Involvement of ryanodine receptors in neurotrophin-induced hippocampal synaptic plasticity and spatial memory formation

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Ryanodine receptors (RyR) amplify activity-dependent calcium influx via calcium-induced calcium release. Calcium signals trigger postsynaptic pathways in hippocampal neurons that underlie synaptic plasticity, learning, and memory. Recent evidence supports a role of the RyR2 and RyR3 isoforms in these processes. Along with calcium signals, brain-derived neurotrophic factor (BDNF) is a key signaling molecule for hippocampal synaptic plasticity and spatial memory. Upon binding to specific TrkB receptors, BDNF initiates complex signaling pathways that modify synaptic structure and function. Here, we show that BDNF-induced remodeling of hippocampal dendritic spines required functional RyR. Additionally, incubation with BDNF enhanced the expression of RyR2, RyR3, and PKMζ, an atypical protein kinase C isoform with key roles in hippocampal memory consolidation. Consistent with their increased RyR protein content, BDNF-treated neurons generated larger RyR-mediated calcium signals than controls. Selective inhibition of RyR-mediated calcium release with inhibitory ryanodine concentrations prevented the PKMζ, RyR2, and RyR3 protein content enhancement induced by BDNF. Intrahippocampal injection of BDNF or training rats in a spatial memory task enhanced PKMζ, RyR2, RyR3, and BDNF hippocampal protein content, while injection of ryanodine at concentrations that stimulate RyR-mediated calcium release improved spatial memory learning and enhanced memory consolidation. We propose that RyR-generated calcium signals are key features of the complex neuronal plasticity processes induced by BDNF, which include increased expression of RyR2, RyR3, and PKMζ and the spine remodeling required for spatial memory formation.

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tivity-generated neuronal Ca2+ signals promote gene transcription and protein synthesis, two essential events of long-lasting synaptic plasticity and memory formation (1). Hippocampal neuronal plasticity requires de novo protein synthesis for the generation and growth of dendritic spines (2). Brain-derived neurotrophic factor (BDNF) is a key signaling factor implicated in activity-dependent neuronal changes underlying memory acquisition and persistence of long-term memory storage (3). At the cellular level, BDNF enhances synaptic transmission in rat hippocampus by inducing local protein synthesis in postsynaptic dendrites (4). A central feature of the responses elicited by BDNF is cyclic AMP-responsive element binding (CREB) activation (5), which occurs via BDNF-induced signaling pathways that promote the expression of genes that prompt the formation, elongation and stretching of dendritic spines (4, 6). BDNF binding to specific TrkB receptors induces Ca2+ release through inositol 1,4,5-trisphosphate receptors (InsP3R) and stimulates calcium-dependent kinases in hippocampal neurons (6), which contribute to induce and maintain long-term potentiation (LTP) (7).

Besides BDNF, other signaling molecules are essential for late-LTP and long-term memory formation. Among them, protein kinase Mζ (PKMζ), an atypical protein kinase C isoform with constitutive activity (8), is both necessary and sufficient for hippocampal late-LTP maintenance (9, 10) and long-term memory retention (11). Translation of PKMζ is rapidly up-regulated during LTP induction (12), whereas selective PKMζ inhibition reverses hippocampal LTP maintenance in vivo and disrupts long-term memory retention elaborated in the hippocampus (13).

The essential role of Ca2+ signals in long-term neuronal plasticity processes is well established (14). Ca2+ release through ryanodine receptors (RyR) and InsP3R present in the endoplasmic reticulum of axons and dendrites produces Ca2+ signals (15) that make an important contribution to synaptic plasticity processes in central neurons (16). In particular, RyR-mediated Ca2+ release (CICR) provides a major fraction of the Ca2+ increase induced in hippocampal CA1 spines by synaptic activity (17). High-frequency field stimulation of primary hippocampal neurons produces Ca2+ signals primarily derived from RyR-mediated Ca2+ release (18), while caffeine-induced RyR-mediated Ca2+ release promotes dendritic spine elongation in primary hippocampal neurons (19). Several reports support RyR participation in hippocampal LTP and memory processes (20–22). Thus, RyR inhibition before tetanic stimulation suppresses hippocampal long-lasting LTP, and RyR activation converts early LTP into late-LTP (23). Hippocampal expression of the RyR2 isoform increases after spatial memory training (24), but selective knockdown of RyR2 and RyR3, but not of RyR1, impairs avoidance memory processes (20). Inhibition of overall brain RyR activity with dantrolene decreases associative memory in chicken (21), whereas the RyR agonist 4-chloro-m-cresol (4-CMC) administered to whole brain 30 min before a passive avoidance task enhances memory retention in mice (20) and promotes long-term memory consolidation in young chicks (22).

