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Pim kinase expression is induced by LTP stimulation and required for the consolidation of enduring LTP

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In animals and several cellular models of synaptic plasticity, long-lasting changes in synaptic strength are dependent on gene transcription and translation. Here we demonstrate that Pim-1, a serine/threonine kinase closely related to Pim-2 and Pim-3, is induced in hippocampus in response to stimuli that evoke long-term potentiation (LTP). Mice deficient for Pim-1 show normal synaptic transmission and short-term plasticity. However, they fail to consolidate enduring LTP even though Pim-2 and Pim-3 are constitutively expressed in the hippocampus and Pim-3 expression is similarly induced by synaptic activity. Thus, expression of Pim-1 is required for LTP. Its level of expression and, consequently, its capacity to phosphorylate target proteins in dendritic and nuclear compartments of stimulated neurons might be a determining factor for the establishment of long-lasting changes in synaptic strength.
Keywords: gene induction/knockout/LTP/Pim kinase/synaptic plasticity

Introduction

Neurons have the capacity to undergo activity-dependent changes in their molecular composition and structure in order to adjust their synaptic strength. Such synaptic plasticity appears to contribute to a variety of physiological and pathological processes in the adult brain, including learning and memory, epileptogenesis, drug abuse and neurological diseases (Nestler and Aghajanian, 1997; Kuhl and Skehel, 1998; Milner *et al*., 1998). The cellular and molecular mechanisms underlying synaptic plasticity have been studied intensively in the context of memory storage. Long-term potentiation (LTP) is an activity-dependent and persistent enhancement of synaptic efficacy that may underlie certain forms of long-term memory (Bliss and Collingridge, 1993). Like memory, LTP can exist in both short- and long-term forms. Short-term forms of LTP (early LTP) appear to rely on the covalent modifications of pre-existing proteins, and several protein kinases have been shown to contribute to these processes (Grant, 1994; Grant and Silva, 1994; Huang *et al*., 1996; Roberson *et al*., 1996). In contrast, enduring forms of LTP (late LTP) require RNA and protein synthesis and are associated with the establishment of new synaptic connections (Bailey *et al*., 1996). Functional plasticity might therefore be achieved by activity-dependent changes in the expression of specific genes (Goelet *et al*., 1986; Curran and Morgan, 1987; Sheng and Greenberg, 1990; Kuhl, 1999). Examples of these include transcription factors (Morgan *et al*., 1987; Saffen *et al*., 1988), as well as a small set of proteins that have the potential to transduce synaptic activity directly into immediate changes of neuronal function (Nedivi *et al*., 1993; Qian *et al*., 1993; Yamagata *et al*., 1993; Link *et al*., 1995; Lyford *et al*., 1995; Frey *et al*., 1996; Brakeman *et al*., 1997).

In the studies described here, we identify the proto-oncogene *Pim-1* as a gene that is induced rapidly by plasticity-producing stimulation in the brain and is instrumental in the formation of enduring LTP. Previously, Pim-1 (provirus integration site for Moloney murine leukemia virus) and Pim-2 were identified as serine/threonine kinases with oncogenic potential in cells of different hematopoetic lineages (Jonkers and Berns, 1996; Berns *et al*., 1999). In addition, by homology screening, we identified a third member of this family, Pim-3. We find that the Pim kinases differ in their regional expression in the brain and their responses to physiological and pathological forms of synaptic activity. Whereas Pim-2 is unresponsive to synaptic activity, the expression of both Pim-1 and Pim-3 is induced by such stimulation. Pim-1 induction is already detectable following weak activity, while that of Pim-3 requires higher intensity stimulation. Notably, Pim-1 RNA and protein are virtually undetectable in the unstimulated hippocampus but strongly induced by synaptic activity, the protein being distributed to the nuclei and dendrites of activated neurons. Pim-1 was induced consistently with LTP-producing stimulation, suggesting that it may be required for this potentiation. To examine the functional role of the Pim-1 protein kinase in LTP, we analyzed genetically engineered mice deficient for Pim-1. These animals develop normally, are fertile, have a normal life span (Laird *et al*., 1993), and our histological analysis does not reveal any gross anatomical abnormalities in hippocampus or other regions of the brain. These mice show normal forms of short-term plasticity, but have lost the ability to establish enduring LTP. Therefore, Pim-1 is a nuclear and dendritically localized protein kinase, the expression of which is both induced by and required for LTP. Such properties set Pim-1 apart from other kinases previously implicated in LTP.

Results

cDNA sequences of the Pim kinases

To identify the components of the genetic program that underlies long-term synaptic plasticity, we developed a

