorylates other synaptic vesicle proteins such as synaptophysin (7, 8) and synaptogryrin (18). Furthermore, pp60c-src interacts with and phosphorylates the type 2 *N*-methyl-D-aspartate (NMDA) receptor (NR2), thereby potentiating NMDA receptor currents (19–21). The c-src-mediated NMDA receptor regulation has been shown to be associated with protein kinase C (PKC) activation (22).

Because molecules involved in the regulation of synaptic activity may contribute to memory processing and pp60c-src interacts with the above proteins at synapses, in this study, we investigated pp60c-src and pp60c-src-associated events in the rat brain after spatial learning. Our findings indicate that pp60c-src undergoes training-induced changes in its gene expression, tyrosine phosphorylation, PTK activity, and protein–protein interactions.

Experimental Procedures

Water Maze Task. Male 45- to 60-day-old Wistar rats (200–250 g) were trained with the Morris water maze task, and memory was assessed as described previously (23). Rats were killed at 1 and 24 h, respectively, after a 1-day and a 4-day training trial for measuring changes in different memory stages. Swimming only and naïve rats were used as controls. All rats were killed by decapitation, and their hippocampi were removed rapidly, frozen on dry ice, and stored at −80°C before use. Hippocampi from nine rats from each group were divided into three pools with each pool containing hippocampi from three rats. For *in situ* hybridization and immunohistochemistry studies, the whole brain was removed rapidly after decapitation and frozen on dry ice. Three rats were used for each group in histochemistry studies.

The experiments were carried out under the guidelines of the National Institutes of Health regulations for the Care and Use of Animals for Scientific Purposes.

Preparation of c-src Riboprobe. A 170-bp fragment of cDNA corresponding to bases 289–458 of the previously characterized rat pp60c-src mRNA (GenBank accession no. AF130457) was synthesized by reverse transcription–PCR from rat brain RNA by using specific primers (forward: TCACGGACAGAGACTGACCT; reverse: AAGTACCACTCCTCAGCCTG). This fragment, which shares no homology with any other proteins in the rat brain, was subcloned into the pCR-Blunt II-TOPO vector (Invitrogen). The sense and antisense riboprobes were synthesized by *in vitro* transcription with T7 RNA polymerase (Ambion kit; Austin, TX) by using templates with opposite orientation of

Abbreviations: PTK, protein tyrosine kinase; NMDA, *N*-methyl-D-aspartate; NR2, type 2 NMDA receptor; PKC, protein kinase C; IR, insulin receptor.

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DNA insert in the presence of 2 mM α-[^{35}S]thio-UTP (>1,000 Ci/mmol; NEN).

***In Situ* Hybridization.** *In situ* hybridization on hippocampal slices was performed as described previously (23).

Immunohistochemistry. Frozen brain sections were fixed for 30 min in 4% formaldehyde. After washing with PBS and preincubation with 2% normal goat serum in PBS for 30 min, the slices were incubated for 2 h at room temperature with a mixture of a polyclonal anti-c-src (Santa Cruz Biotechnology) and a monoclonal anti-synaptophysin antibody (Pierce), both diluted to 1:200. In control slices, the primary antibody was omitted. Slices were washed with PBS, followed by incubation with a mixture of anti-rabbit IgG-fluorescein and anti-mouse IgG-Texas red (1:200; Vector Laboratories) in the dark for 1 h at room temperature. After a wash process, signals on slices were observed with fluorescent microscopy.

Preparation of Crude Synaptosomes and Synaptic Membranes. To minimize postmortem changes, frozen hippocampi were used for tissue preparation. Because freeze–thaw may reduce the yield of synaptosomes, we prepared crude synaptosomal and membrane fractions according to previously published procedures (23) that retain the majority of synaptic components.

Protein concentrations were measured by using the Bio-Rad protein assay reagent.

Immunoblotting. Immunoblotting experiments were performed according to previously published procedures (23).

Immunoprecipitation. Immunoprecipitation (ip) was performed by incubating synaptosomal fractions with a given primary antibody in a buffer (ip buffer) containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 1% protease inhibitor mixture. In experiments assessing tyrosine phosphorylation of pp60c-src, 1% tyrosine phosphatase inhibitor mixture (Sigma) also was included in the ip mixture. This protein–antibody mixture (total volume, 200 μl) was incubated with end-to-end rocking at 4°C overnight. Protein G or protein A agarose beads were added to the mixture according to the IgG type of the primary antibody and rocked at 4°C for another 2 h. After wash, the precipitated protein was resolved on SDS/PAGE and detected with immunoblotting procedures.