The present results show that BDNF increased RyR2, RyR3, and PKMζ protein expression and promoted spine remodeling in primary hippocampal neurons; these effects required functional RyR. Intrahippocampus BDNF injection and memory training also enhanced RyR2, RyR3, and PKMζ expression, while intrahippocampus ryanodine injection at concentrations that stimulate RyR-mediated Ca2+ release facilitated memory acquisition and consolidation.

Results

BDNF Stimulates Hippocampal Expression of the Ca2+ Release Channel Isoforms RyR2 and RyR3 and of the Plasticity-Related Protein PKMζ

Throughout this work, we used primary hippocampal cultures at 14


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and in vitro (DIV) highly enriched in pyramidal neurons (Fig. S1A). These cultures had similar RyR2, RyR3, and PKMζ and twofold higher InsP3R1 protein content as a whole hippocampus from 2.5-
mo-old rats (Fig. S1B), and displayed significant neuronal immuno-
nostaining for RyR2 (Fig S1C) and RyR3 (Fig S1D). Incubation of cultures with BDNF (50 ng/mL, 6 h) increased RyR2 (6.8-fold) and RyR3 (2.9-fold) mRNA levels over controls (Fig. 1 A and B), along with RyR2 (1.8-fold) and RyR3 (2-fold) protein content (Fig. 1 D and E). Actinomycin D prevented the increase in RyR2 and RyR3 mRNA, suggesting stimulation at the transcriptional level. BDNF acted through binding to TrkB receptors because preincubation with TrkB-Fc (20 nM, 1 h), a BDNF scavenger re-
combinant peptide that prevents BDNF receptor activation (4), suppressed the increments in RyR2 and RyR3 mRNA and protein induced by BDNF (Fig. 1). In agreement with the above results, representative confocal images show increased RyR2 (Fig. 2A) and RyR3 (Fig. 2B) immunoreactivity in hippocampal neurons in-
cubated with BDNF; absence of primary or secondary antibodies produced only background staining. The BDNF-induced RyR2 protein increase was concentration- and time-dependent (Fig. S2A and B) and was abolished by Xestospongion B, an InsP3R inhibitor (Fig. S2C), suggesting that the RyR2 increase induced by BDNF requires InsP3R-mediated Ca²⁺ release.

Of note, incubation of primary cultures with BDNF (50 ng/mL) increased transiently PKMζ mRNA levels, with a peak at 3 h followed by a 50% decrease relative to the controls at 6 h (Fig. 1C), and after 6 h also increased 1.7-fold PKMζ protein content (Fig. 1F).

Ryanodine is a plant alkaloid that selectively activates RyR at low concentrations but is inhibitory at higher concentrations. According to, incubation of hippocampal cultures with 1 μM ryanodine stimulates Ca²⁺ release (Fig. S3A), whereas prolonged incubation with 50 μM ryanodine suppresses it (18, 25). Here, we show that cultures preincubated with inhibitory rya-
nodine (50 μM, 1 h) did not exhibit the above changes in RyR2, RyR3, and PKMζ mRNA, and protein contents produced by 6 h incubation with BDNF (Fig. 1).

Addition of the specific RyR agonist 4-CMC to cultures loaded with the fluorescent Ca²⁺-sensitive dye Fluo-4 AM generated larger fluorescence signals in neurons preincubated with BDNF than in controls (Fig. 2C). The endoplasmic reticulum calcium content was similar in controls and BDNF-treated neurons, as reflected by the similar Ca²⁺ signals generated by addition of thapsigargin (5 μM) to empty these stores (Fig. S3B). The Ca²⁺ signals generated by 4-CMC were RyR-mediated, because preincubation for 6 h with 50 μM ryanodine suppressed them in control neurons (25) or in neurons present in BDNF-
treated cultures (Fig. 2C). These results strongly suggest that the larger Ca²⁺ signals displayed by BDNF-treated neurons reflect their increased RyR-content.

