Effects of cerebral ischemia in mice deficient in *Persephin*

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Persephin **(Pspn), a recently cloned member of the transforming growth factor-β superfamily (TGF-β) and glial cell line-derived neurotrophic factor (GDNF) subfamily, is distributed throughout the nervous system at extremely low levels and is thought to function as a survival factor for midbrain dopaminergic and spinal motor neurons *in vivo*. Here, we report that mice lacking Pspn by homologous recombination show normal development and behavior, but are hypersensitive to cerebral ischemia. A 300% increase in infarction volume was observed after middle cerebral artery occlusion. We find that glutamate-induced Ca^{2+} influx, thought to be a major component of ischemic neuronal cell death, can be regulated directly by the *Persephin* protein (PSP) and that PSP can reduce hypoxia/reperfusion cell death *in vitro*. Neuronal cell death can be prevented or markedly attenuated by administration of recombinant human PSP *in vivo* before ischemia in both mouse and rat models. Taken together, these data indicate that PSP is a potent modulator of excitotoxicity in the central nervous system with pronounced neuroprotective activity. Our findings support the view that PSP signaling can exert an important control function in the context of stroke and glutamate-mediated neurotoxicity, and also suggest that future therapeutic approaches may involve this novel trophic protein.**

Neurotrophic factors are important for the proper development and maintenance of the nervous system. These factors promote neuronal survival and can prevent the neuronal degeneration associated with injury, toxins or disease. Four trophic factors in the GDNF protein subfamily have been identified. They are glial cell line-derived neurotrophic factor (GDNF), *Neurturin*, *Artemin*, and *Persephin*, which bind preferentially to glycosylphosphatidylinositol (GPI)–linked *GDNF* family α receptors, termed GFRα1, GFR α2, GFRα3, and GFRα4, respectively, to induce transmembrane receptor tyrosine kinase Ret (cRet)-dependent activity or cRet-independent activity (1, 2).

GDNF, *Neurturin*, and *Artemin* have been reported to play important roles in neuroprotection. For example, GDNF maintains the survival and function of midbrain dopaminergic neurons *in vitro* (3) and attenuates the neuronal damage induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA; ref. 4), brain ischemia (5), and axotomy (6) *in vivo*. *Neurturin* supports the survival of ventral midbrain dopaminergic and motor neurons and induces neurite outgrowth in spinal cord (7, 8). *Artemin* increases the survival of sensory and sympathetic neurons and dopaminergic midbrain neurons in culture (9, 10). Injection of lentiviral vectors carrying *Artemin* cDNA increased dopamine neuronal density in nigra and tyrosine hydroxylase (TH) immunoreactivity in striatum in 6-OHDA-lesioned rats (10). Taken together, these data suggest that agonists to GFRα1, GFRα2, and GFRα3 have neuroprotective effects. The enhancement of expression of these trophic factors during neuronal injury (11–13) further indicates that they are endogenous protective agents that can be up-regulated during injury to reduce insults.

The physiological functions of *Persephin* protein (PSP) are less clear. PSP promotes survival of midbrain dopamine neurons and spinal cord motor neurons *in vitro* and *in vivo* (8, 14). In contrast to GDNF or *Neurturin*, however, PSP has no survival-promoting effect on peripheral neurons, including superior cervical ganglion (SCG), sensory neurons in dorsal root ganglion, and enteric neurons (14), and, at high dose (100 ng/ml), actually enhances glutamate toxicity induced by threohydroxyaspartate, a potent glutamate transport inhibitor, in spinal cord motor neuron culture (15).

To investigate the neurobiological function of PSP, we created a mouse model lacking *Persephin* gene (Pspn) by genetic manipulation as well as used exogenous PSP in different pharmacological protocols. We now report that Pspn-deficient mice show normal development and behavior, but are hypersensitive to focal cerebral ischemia or stroke. Moreover exogenous PSP shows neuroprotective activity in ischemia/neurotoxicity models both *in vitro* and *in vivo*. Our findings support the postulate that PSP signaling can exert an important function in the context of stroke and glutamate-mediated neurotoxicity

Materials and Methods

Generation of Pspn Knockout Mice. A targeting vector was designed to replace the coding exon for the mature peptide of the PSP preproprotein with a Pgk-Neo resistance expression cassette in reverse orientation to Pspn expression. R1 embryonic stem cells were grown and selected with G418 and gancyclovir. Homologous recombinants were identified by Southern blot analysis and used to generate the various genotypes.

Fluorescence *in Situ* Hybridization (FISH). FISH analysis was performed on slides with metaphase chromosome spreads derived from mouse embryo fibroblast cells, by using a digoxigenin dUTP-labeled bacterial artificial chromosome (BAC) clone (containing both coding exons for the murine Pspn gene), according to standard methods.