Protein–protein Interaction Detection. Coimmunoprecipitation was performed as a means of measuring interactions of pp60c-src with other synaptic proteins, during which anti-c-src antibody was used to immunoprecipitate pp60c-src from tissue fractions. Proteins coprecipitated with pp60c-src were respectively identified on Western blots by antibodies against each individual protein. Alternatively, a reverse process was conducted to confirm the detected protein–protein interaction, in which an antibody against a protein that coprecipitated with pp60c-src was used for immunoprecipitation. Subsequently, coprecipitated pp60c-src was detected on Western blots by anti-c-src antibody.

***In Vitro* Phosphorylation Reactions.** *In vitro* serine/threonine (Ser/Thr) phosphorylation was carried out in a total volume of a 100-μl reaction mixture containing 100 μg of hippocampal synaptosomal proteins, 50 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, 2 mM EGTA, 1% protease inhibitor mixture, and 1% Ser/Thr phosphatase inhibitor mixture (Sigma). For tyrosine phosphorylation, 1 mM $MnCl_2$ was included, and the Ser/Thr phosphatase inhibitors were replaced with 1% tyrosine phosphatase inhibitor mixture (see above). The reaction was initiated by the addition of ATP to a final concentration of 100 μM, and terminated after incubating at 37°C for 3 min, by placing the reaction tubes onto ice and adding 100 μl of 2× ip buffer. The phosphorylated samples then were subjected to immunoprecipitation in the presence of a given primary antibody according to the purpose of the experiment.

c-src PTK Activity Assay. c-src PTK activity assays were performed with the src kinase assay kit (Pierce) by using poly(Glu,Tyr) 4:1 (Sigma) as substrate.

Data Analysis. All biochemical experiments were repeated at least three times for each pool of hippocampal tissues. Signals from immunoblotting and *in situ* hybridization histochemistry experiments were analyzed by measuring the optic mean density using the NIH IMAGE program. Values from the swimming controls and trained animals from each experiment were normalized against values from naïve animals and subjected to either one- or two-way ANOVA.

Results

Distribution of c-*src* mRNA and Protein in the Rat Brain. *In situ* hybridization results showed that the c-*src* mRNA signals were highly concentrated in specific brain regions of the rat, such as the piriform cortex, and the hippocampal structures, particularly the CA3 area and the dentate gyrus (Fig. 1*A*). High concentrations of signals were also present in the outer layers (layer II and III) of cerebral cortex, the thalamic nuclei, the amygdala, and the entorhinal cortex. In contrast, relatively low levels of the c-*src* mRNA signals were seen in the cerebellar cortex.

Immunohistochemistry staining in the hippocampus (Fig. 1*B*) revealed that most of the c-src protein was localized in the pyramidal cell somata-free layers, forming clear laminae around the CA1 and CA3 of the hippocampus (Fig. 1*B*, green) that correspond to the stratum oriens (SO), stratum radiatum (SR), and striatum lucidum (SL). When compared with the distribution of synaptophysin (Fig. 1*B*, red), which is also a presynaptic localized protein, c-src showed a particularly high concentration in the stratum lucidum, a region occupied by the mossy fiber axons originating from the dentate gyrus.

Water Maze Training Effects. As shown in Fig. 2*A*, rats spent an average of 1.5 min to find the hidden platform in the first training trial. The escape latency was reduced rapidly in subsequent trials, demonstrating clear evidence of learning. A one-way ANOVA showed a significant training effect [$F(15, 224) = 27.32, P < 0.0001$].

Changes in Expression of c-*src* mRNA and Protein in Memory Formation. To measure changes in pp60c-src expression, equal amounts of synaptosomal proteins from each group were resolved on a gradient SDS gel. The amount of pp60c-src was assessed with anti-c-src antibody on a Western blot. As shown in Fig. 2*B*, the amount of pp60c-src in synaptosomal fractions of the hippocampus was increased significantly after training [$F(1, 16) = 196.1, P < 0.001$, two-way ANOVA], but was reduced in the swimming controls.