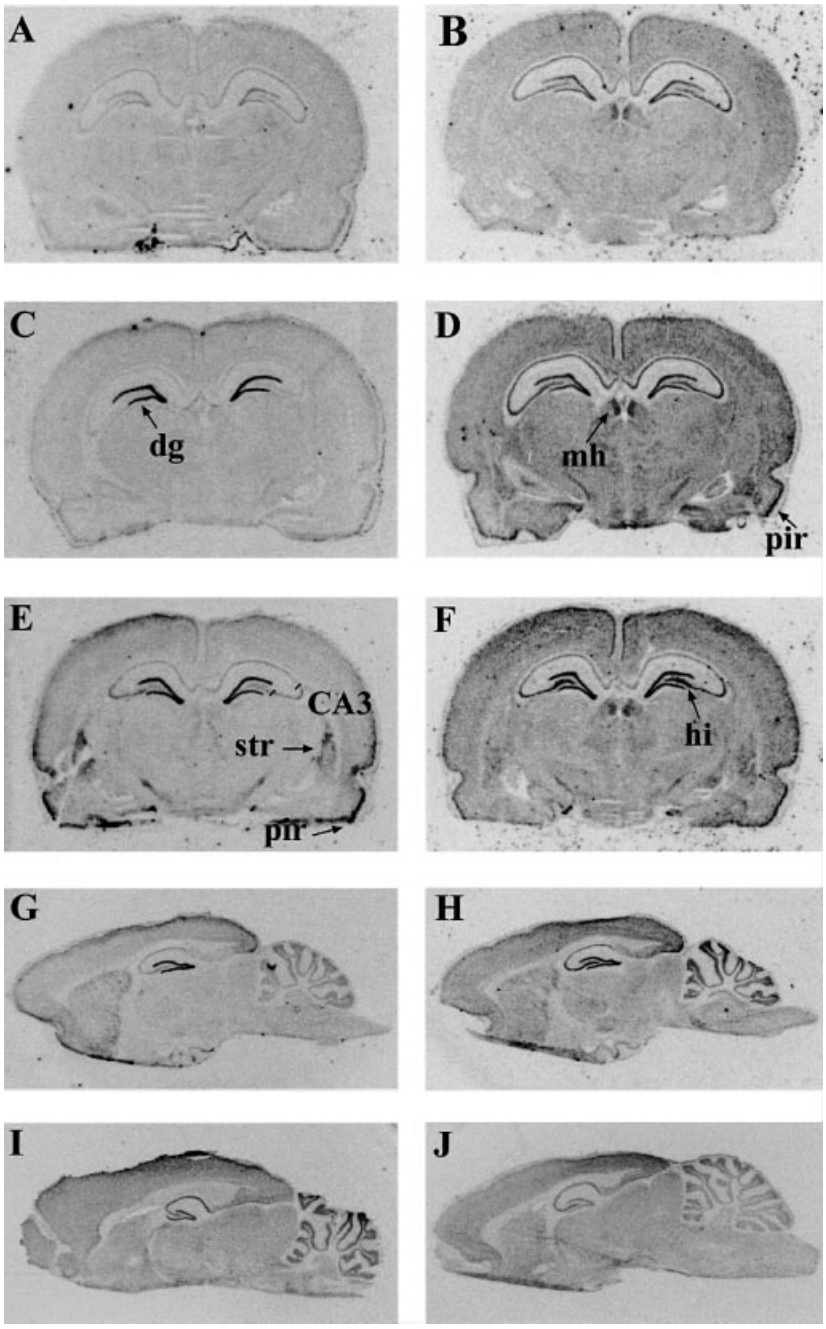

Fig. 1. Comparative analysis of brain Pim-1, Pim-2 and Pim-3 mRNA levels before and after seizure. Coronal and sagittal sections were assayed for Pim-1 mRNA (**A**, **C**, **E** and **G**), Pim-2 mRNA (**I** and **J**) and Pim-3 mRNA (**B**, **D**, **F** and **H**) using *in situ* hybridization with gene-specific antisense probes. (A, B and I) Control rat; (C and D) rat sacrificed 1 h after PTZ-induced seizure; (E, F, G, H and J) rat sacrificed 4 h after KA-induced seizures. Pim-1 and -3 mRNA levels following seizures were quantified in independent Northern analyses of RNA harvested at different time points from cortices and hippocampi of control and PTZ seizure rats (data not shown). dg, dentate gyrus; mh, medial habenula; pir, piriform cortex; CA3, CA3 field of the hippocampus; str, striatum; hi, hilus.

subtractive cloning methodology that overcomes many of the problems encountered with conventional, as well as PCR-based strategies (Konietzko and Kuhl, 1998). Using this technology for identification of induced transcripts and a pentylenetetrazole (PTZ) seizure as stimulus, in the present study we detected Pim-1 to be induced with synaptic activity in the brain. Pim-1 and the closely related Pim-2 are serine/threonine-specific protein kinases that play important roles in lymphomagenesis (Cuypers *et al*., 1984; Selten *et al*., 1986; van Lohuizen *et al*., 1989; van der Lugt *et al*., 1995). Using a homology screen, we have identified a third cDNA of 2335 bp encoding a protein, designated Pim-3, that shares 69 and 53% identity with rat Pim-1 and mouse Pim-2, respectively (DDBJ/EMBL/GenBank accession No. AF086624). Notably, Pim-3 is significantly more closely related to Pim-1 (69%) than is Pim-2 (53%). All serine/threonine kinase-specific residues are conserved in Pim-3, and the recombinant protein displays a Zn^{2+}-sensitive autophosphorylation activity (not shown) similar to that of Pim-1 and -2 (Saris *et al*., 1991). Recently, an apparently identical gene, induced in PC12 cells by forskolin treatment, has been reported and termed

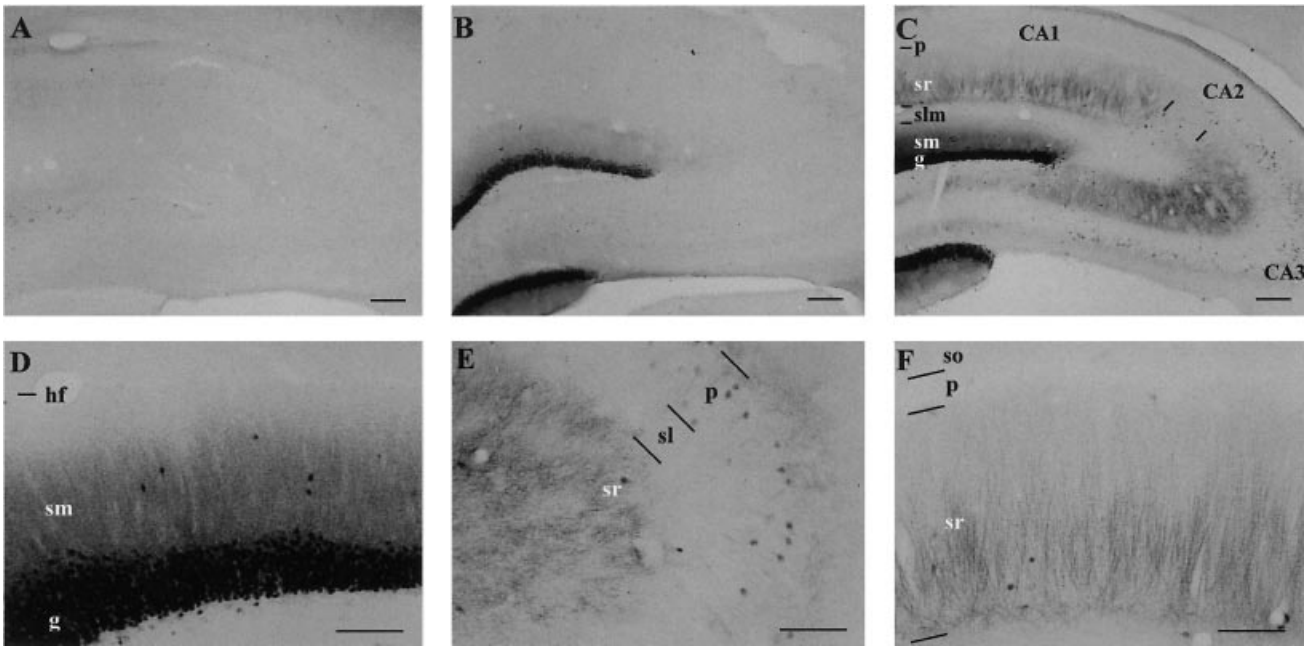

Fig. 2. Pim-1 protein is localized to neuronal soma and dendrites of stimulated hippocampal neurons. Hippocampus, cortex, piriform cortex and striatum of control and PTZ- or KA-stimulated rat brain (4 h survival). (**A**) In control hippocampus, a few somata and dendrites show immunoreactivity. (**B**) After PTZ-induced seizure, Pim-1 immunoreactivity is increased dramatically in the granule and molecular layer of the dentate gyrus. (**C**) After KA-induced seizures, immunostaining can also be detected in region CA1 and CA3 with prominent staining of dendritic processes. (D–F) High power views of Pim-1 immunoreactivity after KA-induced seizures in (**D**) granule cells of the dentate gyrus, (**E**) hippocampal field CA3 and (**F**) hippocampal field CA1. Sections from the same animal incubated with a serum depleted of Pim-1-specific antibodies had no staining (not shown). Scale bars: (A–C) 200 μm; (D–F) 100 μm. g, granular layer; hf, hippocampal fissure; p, pyramidal cell layer; sl, stratum lucidum; slm, stratum lacunosum moleculare; sm, stratum moleculare; so, stratum oriens; sr, stratum radiatum.