BDNF Induces Changes in Spine Morphology. We investigated by confocal microscopy BDNF-induced changes in spine morphol-
y in primary cultures loaded with calcein (19, 25). Addition of BDNF (50 ng/mL) induced elongation of preexisting spines and formation of new ones within 60 min; these changes did not occur in neurons preincubated for 6 h with 50 μM ryanodine (Fig. 3A). Quantification of changes in spine length (Fig. 3B) and number (Fig. 3C) shows that spine remodeling occurred as early as 15 min following BDNF addition, and confirms the inhibitory effects of ryanodine on BDNF-induced spine remodeling. Longer incubation with BDNF (6 h) significantly increased (1.7-fold) spine density relative to the control (Fig. 3D); preincubation with ryanodine (50 μM, 1 h) before BDNF addition not only suppressed this increase (Fig. 3D), but significantly reduced spine density in both control and BDNF-treated neurons (Fig. 3E).

Increased Expression of RyR2, RyR3, and PKMζ After Intrahippocampus BDNF Injection or Spatial Memory Training. To test if BDNF en-

hanced RyR2, RyR3, and PKMζ expression in vivo, we injected BDNF intrahippocampus using an amount (0.25 μg) that enhances memory persistence, as previously reported (3). The hippocampus of rats injected with BDNF and removed 6 h after the injection displayed significantly increased RyR2, RyR3, PKMζ, InsP3R1, and BDNF protein over saline-injected controls (Fig. 4A).

Previous reports indicate that spatial memory training in a Morris water maze increases hippocampal BDNF protein expression (26), which may enhance in turn RyR and PKMζ expression. To examine this possibility, we measured the expression of these proteins after training rats in a Morris water maze, a protocol that significantly increased spatial memory acquisition with time (solid symbols, Fig. S4A) as well as memory consolidation tested at day 9 (Fig. S4B). As detected in immunoblots from hippocampal extracts obtained at the fifth training day, BDNF protein content increased significantly (Fig. S4C), and so did RyR2 and RyR3 protein contents (Fig. S4D). Analysis by qRT-

PCR of hippocampal extracts isolated 6 h after testing at day 9 showed significantly increased RyR2 (sixfold) and RyR3 (three-

fold) mRNA levels, and no changes in RyR1 mRNA levels (Fig.

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**Fig. 1.** BDNF stimulates RyR2, RyR3, and PKMζ mRNA and protein expression. Relative mRNA levels for RyR2 (A), RyR3 (B), and PKMζ (C) were determined by qRT-PCR; protein contents for RyR2 (D), RyR3 (E), and PKMζ (F) were quantified from immunobots and normalized relative to β-actin. Preincubation for 1 h with 50 μM rya-
nodine (Rya) or TrkB-Fc (20 nM) prevented BDNF-induced changes. Data (Mean ± SE; n ≥ 3) represent ratios be-
tween experimental cultures and controls. Statistical significance was analyzed by one-way ANOVA followed by Newman-Keuls post test; *P < 0.05, **P < 0.01, ***P < 0.001.
parallel immunoblot analysis revealed that RyR2 and RyR3 protein contents increased seven- and sixfold, respectively (Fig. 4C). The RyR2 protein increase was significantly higher than previously reported (24). Immunofluorescence analysis of brain slices obtained 6 h after testing at day 9 revealed a marked in situ RyR3 increase in the hippocampal CA1 region of trained rats compared with controls (Fig. S4E). RyR2 was not assayed by immunohistochemistry because, in contrast to RyR3, its increase after spatial memory training was described previously (24). In addition, we made the previously unreported observation that the mRNA and protein levels of PKMζ, a protein that plays an essential role in synaptic plasticity and memory, increased 2.4- and 2-fold, respectively, when measured after the spatial memory consolidation test (Fig. 4B and C).