On the other hand, results from *in situ* hybridization revealed subregional changes in c-*src* mRNA levels after training (Fig. 2*C*). At 24 h after 1-day training, c-*src* mRNA levels were increased significantly [$F(2, 6) = 26.8, P = 0.001$] in the CA3 area, whereas a reduction in c-*src* mRNA signals was seen in the swimming controls. In the CA1 area, c-*src* mRNA signals from both swimming controls and trained animals appeared to be reduced. No apparent changes were detected in dentate gyrus. Fig. 2*D* illustrates emulsion autoradiography revealed as reduced silver grains localized inside the CA1 and CA3 pyramidal cells. Consistent with Fig. 1*A*, a marked up-regulation of c-*src* mRNA was observed in the CA3 region after training. Because CA3 neurons send out projections to the CA1 as well as the CA3 regions, increases in c-*src* mRNA in the CA3 region may be sufficient to account for the training-specific increases of pp60c-src.

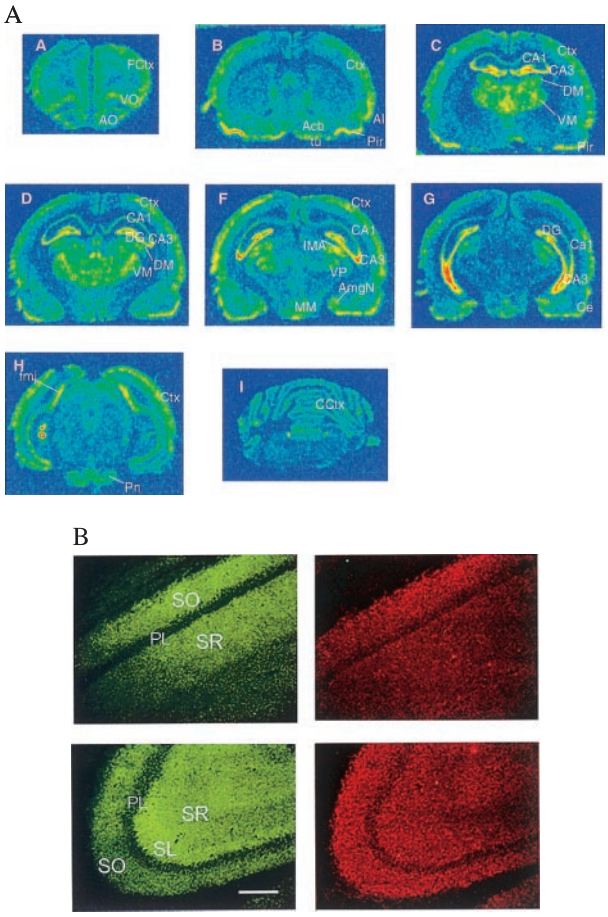

Fig. 1. (*A*) Distribution of c-*src* mRNA in the rat brain. c-*src* mRNA signals in the rat brain were revealed by *in situ* hybridization by using a specific riboprobe. The levels of the signal in each brain region were represented by the intensities of color. Brain regions were defined according to *The Rat Brain* atlas by Paxinos and Watson (24): FCtx, frontal cortex; VO, ventral orbital cortex; AO, anterior olfactory nuclei; Ctx, cerebral cortex; AI, agranular insular cortex; Pir, piriform cortex; Acb, accumbens nucleus; tu, olfactory tubercle; CA1, CA1 area of the hippocampus; CA3, CA3 area of the hippocampus; DG, dentate gyrus; DM, dorsal thalamic nucleus medial; VM, ventromedial thalamic nucleus; IMA, interanteromedial thalamic nucleus; VP, ventroposterior thalamic nuclei; AmgN, amygdaloid nucleus; MM, medial mammill nucleus; Ce, entorhinal cortex; fmj, forceps major corpus callosum; Pn, paranigral nucleus; CCtx, cerebellar cortex. (*B*) Distribution of pp60c-src (green) and synaptophysin (red) in the CA1 and CA3 areas of the rat hippocampus revealed by fluorescent double labeling. Both proteins are localized mainly in the pyramidal cell-free areas of the hippocampus.