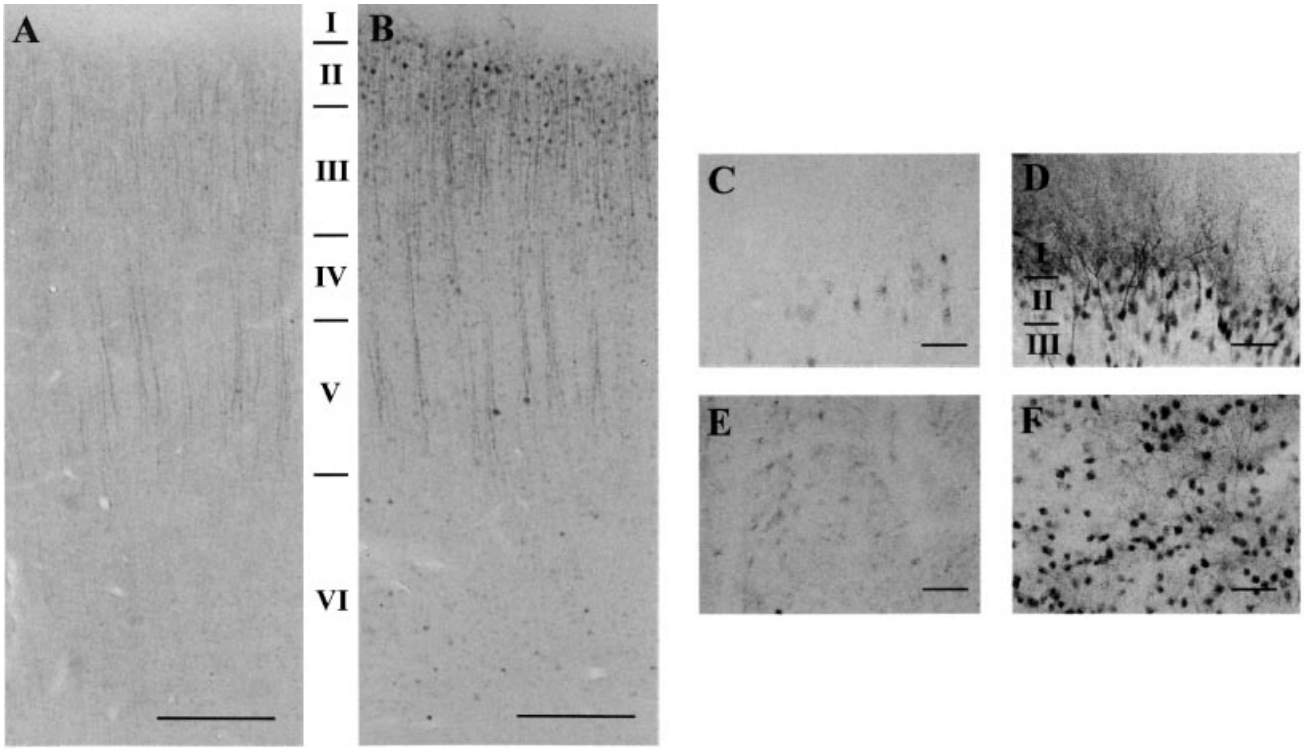

Fig. 3. Pim-1 protein is localized to neuronal soma and dendrites of stimulated cortical and striatal neurons. (**A**) Parietal cortex of a control rat, representative of all neocortical areas. Pim-1 immunoreactivity is evident in the long apical dendrites of layer V pyramidal cells and in layers II/III. (**B**) After KA-induced seizures, immunostaining is increased in cortical layers II/III. (**C**) Weak immunostaining is seen in the piriform cortex under control conditions. (**D**) KA seizures induce strong immunostaining in deep and superficial pyramidal cells and apical dendrites within layer I. (**E**) Neurons of the striatum under control conditions and (**F**) after KA-induced seizures. Immunostaining is localized to both somata and dendrites. Scale bars: (A and B) 200 μm; (C–F) 50 μm. Roman numerals indicate layers in the parietal and piriform cortex.