Histological Analysis. Adult mice were fixed by perfusion in 4% paraformaldehyde, 30% sucrose equilibrated, embedded, sectioned, and processed for either cresyl violet, hematoxylin/eosin staining, or prepared for indirect immunohistochemistry according to standard protocols. Antisera and dilutions were as follows: tyrosine hydroxylase (Pel-Freez, Rogers, AK) 1:1000. The secondary antiserum was rhodamine-conjugated sheep anti-rabbit/mouse immunoglobin (BMB) 1:500. Sections were examined on

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Abbreviations: Pspn, *Persephin* gene; PSP, *Persephin* protein; GDNF, glial cell line-derived neurotrophic factor; GFRα, GDNF family α receptor; MCA, middle cerebral artery; rh, recombinant human; rm, recombinant mouse; TTC, triphenyltetrazolium chloride; rCBF, relative cerebral blood flow; LDH, lactate dehydrogenase; i.c.v., intracerebroventricular.

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a microscope equipped with epifluorescence optics (Nikon Microphot).

Triphenyltetrazolium Chloride (TTC) Staining. One (mice) and two (rats) days after reperfusion, animals were deeply anesthetized, killed, and perfused intracardially with saline. The brain was then removed, immersed in cold saline for 5 min, and sliced into consecutive 1.0-mm (mice) or 2.0-mm (rats) thick sections, by using a mouse or rat brain matrix. The brain slices were incubated in 2% TTC (Sigma) dissolved in normal saline for 15 min at 37°C, and then transferred into a 5% formaldehyde solution for fixation. The area of infarction in each slice was measured by using a digital scanner and the IMAGE TOOLS program (University of Texas Health Sciences Center in San Antonio) as described previously (5). The volume of infarction in each animal was obtained from the product of average slice thickness (mice 1 mm, rat 2 mm) and sum of infarcted areas in all brain slices.

Surgery. Cerebral ischemia was produced by middle cerebral artery (MCA) ligation in anesthetized animals according to standard protocols (16, 17). Briefly, the right MCA and both common carotids (CCAs) were ligated with 10-O suture and two nontraumatic arterial clips, respectively. Ligatures were removed 30 min (mice) and 60 min (rats) later to produce a reperfusional injury to the ischemic brain area. Body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad during anesthesia. After recovery from the anesthesia, body temperature was maintained at 37°C with a heat lamp.

Behavioral Analysis. Vertical locomotor activity was measured in an Omnitech Digiscan apparatus (AccuScan Instruments, Columbus, Ohio). Details for the neuropsychological tests conducted are available on request.

Cerebral Blood Flow Measurements. Changes in relative cerebral blood flow (rCBF) were evaluated in mice with a laser Doppler flowmeter (LASERFLO BPM2, Vasamedic, Minneapolis). The laser Doppler probe was placed on a cranial window above the center of the MCA perfusion zone of cortex (1.3 mm posterior and 2.8 mm lateral from the bregma). The rCBF was monitored continuously from 10 min before until 30 min after ischemia.

Cell Culture and Quantification of Intracellular Ca^{2+} Levels. Dissociated embryonic day E17.5 cortex was isolated from mice according to standard protocols (18). Intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) were quantified by fluorescence imaging of the calcium indicator dye Fura-2 as described previously (18). Briefly, cells were incubated for 15 min in the presence of 2 μM Fura-2 (Molecular Probes). Immediately before imaging, dishes were washed once in Luke's buffer (154 mM NaCl/5.6 mM KCl/2.3 mM $CaCl_2$/1 mM $MgCl_2$/3.6 mM $NaHCO_2$/5 mM Hepes/20 mM glucose). Thereafter, recombinant human (rh) PSP, recombinant mouse (rm) PSP and L-glutamate were added at different time points and conditions as described above. Cells were imaged on a Zeiss Axiovert microscope (40× oil immersion objective) coupled to an Attofluor imaging system. The average $[Ca^{2+}]_i$ in 30–64 neuronal cell bodies per microscope field was quantified in two to three separate cultures per treatment condition. The *in vitro* neurotoxicity (hypoxia) experiments were conducted as described previously (19). Leakage of lactate dehydrogenase (LDH) into the medium was monitored with a LDH Cytotoxicity Detection Kit (Roche) according to the manufacturer's instructions. The average LDH release in 10–14 wells was quantified in three different cultures per treatment condition.