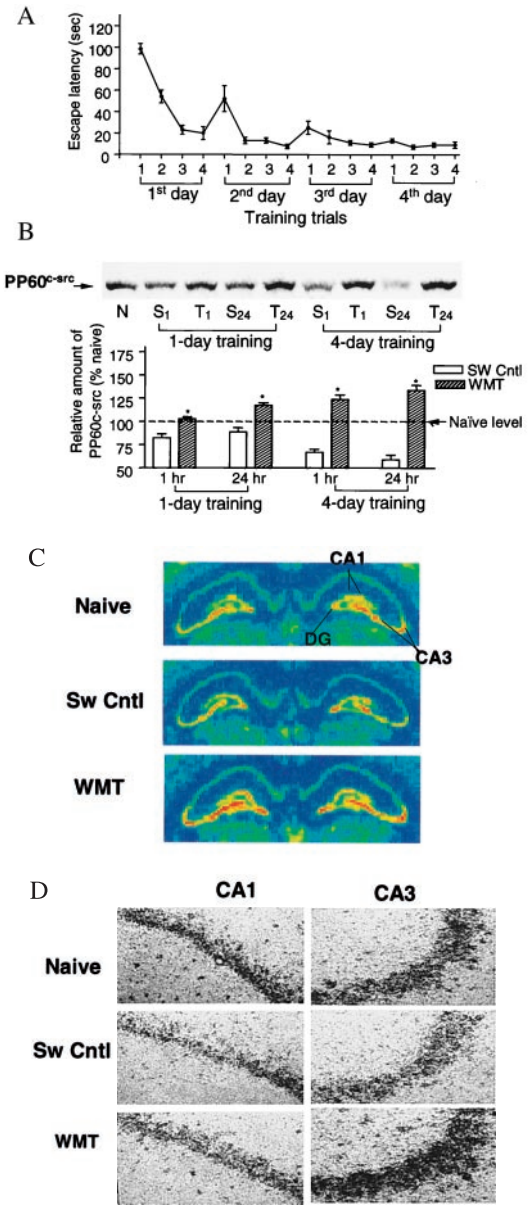

Fig. 2. (*A*) Escape latencies of rats during a 4-day water maze training. (*B*) Changes in amounts of pp60c-src in the hippocampal synaptosomes after training. The upper image shows representative immunoblotting results, and the bar graph below indicates quantified results from three sample pools ($n = 3$; *, $P < 0.001$). S1 and S24, swimming controls sampled at 1 and 24 h after swimming; T1 and T24, trained rats sampled at 1 and 24 h after training tasks. (*C*) Representative images showing training-induced changes in c-*src* mRNA expression. SW Cntl, swimming controls; WMT, water maze trained. (*D*) Representative images for c-*src* mRNA signals in the CA1 and CA3 pyramidal cells revealed by emulsion autoradiography. In each condition, the CA1 and CA3 images were from the same brain slice obtained at 24 h after 1-day training.

Changes in c-src PTK Activity and Tyrosine Phosphorylation After Training. Results from kinase activity measurements (Fig. 3*A*) showed that c-src PTK activity in the hippocampal synaptosomal fraction was increased more than 2-fold shortly (1 h) after training, but was reduced toward the control level at 24 h after training. There were no apparent differences between the trained and control animals, although the rats experienced more training trials over the next 3 days. A two-way ANOVA indicated significant group [$F(1, 16) = 90.88$, $P < 0.001$] and time effects [$F(3, 16) = 30.96$, $P < 0.001$]. In the synaptic membrane fraction (Fig. 3*B*), there was also an increase in c-src PTK activity at 1 h after the first day of training [$F(1, 6) = 6.74$, $P < 0.001$]; no significant differences were detected at other times after training. These results suggest that c-src activity is increased in an early stage of memory formation.

We next assessed changes in tyrosine phosphorylation of pp60c-src. *In vivo* Tyr-phosphorylated pp60c-src was immunoprecipitated from hippocampal synaptosomes and synaptic membranes with an anti-phosphotyrosine antibody (Py20; Santa Cruz Biotechnology), and the amount of precipitated phospho-pp60c-src then was detected with immunoblotting. In the synaptosomal fractions pp60c-src showed significantly higher Tyr phosphorylation of pp60c-src in trained animals at later stages of memory (Fig. 3*C*). A two-way ANOVA indicated significant group [$F(1, 16) = 67$, $P < 0.001$], and time [$F(3, 16) = 8.98$, $P = 0.0025$] effects. In P_2M fractions, a significant Tyr dephosphorylation was observed at 1 h after training