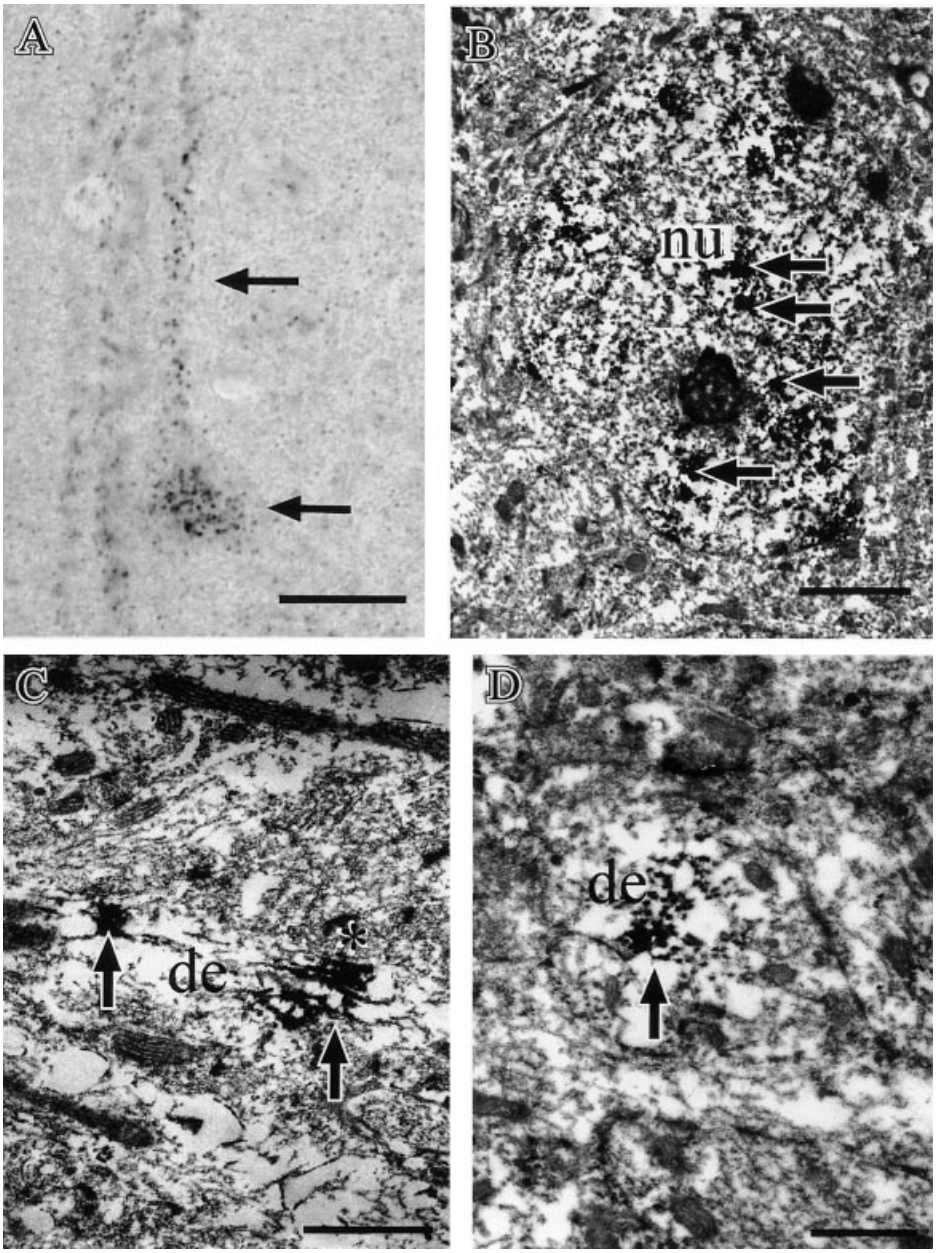

Fig. 4. Pim-1 protein is localized to the nucleus and the microtubles of dendrites in activated neurons. (**A**) High power view of a layer V pyramidal cell. Immunoreactivity is accumulated in discrete patches along the perimeter of the dendrite; in the soma, immunoreactivity is localized in the nucleus. (B–D) Electron photomicrographs show the subcellular localization of Pim-1 immunoreactivity. (**B**) Nucleus of a granule cell of the dentate; arrows indicate immunoreactivity. (**C** and **D**) Patches of immunostaining (arrows) are visible in close apposition to microtubules in granule cell dendrites cut on (C) longitudinal and (D) oblique planes; the asterisk denotes a presynaptic bouton in close proximity to dendritic immunoreactivity in (C). Scale bars: (A) 20 μm; (B) 2 μm; (C and D) 1 μm. de, dendrite; nu, nucleus.

Kid-1 (Feldman *et al*., 1998b). However, given the structural and biochemical similarities of the proteins, we propose that the name *Pim-3* is more appropriate. In addition, other features such as the presence of a CUG initiation codon upstream of the Kozak consensus sequence are shared between *Pim-3*, *Pim-1* and *Pim-2* (Saris *et al*., 1991; van der Lugt *et al*., 1995).

Levels of the Pim mRNAs are differentially regulated by neuronal activity

In situ hybridization analysis demonstrated that Pim-1, Pim-2 and Pim-3 differ in their regional expression patterns and responses to different intensities of synaptic activity in the brain (Figure 1). Constitutive expression of Pim-1 is low (Figure 1A), whereas Pim-3 is expressed at moderate levels throughout the brain (Figure 1B). Following a PTZ-induced seizure, Pim-1 is strongly induced in the dentate gyrus within 1 h (Figure 1C); Pim-3 is induced more broadly (Figure 1D), but to a lesser extent. Increases in Pim-1 and Pim-3 expression follow a similar time course, being observable for at least 4 h and returning to baseline levels by 10 h after the seizure (not shown). In contrast to the effects of PTZ, kainic acid (KA)-induced seizures develop more slowly and recur for several hours. This

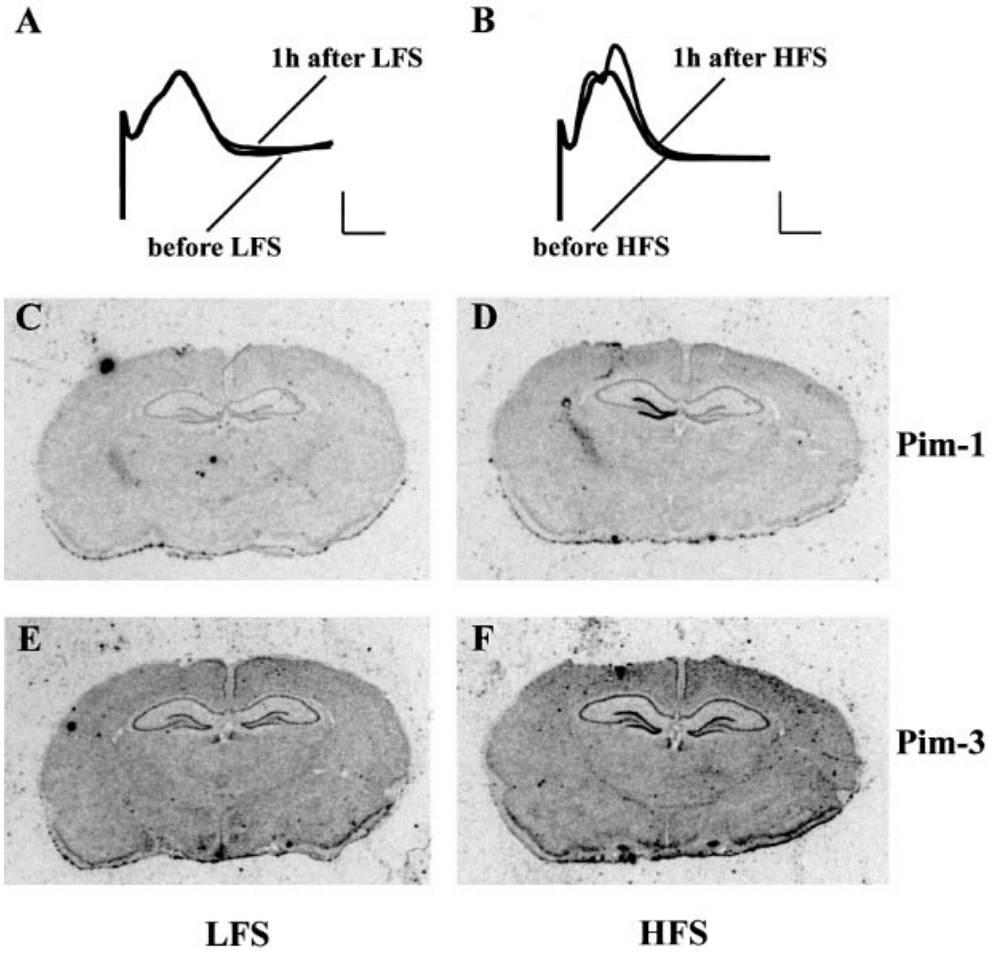

Fig. 5. Induction of LTP and Pim mRNA in dentate gyrus granule cells in freely moving rats. Coronal sections were assayed for Pim-1 and Pim-3 mRNA using *in situ* hybridization with gene-specific antisense probes. (**A** and **B**) Superimposed field potentials before and 1 h after (A) low frequency stimulation (LFS) and (B) high frequency stimulation (HFS) showing the induction of LTP with the latter. (**C** and **D**) Pim-1 RNA levels 1 h after unilateral application of (C) LFS or (D) HFS. (**E** and **F**) Pim-3 RNA levels 1 h after unilateral application of (E) LFS or (F) HFS. Scale bar in (A and B) is 5 mV and 2 ms.

stronger seizure episode resulted in incremental Pim-1 mRNA levels in the piriform cortex, striatum, the dentate gyrus hilus, and hippocampal CA1 and CA3 stratum pyramidale (Figure 1E and G, see also Figure 2); Pim-3 mRNA levels were augmented further in the hilus only (Figure 1F and H). Similar effects of KA were reported recently by Feldman *et al*. (1998a,b). Neither PTZ nor KA affected Pim-2 expression, which remained at moderate levels throughout the brain (Figure 1I and J). These results demonstrate that the three Pim kinase genes are expressed by distinct but overlapping populations of neurons and are differentially regulated by neuronal activity in brain. Seizure effects on Pim kinase expression were not influenced by adrenalectomy (not shown), thereby demonstrating that corticosterone released during the seizure episode (Sun *et al*., 1993) does not account for the changes in gene expression described here.