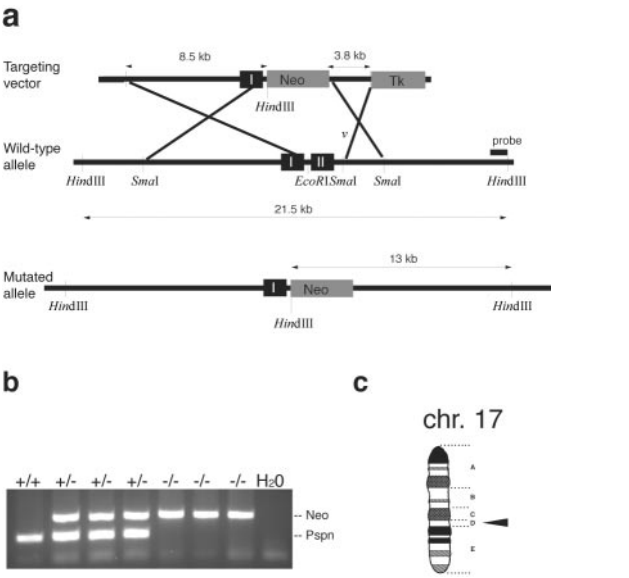

Fig. 1. Generation of Pspn-deficient mice. (*a*) Targeting strategy for disrupting the Pspn genomic locus. (b) Detection of various genotypes by PCR-based analysis of representative tail biopsies from a litter of one heterozygous breeding pair. (*c*) A schematic drawing of mouse chromosome 17. A genomic PSP bacterial artificial chromosome (BAC) probe, mapped on mouse chromosome 17D, indicated by an arrowhead.

Baculovirus-Expressed rmPSP. The cDNA for the coding region of mouse Pspn was obtained as an *Xho*I/*Pac*I-*Hin*dIII fragment by reverse transcription/PCR of poly(A) RNA from mouse brain. After transfer to Bluescript KSII (Stratagene) and ABI 377 (Applied Biosystems) sequence verification, the cDNA was inserted into the baculovirus expression vector pBioGreen-His (PharMingen). Recombinant protein, rmPSP, was expressed in a baculovirus expression system. Insect cells (Sf9) were infected with the recombinant virus. The infected cells were harvested 48 hr after infection and affinity-purified by using Ni-NTA Agarose (Qiagen, Valencia, CA), as recommended by the manufacturer, and finally dialyzed with 1× PBS.

Results

Generation of the Pspn Knockout Mutant Line. The murine Pspn gene was mapped by fluorescence *in situ* hybridization to chromosome 17 in mice (Fig. 1*c*), and disrupted by replacing the entire second coding exon of the PSP protein with a cassette expressing the selectable marker neomycin phosphotransferase (Neo) by homologous recombination in embryonic stem (ES) cells (Fig. 1*a*). The targeted clone was injected into C57BL blastocysts to generate Pspn mutant mice (Fig. 1*b*). Crosses of heterozygous Pspn +/− mice resulted in approximately the expected Mendelian ratio of wild-type (633 of 2,269, 28%), heterozygous (1,115 of 2,269, 49%), and homozygous (521 of 2,269, 23%) animals at weaning. Offspring from homozygous Pspn −/− crosses were also viable and fertile. Animals from +/+, +/−, and −/− crosses were indistinguishable by visual inspection, indicating that PSP is not required for normal embryonic and postnatal gross development.

Pspn Null Mutant Mice Do Not Display Noticeable Anatomical, Histological, or Functional Defects. Based on the ubiquitous, but extremely low, pattern of Pspn expression during embryogenesis and adulthood (14, 20), and its reported ability to support dopaminergic neurons in culture and *in vivo* (14), we examined whether PSP might modulate the morphological appearance of dopaminergic or noradrenaline (NE) neurons. However, based

on tyrosine hydroxylase immunocytochemistry (see Fig. 5 *c–f*, which is published as supporting information on the PNAS web site, www.pnas.org), no significant difference between wild-type and null mutated homozygous mice was detected. These findings indicate that PSP is not an essential survival/maintenance growth factor for dopaminergic midbrain or locus coeruleus neurons *in vivo*. Further, no gross anatomical deficits were identified in the striatum (see Fig. 5 *a* and *b*), neocortex (see Fig. 5 *g* and *h*), or basal forebrain. In terms of peripheral tissue and innervation, no abnormalities were seen in the intestinal tract enteric nervous system, kidneys, liver, gonads, heart, or skeletal muscle (data not shown). These findings strongly suggest that PSP does not act as a typical survival or growth factor for central nervous system or peripheral tissues. To test for a potential red/white blood cell or major chemistry dysfunction, a complete blood count (CBC) and a complete chemical panel were performed on blood collected from adult mice of +/+, +/−, and −/− Pspn genotypes. There were no significant differences between groups (data not shown).