**RyR3 injection into the hippocampus Increases Spatial Memory Formation and Spatial Acuity in Rats.** To investigate the contribution of RyR-mediated Ca\(^{2+}\) release to spatial memory processes, [\(^3\)H]-ryanodine (10 nmol) was injected bilaterally, and the ryanodine concentration present 24 h later in different brain regions was inferred from [\(^3\)H] radioactivity measurements of tissue homogenates. The hippocampus of both hemispheres had a ryanodine concentration of 1.2 ± 0.1 μM (mean ± SE) 24 h after injection; other brain regions had significantly lower levels (Fig. S4). No traces of radioactivity were found in these regions 5 d after [\(^3\)H]-ryanodine injection. Consequently, to produce stimulatory ryanodine concentrations within the range (~1 μM) that promote Ca\(^{2+}\) release in primary hippocampal cultures (Fig. S3A), rats were injected 24 h before training with 10 nmol of cold ryanodine. Controls (sham) were injected with vehicle. Rats injected with ryanodine exhibited a significant reduction in escape latencies on the last 2 d of training compared with sham (Fig. 5B). In contrast, rats injected with ryanodine 96 h before training showed escape latencies similar to un.injected controls (open symbols, Fig. S4A). Spatial acuity, a parameter that represents the probability of finding the rat in a region around the hidden platform, is a sensitive parameter to measure spatial learning (27). Rats injected with ryanodine 24 h before training showed increased spatial acuity, with low escape latency values and high spatial-acuity scores (Fig. 5C). After platform removal from zone B, quadrant 4, rats injected with ryanodine spent a higher percentage of time in zone B compared with sham (Fig. 5D). Motor activity measured as swimming speed average did not change during the test in control rats (sham) or after ryanodine injection (Fig. S4F). These combined results suggest that injection of stimulatory ryanodine enhances spatial memory formation and consolidation in the hippocampus.

**Discussion**

The results presented here show that BDNF enhanced RyR2, RyR3, and PKMζ expression and promoted spine morphology changes in many primary hippocampal neurons. Functional RyR-mediated Ca\(^{2+}\) release was likely required for these changes, because inhibitory ryanodine concentrations prevented these responses. Intrahippocampal BDNF injection or spatial memory training also increased RyR2, RyR3, and PKMζ hippocampal expression; the increments produced by spatial memory training possibly reflect BDNF-induced signaling cascades activated during training (3, 6, 28–30).

**Stimulatory Effects of BDNF in Primary Cultures.** BDNF stimulates neurotransmission via activation of Ca\(^{2+}\)-dependent signaling cascades that promote gene transcription, synthesis of new proteins, and spine remodeling (7). The present results confirm that BDNF increases hippocampal RyR2 transcription (31). Additionally, we report here that BDNF stimulated RyR3 transcription and increased RyR2, RyR3, and PKMζ protein content in primary hippocampal cultures; RyR inhibition abolished these effects, implicating RyR-mediated Ca\(^{2+}\) release in these increments. In primary hippocampal neurons, BDNF-induced Ca\(^{2+}\) signals require functional TrkB and InsP3R plus Ca\(^{2+}\)-filled intracellular stores (32, 33). In addition, TRPC3 channels have been proposed as novel mediators of BDNF-mediated Ca\(^{2+}\) signal generation and dendritic remodeling (33). Our results point to RyR-mediated Ca\(^{2+}\) release as an additional source of Ca\(^{2+}\) underlying BDNF-induced RyR2 and RyR3 transcription upregulation. Through CICR, BDNF is likely to stimulate RyR-mediated Ca\(^{2+}\) release downstream of InsP3 receptor-mediated Ca\(^{2+}\) release, generating larger Ca\(^{2+}\) signals that modify gene expression. Several studies have shown that BDNF enhances calcium-dependent gene transcription (28, 29), including stimulation of transcription mediated by Nuclear Factor of Activated T Cell (NFAT) in cultured CA3–CA1 hippocampal neurons that involves Ca\(^{2+}\) release from intracellular stores (30). Our in silico analysis of published sequences revealed that RyR2 and RyR3 gene promoters contain CRE and NFAT calcium-sensitive response elements, respectively. Hence, stimulation of RyR-mediated CICR by BDNF possibly stimulates calcium-dependent transcriptional regulators implicated in synaptic plasticity, such as CREB and NFAT, which in turn would promote RyR2 and RyR3 transcription. BDNF also increased RyR2 and RyR3 protein content; the newly expressed RyR were functional, as neurons incubated with BDNF displayed higher Ca\(^{2+}\) release signals than controls. Fast and high amplitude intracellular Ca\(^{2+}\) signals induce hippocampal LTP, and smaller but more prolonged Ca\(^{2+}\) signals produce long-term depression (34). Our results suggest that stimulation of RyR by BDNF via CICR is part of a positive feedback system underlying BDNF-induced long-lasting plasticity changes.