Pim-1 protein is induced rapidly and enriched in nuclei and dendrites of activated neurons

The fold of induction in mRNA following neuronal activity was highest for Pim-1. We therefore decided to focus in the following on Pim-1, and determined whether the strong induction of Pim-1 mRNA following seizure results in a corresponding increase in Pim-1 protein. The distribution of Pim-1 immunostaining precisely reflected its mRNA expression (Figures 2 and 3). In hippocampus of control rats, Pim-1 immunoreactivity was localized to a few scattered somata and, at low basal levels, in dendritic layers (Figure 2A). Following PTZ-induced seizures, the dentate gyrus granule cell somata and dendrites were densely immunoreactive (Figure 2B). Recurrent KA-induced seizures additionally increased Pim-1 immunostaining in CA1, CA3 and the hilus, but not in field CA2 (Figure 2C–F). In the hippocampus proper of KA-treated rats, immunoreactivity was most dense within apical dendrites in the stratum radiatum and scattered somata in field CA3 (Figure 2E and F). Pim-1 immunoreactivity was low within the majority of pyramidal cell somata, the stratum oriens containing pyramidal cell basal dendrites and the lucidum containing the mossy fibers and the proximal apical dendrites of regio inferior pyramidal cells. In neocortex, Pim-1 immunoreactivity was present at low levels in apical dendrites of layers II/III and V pyramidal cells in control rats and was increased by seizures in layers II/III (Figure 3A and B). In piriform cortex,

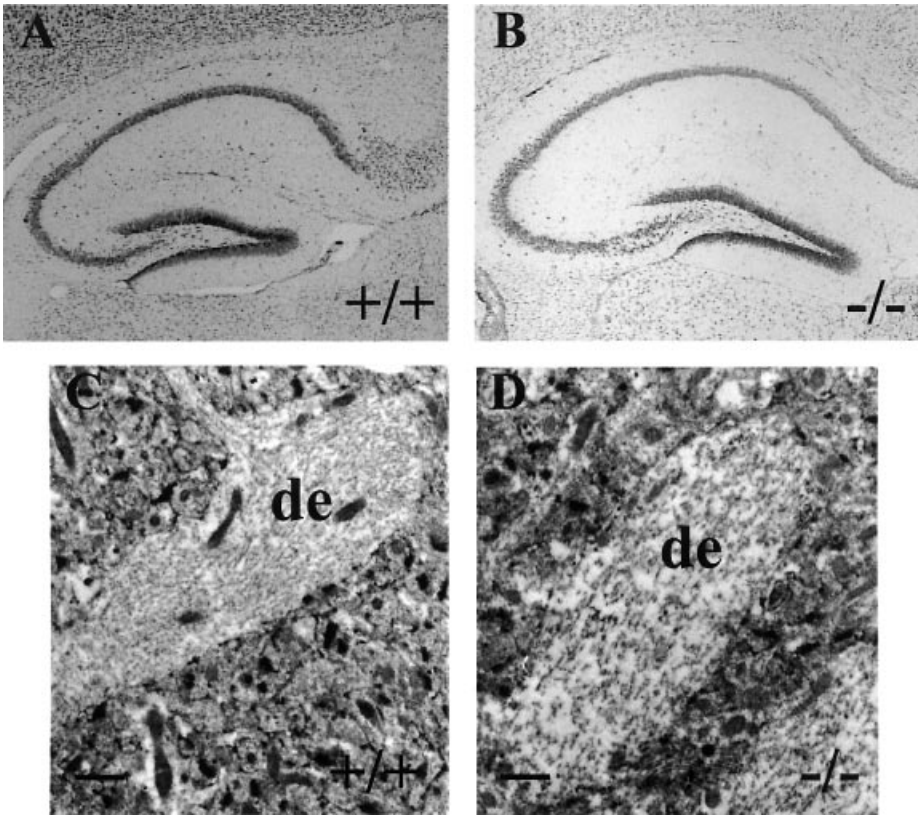

Fig. 6. Pim-1 knockout mice exhibit no obvious morphological abnormalities. Light and electron microscopic photomicrographs of tissue from wild-type (+/+) and Pim-1 knockout (–/–) mice. (**A** and **B**) Nissl-stained sections through the hippocampus of (A) wild-type and (B) Pim-1 knockout mice. The dentate gyrus and hippocampus proper show no gross structural abnormalities. (**C** and **D**) Oblique sections through granule cell dendrites of (C) wild-type and (D) Pim-1 knockout mice showing comparable ultrastructure. No morphological abnormalities of the microtubules or other structures were apparent. Scale bar in (C) and (D) is 1 μm. de, dendrite.

immunostaining was strongly induced in neuronal perikarya of layers II/III and their dendrites within layer I (Figure 3C and D). Strong seizure induction was also observed in the majority of striatal neurons (i.e. probable GABAergic projection neurons innervated by cortex) (Figure 3E and F); and in the medial, basolateral and cortical nuclei of the amygdala (not shown). In all areas, Pim-1 immunoreactivity was enriched most prominently in dendrites, with immunostaining localized to puncta decorating the perimeter of the processes (Figure 4A). In addition, immunostaining was detected within neuronal nuclei (Figure 4A and B). At the ultrastructural level, the reaction product appeared to be closely associated with microtubular structures (Figure 4C and D). This association was not uniform along microtubles but occurred in a punctate pattern, similar to that seen at the light microscopic level.

Pim-1 mRNA level is increased by LTP-producing stimulation

LTP can be induced at synapses within the hippocampus by high frequency orthodromic stimulation (Bliss and Collingridge, 1993). Induction of LTP is accompanied by increases in Pim kinase expression (Figure 5). Granule cells of the adult hippocampus were stimulated synaptically by activating their major afferent projection from the entorhinal cortex using a chronically implanted stimulating electrode (Staubli and Scafidi, 1997). Stimulation of the perforant path at the intensity required to produce a population spike, when administered at low frequency, did not result in LTP or an increase in Pim-1 or Pim-3 mRNA levels (Figure 5A, C and E). In contrast, when LTP was evoked in the granule cells by delivering the same intensity stimuli at high frequency, Pim-1 but not Pim-3 was induced consistently in the ipsilateral dentate gyrus (Figure 5B, D and F). Pim-1 RNA was induced in each of four rats sacrificed 1 h after the LTP stimulation, whereas induction of Pim-3 was weak (one animal) (Figure 5F) or not detectable (three animals, not shown). Similarly, different thresholds for the activation of immediate early genes have been demonstrated. For example, c-*fos*, which was not induced by the stimulation parameters used here (not shown), can be induced when stimulation intensity is increased and more persistent forms of LTP are generated (Abraham *et al*., 1993; Worley *et al*., 1993).