The extracellular moiety of the PSP receptor, GFRα4, is expressed in many areas in both embryonic and adult rodents (21, 22). GFRα4 is also expressed at high levels in the thyroid gland, mainly by calcitonin-expressing C cells (21) and in patients with malignant thyroid medullary cancer (23). However, no differences were seen in thyroid or parathyroid gland morphology (see Fig. 5 *i* and *j*) between wild-type and null mutated animals nor in serum calcium levels (data not shown). Cortex and hippocampus also show significant levels of GFRα4 and Pspn expression (21, 22), which could suggest a potential function for PSP in motor function, memory, and learning. To address this result, we first investigated the gross morphological appearance of hippocampus and cortex in adult mice by hematoxylin/eosin staining (see Fig. 5 *g* and *h*) and by gamma-aminobutyric acid (GABA) and glutamic acid decarboxylase (GAD) immunohistochemistry (data not shown). Again, there were no differences seen in mice of +/+, +/−, or −/− Pspn genotypes. Second, a behavioral examination was performed on adult Pspn +/+ ($n = 13$) and Pspn −/− ($n = 11$) animals, consisting of 20 different assessments (Table 1). Some assessments contained multiple measurements. Overall, the appearance and behavior of the two groups of mice were similar. There were few significant ($P < 0.05$) differences but no data to suggest that PSP plays a prominent role in motor control or cognitive behavior (Table 1).

Functional Ablation of the Pspn Gene Increases Sensitivity to Stroke. Given the hypothesized role of many TGF-β family members as neuroprotective factors, we next examined whether mice lacking Pspn are more sensitive to central nervous system insults. We used MCA occlusion, followed by reperfusion, in mice to mimic effects seen in humans with focal stroke and to determine the potential ischemic neuroprotective efficacy of PSP. Mice were divided into 2 groups for behavioral analysis [Pspn +/+ ($n = 8$), Pspn −/− ($n = 10$)]. Because both the Pspn +/+ and Pspn −/− groups had some variation in age and size, all poststroke locomotor data were normalized to the degree of prestroke activity in the same animal group. We found that all stroke animals developed a marked reduction in vertical activity on day 2, followed by spontaneous improvement from day 2 to day 30, after stroke. There was a significant diminution in the degree of recovery of vertical activity in the Pspn−/−, compared with the Pspn +/+, animals from day 2 to day 30 after MCA (Fig. 2*a*, $P < 0.03$, two-way ANOVA + Newman–Keuls test).

TTC staining (which indicates devitalized brain areas) was carried out in separate groups of Pspn +/+ ($n = 8$) and Pspn −/− ($n = 10$) animals 1 day after reperfusion, and the infarct volume was determined by a blinded investigator (Fig. 2 *b–d*). The total infarct volume was 13.72 ± 2.10 mm^3 in Pspn +/+

Table 1. Behavioral tests

Examination	Group mean +/+	Group mean −/−	Significant?
Appearance and reflexes			
Initial assessment			
Body weight	30.8 g	29.6 g	No
Body length	9.5 cm	9.5 cm	No
Tail length	6.4 cm	6.6 cm	No
Head width	6.4 cm	6.6 cm	No
Vibrissae condition	0.92	0.64	No
Eye assessment	0	0	No
Muscle tone	0	0	No
Tail hang			
Turn	1.5	1.2	No
Forelimbs	0.92	0.46	No
Hindlimbs	0.69	0.18	No
Digit extension	0	0	No
Grip strength			
Forelimb	459.6	443.2	No
Pain responses			
Heat and cold response			
Heat	24	26.4	No
Cold	22.5	14.4	No*
Von Frey hairs			
Withdrawal threshold	5.41	4.97	No
Balance tests			
Beam walking			
Errors	7.4	4	Yes
Balance task			
Latency to right	4.1	4.9	No
Roto-rod task			
Latency	49.5	55.7	Yes
Looping	1.4	1.9	No
Learning tests			
Water maze			
Latency	16	14.6	No
Distance	471.9	447	No
Passive avoidance			
Latency on platform	39	37.8	No
T-maze activity			
Latency in novel area per block	15.6	22.3	No
Context discrimination			
Time on rewarded side	118.2	140.4	No†
Activity tests			
Circadian activity			
Mean cage crosses per block	9.3	8.3	No
Open field activity			
Total distance	274.3	291.2	No
Vertical movements	10.6	8.8	No
Fecal pellets	3.3	4	No
Light/dark transition			
Time in dark	39.6	34.7	No
No. of zone transitions	5.2	7.1	No
Motor skill			
Grid walking			
Hindlimb errors	3.3	2.2	No
Forelimb errors	2.8	2.5	No
Reaching			
Hit %	32.5	25.9	No
No. of reaches	27.2	32.4	No
Reactivity			
Reactivity assessment			
Reactivity	0.115	0.5	Yes

Results from a battery of neuropsychological tests, performed on adult Pspn +/+ ($n = 13$) and Pspn −/− ($n = 11$) mice.