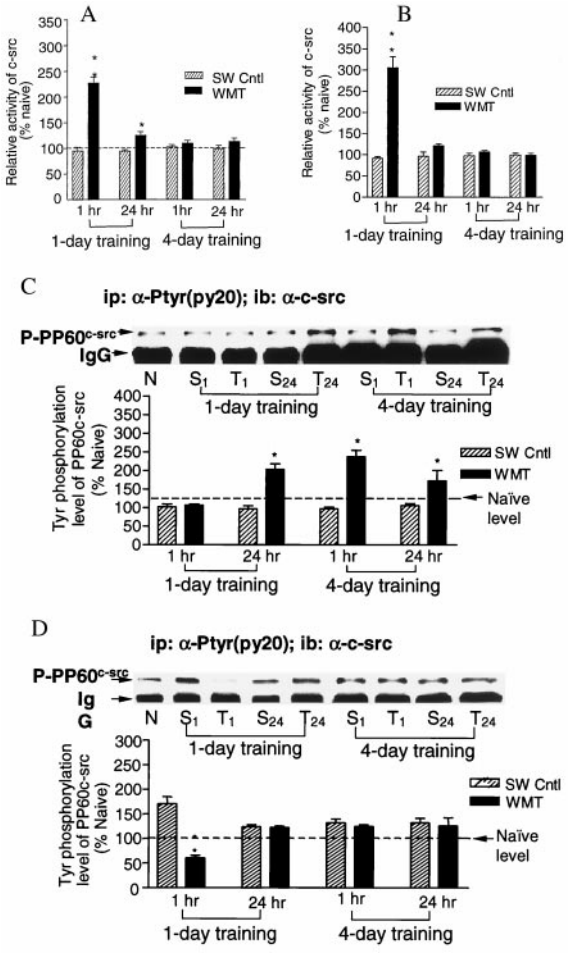

Fig. 3. Changes in c-src PTK activity and Tyr phosphorylation after training. c-src PTK activities were measured in both synaptosomal (*A*) and synaptic membrane (*B*) fractions ($n = 3$; **, $P < 0.001$). Changes in Tyr phosphorylation were measured in synaptosomal (*C*) and synaptic membrane (*D*) fractions by immunoprecipitation by using an anti-phosphotyrosine antibody. The intensity of the protein band correlated with *in vivo* Tyr phosphorylation of pp60c-src (P-pp60c-src) ($n = 3$; *, $P < 0.001$). See Fig. 2 for details on S1, S24, T1, and T24.

$[F(1, 16) = 22.27, P < 0.001]$ (Fig. 3*D*). These results suggest that pp60c-src may undergo different phosphorylation changes in different synaptic subregions, and its Tyr dephosphorylation in the synaptic membrane fraction appears to be correlated with an increase in c-src activity.

Training-Induced Changes in the Interaction of pp60c-src with Other Synaptic Proteins. In this experiment we investigated protein–protein interactions of pp60c-src by using a coimmunoprecipitation procedure. A few proteins were coimmunoprecipitated with pp60c-src from both synaptosomal and synaptic membrane fractions. The first detected protein that coprecipitated with pp60c-src in the synaptic membrane fraction was recognized specifically by anti-NR2 (a/b) antibody (Chemicon) (Fig. 4*A*), indicating that this protein was the type 2 NMDA receptor. The amount of NR2 coprecipitated with pp60c-src was significantly greater $[F(116) = 96.3, P < 0.001]$ in all trained animals. In addition, Tyr phosphorylation of NR2 also was increased $[F(1, 16 = 23.55, P < 0.001]$ at 1 h but not 24 h after training after both 1- and 4-day training experiences (Fig. 4*B*). This short-lasting increase suggests that changes in Tyr phosphorylation of NR2 may be relevant to short-term memory processing.

The second identified protein was synapsin I, a synaptic vesicle protein, whose interaction with pp60c-src was increased significantly $[F(1, 16) = 79.43, P < 0.001]$ after the first day of training and appeared to be increased further after a 4-day training experience (Fig. 4*C*). No apparent changes in the total amount of synapsin I in the hippocampal synaptosomes across groups were detected (data no shown).

We next tested binding of pp60c-src with synaptophysin, another major, integral protein of the synaptic vesicle membrane. The results clearly showed (Fig. 4*D*) that there was a persistent and significant increase in binding of synaptophysin to pp60c-src $[F(1, 16) = 579, P < 0.001]$. Similar to synapsin I, no significant changes in the overall amount of synaptophysin in the hippocampal synaptosomes were detected with the immunoblotting method (data not shown).

Finally, the amount of actin coprecipitated with pp60c-src also was increased in the samples from trained animals but apparently reduced in samples from the swimming controls yoked to a 4-day training task (Fig. 4*E*). A two-way ANOVA showed significant training $[F(1, 16) = 65.4, P < 0.001]$ and time $[F(3, 16) = 12.85, P < 0.001]$ effects. Because actin also is known to bind to synapsin I (25), we examined coimmunoprecipitation of actin with synapsin I. As shown in Fig. 4*F*, the amount of actin coprecipitated with synapsin I also was increased after training but decreased in the swimming controls yoked to the 4-day training task.