Pim-1 expression is required for the consolidation of enduring LTP

LTP-producing stimulation results in a more dramatic increase in Pim-1 expression than that of Pim-3. To determine whether this induction has a functional role in this form of plasticity, we analyzed genetically engineered mice deficient for Pim-1. These animals develop normally,

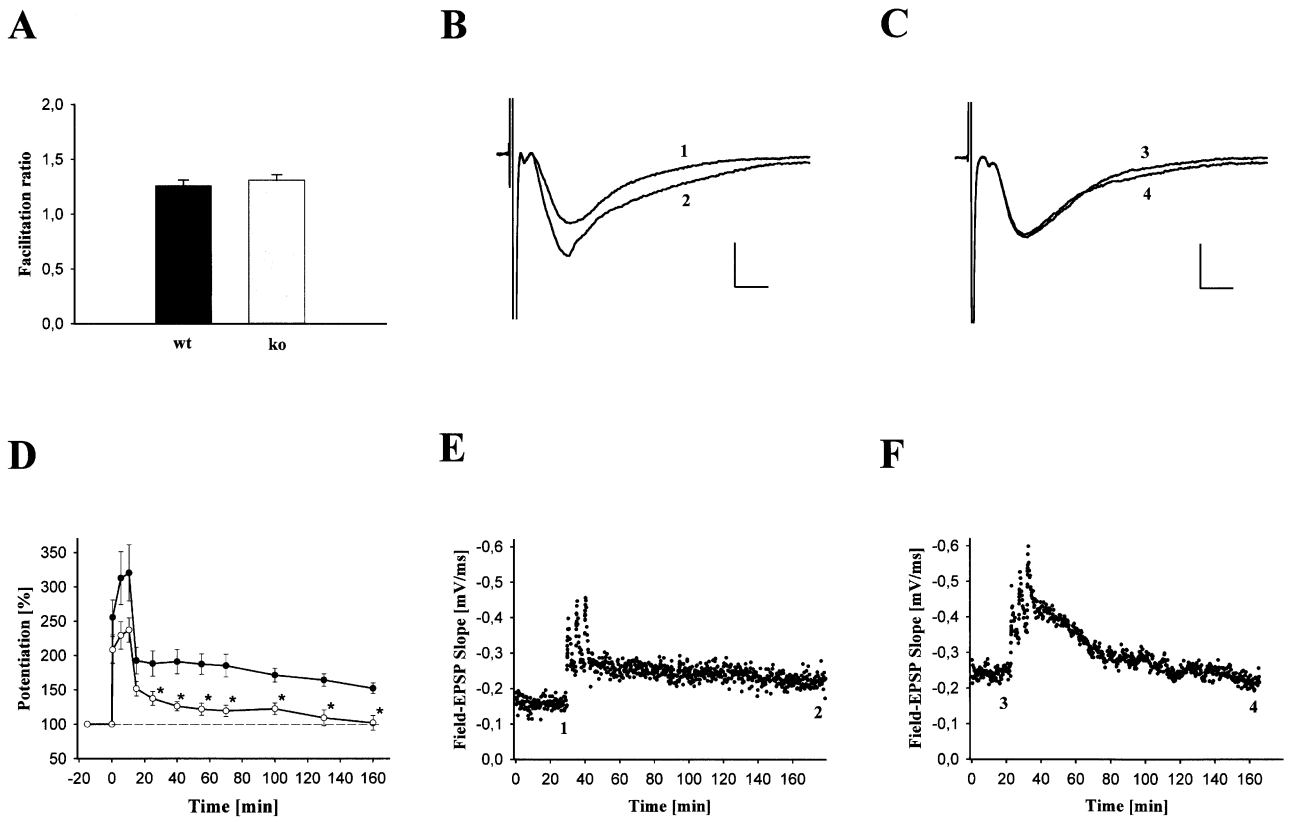

Fig. 7. Electrophysiological analysis of hippocampal slices reveals rapidly decaying LTP in Pim-1 knockout mice. Extracellular field potentials were recorded in the stratum radiatum of field CA1. (**A**) Paired pulse facilitation calculated from the ratio of the second fEPSP slope to the first fEPSP slope at an interpulse interval of 100 ms. Wild-type (wt) mice (n = 10 inputs) and knockout (ko) mice (n = 12 inputs) did not differ significantly in their facilitation ratio. (**B**) Sample fEPSP traces before and 2.5 h after the induction of LTP in wild-type mice taken from the experiment shown in (E). (**C**) Sample fEPSP traces at similar time points in Pim-1 knockout mice taken from the experiment shown in (F). Scale bars in (B) and (C), 0.4 mV and 5 ms. (**D**) Summary graph of mean fEPSP slope measurements up to 2.5 h after the induction of LTP with three 100 Hz tetani. Potentiation is expressed as a percentage of baseline recordings prior to tetanization. The fEPSP slope potentiation of wild-type (n = 7 slices, ●) and knockout (n = 6 slices, ○) mice is plotted against time, with the first tetanus assigned to the zero time point (error bars denote SEM). The first three time points after tetanization refer to the strongest fEPSP response after each tetanus (post-tetanic potentiation). For the following time points, the average of seven traces from each experiment was used for analysis. Potentiation at the time points marked by an asterisk differs significantly between wild-type and knockout mice (p <0.05, Students t-test). In knockout mice, LTP is severely impaired. (**E**) Original recording from a wild-type animal shows stable potentiation of the fEPSP slope after the application of three tetani. Numbers indicate the time points of the traces shown in (B). (**F**) A similar recording from a knockout animal shows intact post-tetanic potentiation and a marked decay in the potentiation of the fEPSP. Numbers indicate the time points of the traces shown in (C).

are fertile and show a normal life span (Laird *et al*., 1993). Histological analyses at the light and electron microscopic levels did not reveal gross structural abnormalities in hippocampus or other regions of the brain (Figure 6A–D). These mice displayed stable baseline synaptic transmission that did not differ from wild-type controls. As an index of short-term plasticity, we measured paired pulse facilitation which is sensitive to presynaptic release probability (Manabe *et al*., 1993) (Figure 7A). The extent of facilitation obtained in field CA1 by delivering two pulses at an interval of 100 ms was indistinguishable between wild-type (1.26 ± 0.05, n = 10 pathways, seven slices from four mice) and mutant mice (1.31 ± 0.05, n = 12 pathways, six slices from three mice). In addition, post-tetanic potentiation, another form of short-term plasticity that was induced by 100 Hz stimulation of the Schaffer collaterals, was not significantly different between wild-type and mutant animals (for example, see Figure 7E and F). Because our results suggested that Pim-1 might play a role in longer lasting forms of synaptic plasticity such as LTP, we next explored the effects of repeated tetanization on the Schaffer collateral pathway in Pim-1 knockout mice. With three 100 Hz trains (1 s duration, spaced 5 min apart), slices from wild-type mice (n = 7 slices from four mice) showed robust LTP that lasted for at least 3 h (Figure 7B, D and E): mean field excitatory postsynaptic potential (fEPSP) slopes were 192 ± 19%, 188 ± 18%, 172 ± 10% and 152 ± 8%, at 5, 15, 90 and 150 min post-tetanization, respectively. In contrast, the same stimulation regimen applied to slices obtained from mutant mice resulted in a rapidly declining potentiation that disappeared within 2.5 h (Figure 7C, D and F). The corresponding values for Pim-1 knockout mice (n = 6 slices from three mice) were 152 ± 11% (p >0.1), 137 ± 10% (p <0.05), 123 ± 9% (p <0.01) and 102 ± 11% (p <0.01). The potentiation observed in slices from mutant animals was significantly below that obtained in wild-types at 2.5 h, and this significance first appears at 15 min post-tetanization (p <0.05; Student's t-test).