*Pspn −/− was slightly more sensitive to the cold plate test, but this difference did not reach statistical significance.

†When the mice were given a reversal test in which the platform was moved to a new location, there was a significant group difference, with the Pspn −/− group learning more quickly than the Pspn +/+ group. The mice were then given two more reversals, but there were no further differences.

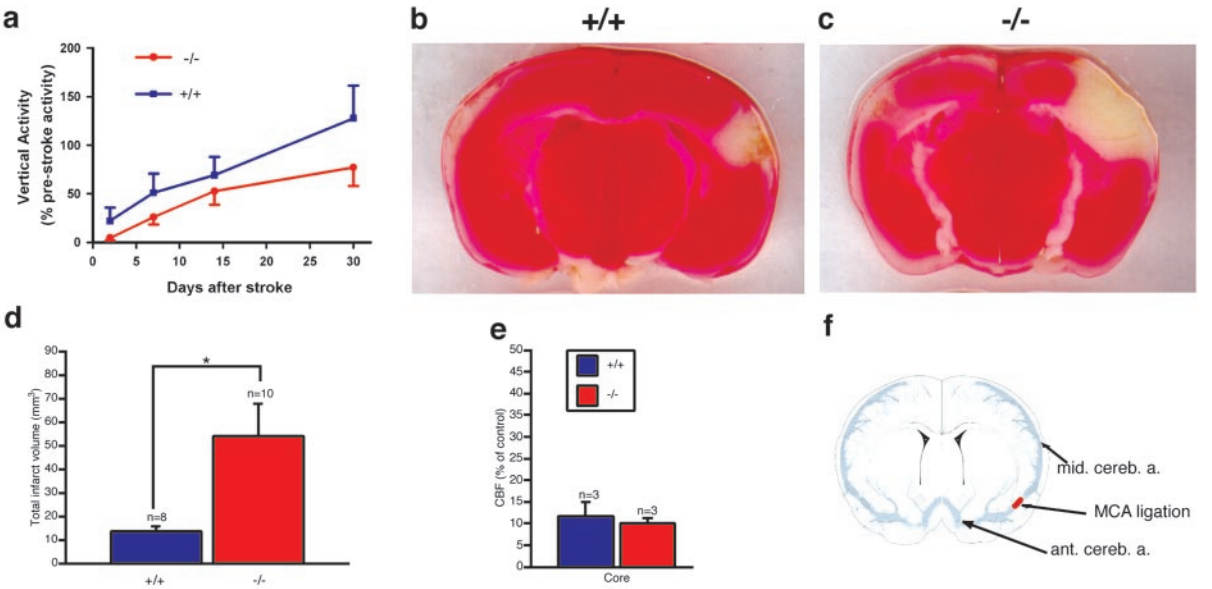

Fig. 2. Stroke/ischemic damage in cortex of Pspn +/+ and −/− mice. (*a*) Vertical locomotor activity after MCA ligation. All animals were individually placed in activity chambers on days 2, 7, 14, and 30 for a 60-min vertical activity measurement. Poststroke activity was normalized by comparison to prestroke activity. There was a significant diminution in the recovery of vertical activity in the −/−, compared with the +/+, animals after MCA ($P < 0.03$, two-way ANOVA + Newman–Keuls test). (*b* and *c*) TTC staining of the cortical regions contralateral (left) and ipsilateral (right) to MCA occlusion in Pspn +/+ and Pspn −/− mice. (*d*) Total infarct volume (mean ± SEM), after 24 hr, in Pspn −/− ($n = 10$, red bar) and Pspn +/+ ($n = 8$, blue bar) mice that received 30-min MCA ligation. (ANOVA; $P < 0.05$, post hoc Newman–Keuls test). (*e*) Depicts rCBF decrease after MCA occlusion in Pspn +/+ (blue) and Pspn −/− (red) mice in the infarct core. The rCBF level was determined by laser Doppler flowmetry ($P < 0.05$, *t* test). (*f*) Schematic drawing of MCA occlusion.

mice and 52.87 ± 13.80 mm^3 in the Pspn −/− mice; thus, there was a 385 ± 101% difference between the groups (Fig. 2*d*, $P = 0.029$, *t* test). Taken together, these data show that Pspn −/− mice developed more cerebral infarction after ischemia. To evaluate the importance of cellular vs. vasculature-mediated mechanisms in this differential sensitivity to ischemia, we studied local blood flow and anatomy of the cerebral vasculature. There were no significant differences in cerebral vasculature, as evaluated by carbon black injection (data not shown). Moreover, rCBF changes measured with laser Doppler flow probes, before, during, or after ischemia showed no significant rCBF alterations in the ischemic core (Fig. 2*e*) between Pspn +/+ (11.7 ± 3.2%, $n = 3$) and Pspn −/− (10 ± 1.2%, $n = 3$) mice ($P < 0.05$, *t* test).