Although pp60c-src has been reported to interact with adhesion proteins such as NCAM and FAK120, we failed to detect these two proteins in our c-src antibody-precipitated samples.

Interaction of P60c-src with Insulin Receptor (IR). pp60c-src is known to interact with and to be phosphorylated by IR (26). We thus studied the IR/pp60c-src interaction and its change after learning in the hippocampal synaptic membrane fractions, where the majority of IR is localized (23). pp60c-src was coprecipitated with IR by anti-IR antibody (Santa Cruz Biotechnology) and then detected in immunoblotting with anti-c-src antibody. In contrast to the effects for proteins reported above, the amount of pp60c-src coprecipitated with IR was decreased markedly after a 1-day training experience but was increased significantly in swimming controls (Fig. 5*A*). After the 4-day training trial, however, binding of pp60c-src to IR was at similar levels among all groups (Fig. 5*A*). A two-way ANOVA showed significant training $[F(1, 16) = 161.8, P < 0.001]$ and time $[F(3, 16) = 12.72, P < 0.001]$ effects. These results indicate that changes in the pp60c-src/IR interaction may be involved in a relatively acute and new behavioral experience, during which pp60c-src binds to IR perhaps in response to increased stress, whereas learning induces dissociation between these two proteins.

Effects of Ca^{2+} and Phorbol Esters on the Protein–Protein Interaction of pp60c-src. In this experiment, we tested effects of Ca^{2+} and activation of PKC on the protein–protein interaction of pp60c-src under different *in vitro* phosphorylation conditions. We found that interactions of pp60c-src with NR2 and synapsin I were reduced in the conditions optimal for Ser/Thr phosphorylation in the absence or presence of Ca^{2+} (Fig. 5*B*, lanes 1–3). The addition of the phorbol ester phorbol 12-myristate 13-acetate, a PKC activator, to the reaction apparently enhanced the pp60c-src/NR2 and pp60c-src/synapsin I interactions (Fig. 5*B*, lane 4). Under conditions favoring Tyr phosphorylation, however, interactions of synaptosomal pp60c-src with NR2 and synapsin I were increased markedly compared with the controls (Fig. 5*B*, lane 5–7). Results from these experiments suggest that interactions of pp60c-src with other synaptic proteins may be regulated by intracellular Ca^{2+} and PKC activity.

Discussion

In the rat, expression of *src* mRNA in the hippocampus increases after birth and remains high throughout life (27). In other brain regions such as the striatum, however, expression and PTK activity