Discussion

Members of the Pim family of serine/threonine kinases were identified originally as cellular genes frequently

activated by integrated proviruses in T cell lymphomas (Jonkers and Berns, 1996). Accordingly, mice deficient for Pim-1 have been examined for defects in the hematopoetic system, although only subtle phenotypic changes have been reported (Domen *et al*., 1993a,b). This lack of an apparent phenotype for Pim-1 knockout mice has been taken as an argument for functional redundancy of the Pim kinases in hematopoetic cell lineages. This does not seem to be the case in neurons.

The present study demonstrates that in brain, Pim-1, -2, and -3 differ in their regional expression patterns and their responses to synaptic activity. In the brains of control animals, Pim-2 and Pim-3 are widely expressed at levels higher than those for Pim-1. While Pim-2 is unresponsive to synaptic activity, the expression of Pim-1 and Pim-3 is induced by the intense activity associated with seizures. However, only Pim-1 was induced consistently with LTP-producing stimulation, suggesting that it may be required for this potentiation. Furthermore, mice deficient for Pim-1 are not able to sustain enduring LTP at the Schaffer collateral–CA1 pyramidal cell synapse despite apparently normal synaptic transmission.

The attenuation of LTP in mutant mice was already apparent at 15 min after the tetanus, and a complete loss of potentiation was observed after 2.5 h. A similar time course of decremental LTP is seen when protein kinases are inhibited by pharmacological or genetic means, where potentiation usually persists only for 30–60 min (Grant, 1994; Grant and Silva, 1994; Huang *et al*., 1996; Roberson *et al*., 1996; Abel *et al*., 1997). The time course of LTP decay in Pim-1 knockout animals is faster than that observed in the presence of some protein synthesis inhibitors which reportedly attenuate only the late phase of LTP occurring at 3–6 h after stimulation (Krug *et al*., 1984; Frey *et al*., 1988; Otani and Abraham, 1989; but see Osten *et al*., 1996). This may indicate a requirement for a basal level of Pim-1 expression in the inductive or early phases of LTP. In this situation, the function of induced levels of Pim-1 expression at later time points of LTP would remain to be determined. However, anisomycin, the inhibitor used in several of these studies, is a potent stimulator of MAP kinases (Hazzalin *et al*., 1998). In view of recent reports that MAP kinase is an important factor in the induction of LTP in hippocampus (English and Sweatt, 1997) and long-term facilitation in *Aplysia* (Martin *et al*., 1997), it is possible that a requirement for protein synthesis at earlier time points may be surpressed by anisomycin stimulation of MAP kinases. Moreover, other studies using emetine, cycloheximide or puromycin to block protein synthesis show severe attenuation or complete inhibition of LTP by 30–100 min after stimulation, suggesting a requirement for protein synthesis at earlier time points (Stanton and Sarvey, 1984; Deadwyler *et al*., 1987; Nguyen and Kandel, 1996). In any event, our results indicate that basal and/or induced Pim-1 expression plays a critical role in the formation of enduring LTP.

What role might this be? Several studies in non-neuronal cells have demonstrated the involvement of Pim-1 in promoting cell proliferation (Domen *et al*., 1993a,b; Buckley *et al*., 1995; Yip-Schneider *et al*., 1995; Liang *et al*., 1996). Although cell proliferation may occur in a small subpopulation of neurons (Kempermann *et al*., 1997; Parent *et al*., 1997), the majority of cells in which we detect Pim-1 induction are post-mitotic. Stimuli that induce seizures and LTP have been associated with activation of gene expression cascades (Kuhl, 1999) and structural changes of activated neurons (Bailey and Kandel, 1993). Pim-1 might contribute directly to both of these processes, as we detect Pim-1 in the nucleus and dendrites of activated neurons. In the nucleus, Pim-1 is likely to activate components of the transcriptional machinery, for example Pim-1 can associate with the transcriptional coactivator p100 to stimulate c-Myb transcriptional activity (Leverson *et al*., 1998). Throughout dendrites, Pim-1 appears to be associated with microtubules. Pim-1 might, therefore, contribute to activity-induced structural rearrangements by phosphorylating microtubules or microtubule-associated proteins. Indeed, phosphorylation of microtubule-associated proteins is regulated by *N*-methyl-D-aspartate (NMDA) receptor activation and may transduce neuronal activity into modifications in dendritic structure (Bading and Greenberg, 1991; Quinlan and Halpain, 1996; Sanchez *et al*., 1997).

More generally, the patchy distribution of Pim-1 in the dendrite is typical of proteins and RNAs targeted to the region of synaptic structures (Sheng and Kim, 1996; Kuhl and Skehel, 1998). Thus, Pim-1 may be involved in phosphorylation of synaptic proteins or fulfill a role in the dendritic transport process itself. In hematopoietic cells, Pim-1 is proposed to play a role in the cross-talk between interleukin and growth factor receptors (Berns *et al*., 1998). Upon its induction by interleukins, it might affect the efficiency of growth factor signaling and in that fashion serve as a gain setter for growth factor responses. In the light of studies on the effects of peptide growth factors on synaptic plasticity (Kang and Schuman, 1995; Korte *et al*., 1995), one may speculate that Pim-1 might fulfill a similar integrative function in neurons.

Both serine/threonine- and tyrosine-specific protein kinases have been implicated in the induction and maintenance of LTP (Grant, 1994; Grant and Silva, 1994; Huang *et al*., 1996; Roberson *et al*., 1996). It has also been proposed that post-translational modifications altering the activity of kinases and kinase-regulating proteins may act in the transfer of a short-lived LTP to longer lasting potentiations (Schwartz, 1993; Lisman, 1994). While Pim-1 can be modified post-translationally by autophosphorylation, the most remarkable feature of Pim-1 regulation is its dramatic induction at the mRNA and protein level in response to synaptic activity. Thus, the history of a neuron's synaptic activity is reflected in the expression level of this kinase. Such mnemonic properties, its localization to the nucleus and dendrites, and the demonstration of its requirement for the consolidation of enduring LTP indicate that Pim-1 may play a pivotal role in regulating the functional changes underlying long-term synaptic plasticity.

Materials and methods

Animal preparation and treatments

Adult male Sprague–Dawley rats, 129/Ola wild-type mice and Pim-1 knockout mice, derived from male 129/Ola ES cells (te Riele *et al*., 1990; Domen *et al*., 1993a,b), and backcrossed with 129/Ola mice for obtaining Pim-1 knockout inbred mice were used. PTZ [in phosphate-

buffered saline (PBS) 50 mg/kg], KA (in PBS, 10 mg/kg) and cycloheximide [CHX in dimethylsulfoxide (DMSO), 120 mg/kg] were administered by i.p. injection. Animals that received CHX and PTZ were injected with CHX 0.5 h prior to PTZ. Control animals were injected with similar volumes of isotonic saline. All animals were sacrificed by decapitation at the appropriate times. For the isolation of RNA, the brain was removed after decapitation and transferred into ice-cold PBS. Cortices and hippocampi were dissected, frozen in liquid nitrogen and stored at –75°C. For *in situ* hybridization, the brains were removed, frozen on dry ice and stored at –75°C. For immunohistochemistry, animals under deep anesthesia were perfused transcardially with 4% paraformaldehyde, for electron microscopic immunohistochemistry, the perfusate additionally contained 0.5% glutaraldehyde. Brains were dissected out and stored in the same fixative overnight.