Pretreatment with Recombinant PSP Alters Ischemic Insults in a Dose-Dependant Manner. We next investigated whether treatment with rhPSP (PeproTech, Rocky Hill, NJ) could reverse this induced Pspn −/− phenotype. Pretreating Pspn −/− mice with 0.1 μg PSP ($n = 8$) intraventricularly 10 min before a 30-min ischemia markedly reduced the volume of infarction by 219 ± 23% as compared with vehicle (10 μl 0.9% NaCl)-treated animals and by 18% below wild-type vehicle-treated animals (Fig. 3*a*; $P = 0.035$, one-way ANOVA; $P < 0.05$, post hoc Newman–Keuls test). Interestingly, treatment with a high dose of PSP (10 μg) actually increased infarct volume (Fig. 3*a*).

To compare our data with the literature on the effects of other trophic proteins on focal ischemia in rats, the protective effect of PSP on infarct size was also studied in 32 adult Sprague–Dawley rats. Eight rats were injected with 0.5 μg/10 μl PSP intracerebroventricular (i.c.v.), 6 received 0.1 μg/10 μl PSP, and 18 were injected with vehicle before a 60-min MCA ligation. Locomotor activity was examined after 2 days. We found that pretreatment with PSP (0.5 μg or 0.1 μg in 10 μl, i.c.v.) significantly enhanced vertical locomotor activity in the stroke animals compared with the vehicle-treated group. The magnitude of the effect was similar to that shown above for mice (data available on request). The total volume of infarction was also significantly reduced by more than 50% (Fig. 3*b*; $P < 0.05$, one-way ANOVA + Newman–Keuls test). When the data on infarction volumes were recalculated by using corrections for cerebral edema, there was still significant neuroprotection by PSP in both mice and rats, with a dose-response relationship similar to that in our original calculations.

PSP Protects Cortical Neurons from Hypoxia-Induced Cell Death *in Vitro*. We investigated whether rhPSP could reverse cell death, measured by LDH release in cortical neurons *in vitro* (19) after hypoxia and reoxygenation. We found that 10 pM rhPSP significantly reduced the induced cell death by up to 50% compared with untreated sister cultures (Fig. 4*a*, ANOVA with Tukey post hoc comparison; $P < 0.05$). Higher doses of rhPSP (100 pM) were much less effective.

PSP Modulates Glutamate-Induced Calcium Influx. Whereas controversy exists about the role of various intracellular mechanisms producing neuronal death after ischemia, there is an extensive literature implicating excessive glutamate release and subsequent elevation of intracellular calcium (24–26). To determine whether PSP could influence such an excitotoxic mechanism, we examined the action of PSP on cortical neurons *in vitro* by intracellular calcium($[Ca^{2+}]_i$) Fura-2-imaging. Recombinant PSP had little direct effect on intracellular calcium levels at either 5, 50, or 500 ng/ml when compared with vehicle (Fig. 4 *b* and *c*). Addition of 70 μM glutamate resulted in a significant increment in intracellular $[Ca^{2+}]_i$ levels. In agreement with our *in vivo* neuroprotection data, low doses of PSP significantly reduced those glutamate-elicited intracellular calcium increments whereas high doses of PSP exacerbated these elevations (Fig. 4 *b* and *c*; rhPSP: 5 ng/ml, 327 ± 43; 50 ng/ml, 657 ± 56; 500 ng/ml, 1,007 ± 83; rmPSP: 5 ng/ml, 264 ± 52; 50 ng/ml, 490 ± 68; 500 ng/ml, 1,189 ± 91; vehicle: 702 ± 34 nM $[Ca^{2+}]_i$; $P < 0.05$, ANOVA with Tukey post hoc comparisons). Chelation of extracellular calcium with EGTA completely eliminated alterations in glutamate-triggered calcium flux by PSP (data not