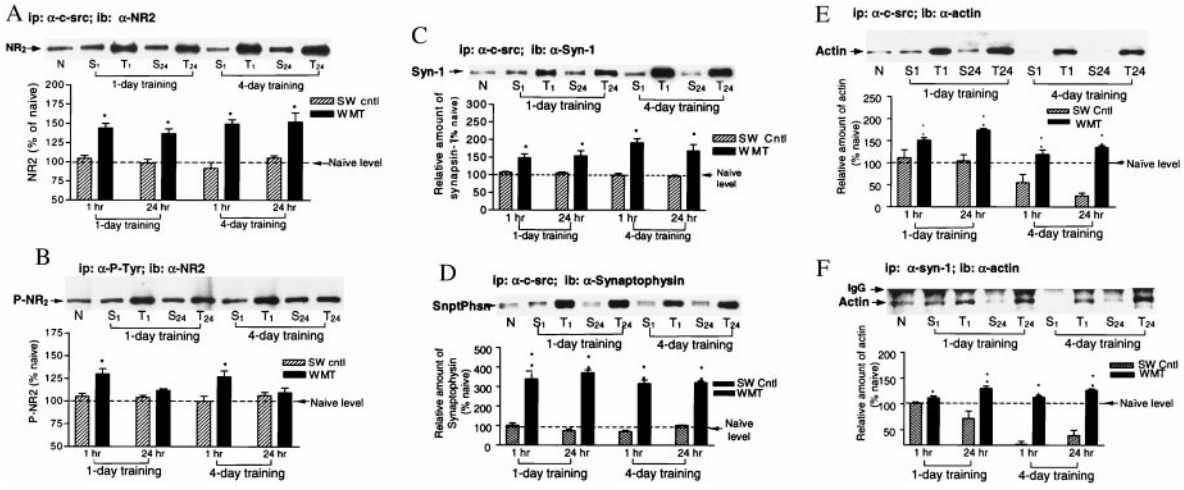

Fig. 4. Changes in pp60c-src/NR interaction and NR2 Tyr phosphorylation. (*A*) NR2 was coprecipitated with pp60c-src by anti-c-src antibody from synaptic membrane fractions from each group and identified with anti-NR2 antibody on Western blots ($n = 3$; *, $P < 0.001$). (*B*) Phosphorylated NR2 was precipitated by anti-phosphotyrosine antibody and subsequently detected by anti-NR2 antibody on Western blots ($n = 3$; *, $P < 0.001$). (*C*) Changes in pp60c-src/synapsin I interactions after training. Synapsin I was coprecipitated with pp60c-src from hippocampal synaptosomes by anti-c-src antibody and detected on Western blots with anti-synapsin I antibody ($n = 3$; *, $P < 0.001$). (*D*) Changes in pp60c-src/synaptophysin interactions after training. Synaptophysin was coprecipitated with pp60c-src from hippocampal synaptosomes by anti-c-src antibody and detected on Western blots with anti-synaptophysin antibody ($n = 3$; *, $P < 0.001$). (*E*) Changes in pp60c-src/actin interactions after training ($n = 3$; *, $P < 0.001$). (*F*) Changes in synapsin I/actin interactions ($n = 3$; *, $P < 0.001$). See Fig. 2 for details on S1, S24, T1, and T24. ip, immunoprecipitation; ib, immunoblotting.

of pp60c-src peaks during embryonic development and then declines in the adult (27). The *in situ* hybridization results in the present study provide more complete localization of c-*src* mRNA in different areas of the adult rat brain. c-*src* mRNA signals are abundantly expressed in neurons of the outer layer of the neocortex, thalamic nuclei, piriform cortex of the olfactory system, entorhinal cortex, and the hippocampus, with particularly high concentrations in the CA3 and dentate gyrus. The protein product (pp60c-src), on the other hand, is localized mostly in the non-neuronal area of the hippocampus, such as the stratum oriens, stratum radiatum, and stratum lucidum, suggesting that the protein product of the c-*src* gene is transported to the nerve terminals and the dendritic regions after synthesis in the somata. That both c-*src* mRNA and protein are shown to be particularly concentrated in the CA3 area may suggest that pp60c-src is more actively involved in the functions of CA3 neurons.

In the present study, a significant up-regulation of c-*src* gene in the CA3 area was triggered after water maze training, accompanied by increases in the amount of pp60c-src. The synthesized protein may be transported to the synaptic terminals via the Schaeffer and recurrent collaterals as well as to the growth cones of dendrites, where it may participate in the regulation of synaptic function during learning.

It was reported previously that knockout of the *src* gene did not reveal detectable histological abnormality in the brain (28), nor did it interfere with expression of the CA1 long-term potentiation (LTP) (29). Because pp60c-src is distributed mostly in the presynaptic compartments such as the synaptic vesicle and growth cone, it would be interesting to know whether c-*src* mutation interferes with LTP in the mossy fiber of the CA3 area, where LTP is thought to be a predominantly presynaptic process (30). Although mutation of Fyn, another *src* family member, has been shown to impair both CA1 LTP and spatial learning (29), no report is available regarding the impact of the c-*src* mutation on learning and memory.

A temporary increase in the c-src tyrosine kinase activity also was detected shortly after training in both synaptosomal and synaptic membrane fractions. In addition, increases in tyrosine phosphorylation of pp60c-src were revealed in the synaptosomal fractions during a late stage of memory formation, whereas Tyr dephosphorylation was detected in the synaptic membrane fraction in the early stage of memory formation. It is not clear whether the dephosphorylated pp60c-src in the P2 M fraction has a presynaptic origin, perhaps in association with docked and/or fused synaptic vesicles, or a postsynaptic origin in association with NR2. Although the increased c-src PTK activity in the P2 fraction did not appear to be correlated with the enhanced Tyr phosphorylation of pp60c-src, it clearly coincided with the Tyr dephosphorylation in the P2 M fraction after training. An important regulatory mechanism underlying c-src PTK activity has been known to be selective phosphor-