Molecular cloning

A subtracted cDNA library strongly enriched for activity-regulated genes was constructed as described in detail (Konietzko and Kuhl, 1998). The cDNA inserts of this library were analyzed in differential Southern blots (reverse Northern blots) using virtual, PTZ/CHX-induced and control cDNA population probes as described (Konietzko and Kuhl, 1998). One of the differential hybridizing cDNAs was identical to *Pim-1*. The entire coding region of *Pim-1* was obtained in an RT–PCR using PTZ/CHX-induced rat hippocampal RNA, primers according to the published sequence (Wingett *et al*., 1992) (DDBJ/EMBL/GenBank accession No. X63675) and the Preamplification System (Gibco-BRL). The 1300 bp amplification product was cloned into pCR™2.1 (Invitrogen). Similarly, the *Pim-2* rat cDNA was obtained using primers based on the published mouse sequence (van der Lugt *et al*., 1995) (DDBJ/EMBL/GenBank accession No. L41495). *Pim-3* cDNA (DDBJ/EMBL/GenBank accession No. AF086624) was obtained in a low stringency screen of a non-subtracted cDNA library prepared from PTZ/CHX-induced animals (Link *et al*., 1995) at 42°C with reduced formamide (40%); low stringency washes were at 60°C with 2× SSC. Total and poly(A)$^+$ RNA isolations, Northern analyses and quantification of Northern blots on a bioimaging analyzer were as described (Qian *et al*., 1993; Link *et al*., 1995; Konietzko and Kuhl, 1998). The cDNA probes for *Pim-1* and *Pim-3* were tested on multiple tissue Northern blots and showed specific hybridization as evidenced by transcripts of different sizes and differential expression in various tissues. Both strands of *Pim-3* cDNA were sequenced as a double-stranded plasmid with multiple synthetic primers by the dideoxy chain termination method (Sanger *et al*., 1977).

In situ hybridization

In situ hybridization was conducted as described (Link *et al*., 1995). Frozen brains were cryostat sectioned at 16 μm on the coronal and sagittal plane. Control and experimental tissue sections were thaw-mounted onto the same slide to ensure identical hybridization conditions. Uridine 5′-α-[^{35}S]thiotriphosphate-labeled antisense Pim-1 RNA was transcribed from the T7 promoter of *Kpn*I linearized pCR™2.1 plasmid (Invitrogen) containing 1300 bp of the *Pim-1* cDNA insert. A sense strand control was generated from *Kpn*I-linearized pCR™2.1 plasmid containing an identical *Pim-1* cDNA insert in reverse orientation. Labeled sense and antisense Pim-2 and Pim-3 RNAs were transcribed from the T7 and SP6 promoter of linearized pSPORT-1 plasmid (Gibco-BRL).

Generation of polyclonal antisera

The entire coding region starting with methionine of Pim-1 and Pim-3 was cloned in pET28a(+) (Novagen). The integrity of the coding region was verified by transcription and translation in the presence of [^{35}S]methionine in a reticulocyte system (TNT, Promega). Recombinant Pim-1 and Pim-3 were expressed as bacterial fusion proteins according to the protocol of the manufacturer (Novagen). The fusion proteins were purified over Ni^{2+}-nitrilotriacetic acid–agarose (Qiagen). The recombinant proteins showed autophosphorylation activity that could be blocked in the presence of 1 mM Zn^{2+}. Rabbits were immunized with recombinant Pim-1 protein using a standard immunization protocol (Eurogentec). Antisera were purified over an affinity column containing Sepharose 4B (Pharmacia) coupled to recombinant Pim-1 protein. Antibodies were eluted in glycine buffer (pH 2.5). The purified Pim-1 antibodies were applied to a column containing Sepharose 4B coupled to recombinant Pim-3 protein and the flowthrough was collected and used in all immunocytochemical analyses. Depletion was achieved by applying this purified serum to a column containing Sepharose 4B coupled to recombinant Pim-1 protein.

Immunohistochemistry

Free-floating 40 μm vibratome sections were pre-incubated in normal horse serum (10%) and then incubated in purified rabbit polyclonal antisera to Pim-1 (1:5; PBS as diluent). Localization of Pim-1 was detected using the Avidin–Biotin Elite system (Vector Laboratories) with nickel/cobalt chloride-intensified diaminobenzidine (DAB) as chromagen (Vanderklish *et al*., 1995). For electron microscopic analysis, sections were transferred to osmium tetroxide (2% in 0.1 M phosphate buffer) for 30 min, embedded in Araldite and cured for 48 h at 60°C. Ultrathin sections were prepared and analyzed with an electron microscope (Zeiss 902A).

Chronic electrophysiology

Adult male Sprague–Dawley rats (250–300 g) were anesthetized with sodium pentobarbital (55 mg/kg) and implanted stereotaxically with a monopolar stimulating electrode in the perforant path and a monopolar recording electrode in the hilus of the dentate gyrus. Physiological recordings were used to find and maximize the evoked positive-going field potential (maximum spike-free amplitude range 7–12 mV). The electrodes were mounted to the skull using dental cement and their leads connected to a permanently affixed headstage. The wound was sutured and analgesics administered, and the animals recovered for at least 7 days before being transferred to a chronic recording cage for the actual experiment involving standard *in vivo* electrophysiological techniques (Staubli and Scafidi, 1997). During the experimental sessions, the current intensity was adjusted (20–70 μA, pulse width 150 μs, biphasic) to produce a baseline response with a minimal population spike of 0.5–1 mV. Test pulses were delivered at 0.07 Hz for at least 20 min to establish stable baseline potentials, after which either high frequency or low frequency stimulation (HFS or LFS) was initiated randomly. The HFS paradigm consisted of five 25 ms long trains at 400 Hz, delivered 1 s apart, and repeated 10 times with a 1 min interval between repetitions. The same number of pulses was delivered for the LFS treatment, but at a rate of 0.2 Hz. Potentials were then monitored at 0.07 Hz for 1 h, following which the rats were decapitated, and the brains removed and placed on dry ice.

Hippocampal slice analysis

129/Ola wild-type mice and Pim-1-knockout mice (te Riele *et al*., 1990; Domen *et al*., 1993a,b) were anesthetized. The brains were removed into ice-cold ACSF (126 mM NaCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 2 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 200 μM ascorbic acid). Then 400 μm thick slices were cut on a vibratome and stored submerged at room temperature for at least 1 h before recording. Slices were submerged in the recording chamber and perfused with ACSF (2.5 ml/min) at room temperature (23–24°C). Two monopolar tungsten stimulation electrodes were placed in the CA1 stratum radiatum, and biphasic current pulses (300 μs, 7–40 μA) were delivered with a stimulus isolator (A&M Systems) at 0.07 Hz. Extracellular field potentials were recorded in the stratum radiatum with glass micropipets filled with 3 M NaCl (2–4 MΩ) using an Axoclamp 2B amplifier. After a 100-fold post-amplification, the signals were digitized with a Digidata 1200 at 10 kHz. To elicit LTP in one pathway, three tetani (1 s, 100 Hz) spaced 5 min apart were delivered at the same stimulation intensity as the baseline stimulation.

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