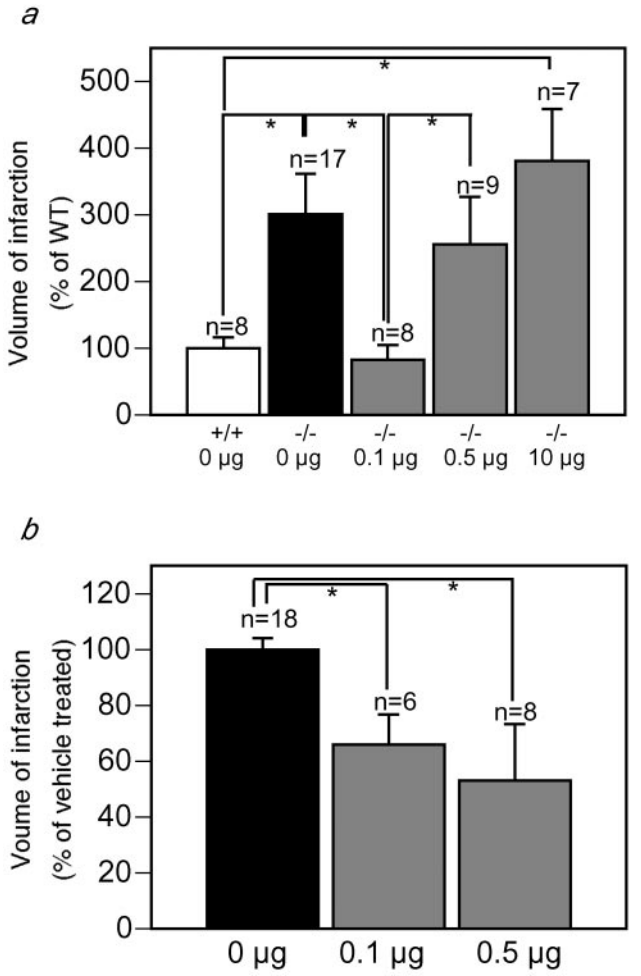

Fig. 3. *In vivo* protection and rescue against cerebral ischemia by PSP i.c.v. injections. (*a* and *b*) Total cerebral infarction volume, based on TTC staining. (*a*) Dose-response for neuroprotection in the Pspn +/+ and Pspn −/− mice by an i.c.v. injection of 10 μl of PSP (0.1, 0.5, or 10 μg) or vehicle (0 μg). (*b*) Reduced cerebral infarction volume in Sprague–Dawley rats, pretreated 10 min before MCA occlusion with PSP (0.1 μg or 0.5 μg/10 μl) or vehicle (0 μg) i.c.v. (one-way ANOVA; $P < 0.05$ post hoc Newman–Keuls test).