The constitutive activity of the atypical PKC isoform PKMζ is both necessary and sufficient for hippocampal long-term memory retention (11, 13). To our knowledge, this article is unique in...
describing stimulation of PKMζ protein expression by BDNF and demonstrating that these effects require functional RyR. Enhanced PKMζ synthesis is the final common target of many signaling pathways involved in long-term potentiation (8); the present results strongly suggest that RyR-mediated Ca2+ release is an integral component of these pathways.

The present results, which show that BDNF-induced spine formation and growth require functional RyR, add to previous reports showing that RyR play key roles in neurite outgrowth at later developmental stages in chick and mouse dorsal root ganglion neurons (35). They also complement our recent work showing that synaptotoxic amyloid β peptide oligomers, which are implicated in impaired synaptic plasticity and memory loss in

significant differences at a given time between controls and BDNF-incubated cells; *, significant differences (P < 0.05) between cells incubated with BDNF and BDNF plus ryanodine. (C) Average changes (mean ± SE) in spine number with time; controls (n = 5), exposed to BDNF (n = 6), preincubated with ryanodine before BDNF addition (n = 5). *P < 0.05. In B and C, results were analyzed by two-way ANOVA plus Newman-Keuls posttest. (D) Representative confocal images of a neurite, visualized with calcein fluorescence, after BDNF addition (50 ng/mL); spine elongation and formation of a new spine can be appreciated. (Lower) Preincubation with ryanodine (Rya, 50 μM for 6 h) prevented BDNF-induced spine formation. (Scale bars, 2 μm.) (E) Average changes (mean ± SE) in spine length with time; controls (n = 8); exposed to BDNF (n = 10); preincubated with ryanodine (Rya 50 μM, 6 h) before BDNF addition (n = 10). *P < 0.05 and ***P < 0.001.
Alzheimer disease, decrease RyR2 protein expression, and prevent RyR-dependent spine remodeling induced by BDNF in hippocampal neurons (25).

Intrahippocampal BDNF Injection and Spatial Memory Training Enhance RyR2, RyR3, and PKMζ Expression. In accord with the stimulation of RyR2, RyR3, and PKMζ expression promoted by BDNF in primary cultures, we found that intrahippocampal BDNF injection stimulated the expression of these three proteins within 6 h, and also increased BDNF and InsP3R1 protein expression. These results suggest that BDNF-induced signaling pathways mediate the RyR2, RyR3, and PKMζ protein increases induced by spatial memory training. The RyR2 and RyR3 isoforms seem to have a prominent role in memory processes, as whole-brain administration of RyR2 and RyR3 but not of RyR1 antisense oligonucleotides impairs associative memory (20). Two studies using RyR3 knockout mice implicate RyR3 in spatial memory (36) and associative memory (37); in contrast, normal learning but impaired ability to relearn a new target was reported in these mice (38). While the three mammalian RyR isoforms are expressed in the hippocampus (39), we show here that spatial memory training selectively increased RyR2 and RyR3 mRNA and protein expression in rat hippocampus. These findings strengthen the proposal that RyR2 and RyR3 participate in long-term spatial memory storage (20). Our results, which show that stimulatory ryanodine concentrations present in the hippocampus when initiating training improved memory acquisition and consolidation, suggest that activated RyR-mediated Ca2+ release underlies the observed memory improvement.

The present results are unique in showing significantly enhanced PKMζ mRNA and protein expression in the hippocampus of rats trained in a Morris water maze. PKMζ is translated from a brain-specific PKMζ mRNA that encodes the catalytic but not the regulatory PKCζ domain (12). Moreover, PKMζ protein levels increase significantly 30 min after LTP induction without concomitant changes in mRNA expression (8, 12). The PKMζ gene promoter contains a CRE response element (12). Accordingly, pathways mediated by Ca2+ and BDNF that promote CRE-dependent activation (32, 33, 40) may contribute to enhance PKMζ transcription in vivo after spatial memory training. The coincident up-regulation of RyR2, RyR3, and PKMζ expression after training suggests that these proteins participate in common or parallel complementary pathways. As an example, PKMζ maintains late-LTP by persistently favoring AMPA receptor insertion into postsynaptic sites (41). An enhanced RyR3 expression, such as that produced by BDNF and training, could complement this pathway because RyR3 knockout mice display diminished LTP and decreased AMPA receptor activity but normal receptor traffic (42).