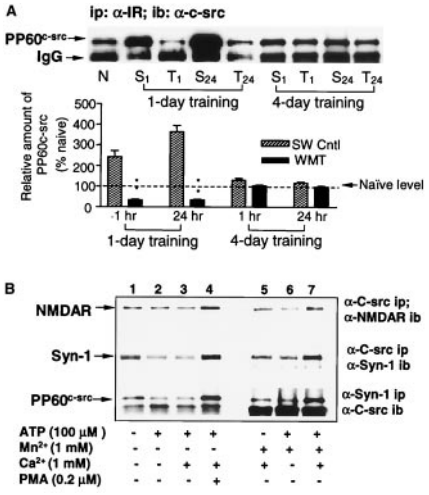

Fig. 5. Binding of pp60c-src to IR after training. (*A*) Synaptic membrane proteins were precipitated with anti-IR antibody, and the coprecipitated pp60c-src was identified on Western blots with anti-c-src antibody ($n = 3$; **, $P < 0.001$). (*B*) Effects of Ca^{2+} and phorbol ester on protein–protein interaction of pp60c-src. Hippocampal synaptosomal proteins were phosphorylated *in vitro* under conditions optimal for Ser/Thr (lanes 1–4) or Tyr (lanes 5–7) phosphorylations in the presence or absence of Ca^{2+} and phorbol 12-myristate 13-acetate. The phosphorylated proteins were immunoprecipitated by anti-c-src antibody followed by identification on Western blots with anti-NR2 (*Top*) and synapsin I (*Middle*), respectively. Samples also were precipitated with anti-synapsin I antibody and subsequently detected with c-src antibody (*Bottom*).

ylation at Tyr-416 for positive regulation vs. Tyr-527 for negative regulation of the c-src PTK activity (31–33). Thus, either phosphorylation at Tyr-416 or dephosphorylation at Tyr-527 would enhance the c-src PTK activity. It is not clear, however, in the present study which sites precisely were phosphorylated or dephosphorylated as a result of training. In addition, other factors, such as the insulin receptor and PKC, are known to phosphorylate pp60c-src at different sites (26, 34), with different consequences on the regulation of c-src PTK activity.

Results from the present studies showed that interactions of pp60c-src with other synaptic proteins might provide important means of regulating a variety of molecules involved in synaptic functions. Associations of several well characterized proteins with pp60c-src have been found to be increased after training. At the presynaptic site, for example, pp60c-src was associated with synaptic vesicle proteins synapsin I and synaptophysin. Synapsin I is a vesicle phosphoprotein that is phosphorylated by multiple protein kinases (35, 36). By association with synaptic vesicle membrane, pp60c-src maintains the stability of the reserve pool of synaptic vesicles that are needed for high-frequency synaptic activity (36). It also regulates the kinetics of neurotransmitter release through an unidentified mechanism (37). Recently, two laboratories have reported the interaction of synapsin I with pp60c-src (16, 17), which enhances c-src TPK activity (17). In an *in vitro* system, pp60c-src has been found to interact and phosphorylate synaptophysin (7), a major, integral synaptic vesicle protein that is required for vesicle fusion and release of neurotransmitters (35).

In addition, pp60c-src interacts with the NR2, a subtype of NMDA receptor that regulates excitatory neurotransmission in the brain and may play a role in synaptic models of memory formation (38–40). By acting on the C-terminal domain of the NR2A receptor subunit (22), pp60c-src was found to enhance the NMDA receptor current (18, 21, 22). The pp60c-src–NMDA receptor function is regulated, at least partly, by PKC activity (22). In the present study we have shown that the enhanced pp60c-src/NR2 interaction occurred together with increases in tyrosine phosphorylation of the NR2 after training. We also showed (Fig. 5) that a PKC activator, phorbol 12-myristate 13-acetate, promotes NR2/pp60c-src binding in an *in vitro* phosphorylation reaction. Past studies have implicated PKC activation in rat spatial learning (41). These results, taken together, appear to reveal a signal-transduction pathway involving PKC-src-NR2 in spatial memory formation.

Increased amounts of the cytoskeletal protein actin were coimmunoprecipitated with both pp60c-src and synapsin I after training. Although pp60v-src is known to be associated with the cytoskeleton during transformation, very low levels of pp60c-src were found to bind to the cytoskeleton (42). Synapsin I, on the other hand, is well known for its actin association (25). It thus remains to be determined whether the training-induced increase in pp60c-src/actin binding detected in the present study is a direct association of these two proteins or a pp60c-src/actin/synapsin I triple complex. In either case, training appears to enhance interactions of pp60c-src and/or synapsin I with cytoskeletal proteins in the synaptic region.

While our results clearly demonstrate training-induced changes in pp60c-src, their functional consequences for synaptic transmission during memory formation require further study.

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