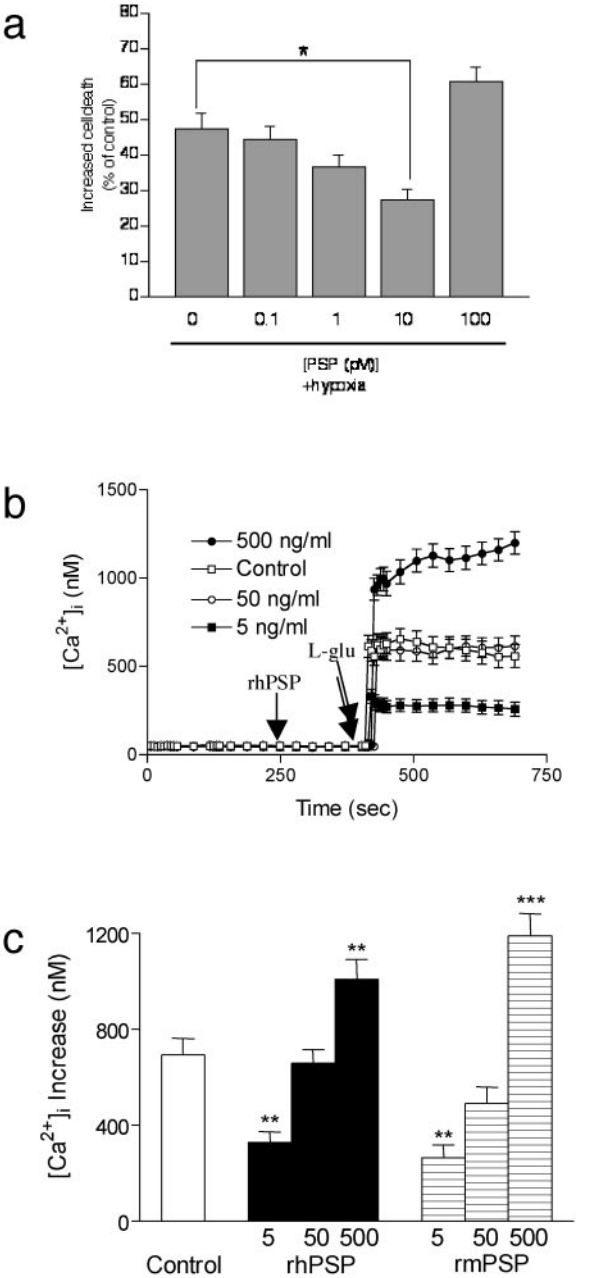

Fig. 4. Effects of PSP on cortical neurons *in vitro*. (*a*) PSP protects cortical neurons after hypoxia/reoxygenation. (*b* and *c*) Effects of PSP on intracellular Ca^{2+}. Calcium concentration changes in primary cultured-cortical neurons pretreated for 2 min with a solution of either 5, 50, or 500 ng/ml PSP or vehicle (indicated by an arrow), which preceded the addition of 70 μM glutamate (at the double arrow). (*b*) Time course of changes in calcium levels in cultures treated with either vehicle or with various concentrations of rhPSP. (*c*) Mean peak calcium concentrations of cortical neurons pretreated with either vehicle or with various concentrations rhPSP or rmPSP. All values represent mean ± SEM; all experiments were repeated at least three times. Asterisk designates a significant difference ($P < 0.05$, ANOVA + Tukey post hoc comparison).

shown), indicating that PSP affected glutamate receptor-mediated calcium influx.

Discussion

In the present study, we generated mutant mice deficient in *Persephin*, a member of the transforming growth factor-β family, to investigate the potential neurobiological role of PSP *in vivo* both during embryonic development and in the adult. We have shown that mice lacking Pspn have markedly increased cerebral infarction after focal ischemia. We have also shown neuroprotection and rescue in both mice and rats given a single injection of rhPSP. The neuroprotection induced by i.c.v. PSP *in vivo* is manifested at doses 100- to 1,000-fold lower than that seen with intracerebral and i.c.v. GDNF (5). The Pspn knockout mice do not display apparent developmental impairments as assessed by their body weight, mortality, gross anatomy, and overall behavior. This finding is very different from those concerning the GDNF (27–29), *Neurturin* (30), or GFRα1–3 (31–35) knockouts generated previously.

Elevations in intracellular calcium levels, induced by high concentrations of glutamate, can be regulated in a biphasic fashion by PSP. High concentrations of PSP augment glutamate-induced calcium entry into cortical cells whereas low concentrations suppress Ca^{2+} influx elicited by glutamate. The *in vivo* and *in vitro* neuroprotection reported here manifested an analogous biphasic PSP dose relationship. The importance and dose-dependence of this regulatory mechanism may explain the tight control of Pspn expression, by alternative splicing (14), and by its ubiquitous but low level of overall expression in the brain (14, 20).

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In the rodent brain, the distribution of PSP, and its preferred receptor GFRα4, has been studied by a variety of *in vitro* techniques (21, 23, 36). Recent studies have shown that the GFRα4 precursor mRNA is alternatively spliced in a tissue-specific manner. One splice variant encodes a putative transmembrane-anchored isoform, whereas a second splice variant encodes a putative truncated isoform, suggesting that the latter protein could also be secreted. Literature before these findings had suggested that GFRα4 is expressed only as a membrane-anchored receptor in the brain. Reverse transcription–PCR studies have shown that mRNA isolated from the whole brain (21), as well as from neurons of the olfactory bulb, cortex, striatum, ventral tegmental area, and cerebellum, contained exclusively the secreted form of the GFRα4 receptor. Therefore, the hybridization signal for soluble GFRα4 mRNA appears to be the isoform predominantly expressed in brain tissue under normal conditions. However, because only the anchored splice variant can interact with cRet (see below), it is important to determine which cell type(s) in cortex normally synthesizes PSP, GFRα4 subtypes, and potential transducing molecules. Although it appears as if a subset of neurons can make the soluble GFRα4 subtype under normal conditions, it is not clear what happens to different GFRα4 subtypes after an injury, a stimulus for up-regulation of other GFR receptors in this subfamily.

The long-held view that PSP simply binds to its receptor GFRα4-cRET complex, causing receptor dimerization, and transcription of genes, may be too simple. Accumulating evidence has indicated that GDNF family members may also activate second messenger-signaling pathways, such as adenylyl cyclase, phosphoinositol 3-kinase, and/or mitogen-activated protein kinase (2, 37, 38).

In conclusion, our data support the contention that endogenous PSP is a critical component in protection against ischemic brain injury induced via glutamate excitotoxicity/dysfunction. Taken together, these data on PSP might have clinical implications for the development of new treatment strategies for preventing the devastating effects of stroke. PSP might also have therapeutic applications in other brain disorders where glutamate/calcium homeostasis may be impaired, such as Alzheimer's disease and Parkinson's disease (39, 40)

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