Taken together, our results support the hypothesis that RyR-mediated CICR contributes to postsynaptic signaling mechanisms responsible for inducing the expression of key genes for long-term memory storage. In response to activity-dependent BDNF-mediated signaling, newly synthesized RyR could generate the strong Ca2+ signals needed to initiate and maintain permanent changes during long-term memory storage. Together with PKMζ, the calcium-sensitive ERK/CREB pathway is critical for both memory consolidation and long-term neuronal plasticity processes (43) and represents an attractive downstream candidate for the RyR-dependent signaling events produced by BDNF.

Materials and Methods

Adult male (2.5 mo old) or embryonic (day 18) Sprague-Dawley rats were used throughout this work. Primary cultures prepared from hippocampus dissected at embryonic day 18 (25) were used at 14 DIV unless specified otherwise. All

![Fig. 5. Hippocampal ryanodine injection improves spatial memory formation. (A) [3H]-Ryanodine (10 nmol per hemisphere) was administered bilaterally intrahippocampus. Radioactivity was determined in different brain regions 24 h later, and ryanodine concentrations (mean ± SE, n = 3) were calculated from tissue weight, assuming a density of 1.0. (Inset) Photomicrograph of a representative coronal brain section showing the injection track ending in the dorsal hippocampus CA1 region; M.O.: Medulla Oblongata. (B) Ryanodine (10 nmol per hemisphere) or sham was injected as above 24 h before training. Escape latency values (mean ± SE) were measured daily during training; *P < 0.05, determined by two-way ANOVA. (C) Time spent in Quadrant 4 and Zone B respect to sham, measured at day 9 (mean ± SE; n ≥ 6). *P < 0.05, determined by unpaired Student’s t test.](image)
protocols were approved by the Bioethics Committee, Faculty of Medicine, Universidad de Chile. Before BDNF (Millipore Corp.) addition, some cultures were preincubated for 1 h with 20 μM recombinant human TrkB-βc Chimera (TrkB-FC; R&D Systems), 50 μM ryano dine (Alexis Corp.), or 1.5 μM Actinomycin D (ActD; Sigma). Total RNA isolation, real-time quantitative PCR (qRT-PCR) and Western blot analysis using antibodies against RyR2 (Affinity Bioreagents), RyR3 (Chemicon), or PKMz (Zymed) were performed as described elsewhere (25). Amplification was performed using the primers and conditions detailed in Table S1. Immunocytochemistry assays were carried out as described in detail elsewhere (18, 25). Brain tissue fixation and immunocytochemistry was performed as described (44), except that coronal slices were incubated at 4 °C overnight with anti-RyR3 antibodies. For further details on Ca2+ signal detection as Fluo-4 fluorescence and morphological analysis of dendritic spines, see SI Materials and Methods.

Water Maze Training. Rats were trained in a circular water maze for 1-min periods three times daily for 6 consecutive days, followed by 2 d off; 1 additional day without platform was added to assess memory retention. The hippocampus was removed 6 h after the last session at day 9. Control animals were subjected for 4 consecutive days to three swimming trials daily with the cued platform; the platform location varied daily. At day 4, the whole hippocampus was removed 6 h after the last trial. To assess spatial acuity, the pool was subdivided by imaginary lines into four equal quadrants and three equidistant concentric zones (A–C); the hidden platform was located in quadrant 4, zone B. The escape latency during each trial and the percentage of permanence in specific regions of the pool was assessed with a software-coupled video camera (HVS imager). Further details are provided in SI Materials and Methods.

Ryano dine and BDNF Injection. Rats under anesthesia were bilaterally injected in the dorsal CA1 region of the hippocampus with 2 μL of 5 mM ryano dine or [1H]-ryano dine, or with 0.25 μg BDNF. Ryano dine injection was performed 96 or 24 h before training; sham controls were injected with 2 μL of saline. Radioactivity was determined 24 h or 96 h after injection in different brain regions as detailed in SI Materials and Methods.

Statistical Analysis. Results are expressed as mean ± SE. Significance was evaluated with the GraphPad Software, using the unequal Student’s t test or ANOVA analysis followed by Newman-Keuls posttest, unless specified otherwise.

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