

Stanniocalcin: A molecular guard of neurons during cerebral ischemia

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Stanniocalcin (STC) is a glycoprotein hormone originally found in bony fish, in which it regulates calcium/phosphate homeostasis and protects against hypercalcemia. The recently characterized human STC shows about 70% homology with fish STC. We previously reported a constitutive expression of STC in terminally differentiated neurons. Here, we show that exposure of human neural-crest-derived cell line Paju to hypercalcemic culture medium induced expression of STC. Treatment of Paju cells with recombinant human STC increased their uptake of inorganic phosphate. Paju cells expressing STC by cDNA transfection displayed increased resistance to ischemic challenge and to elevated intracellular free calcium induced by treatment with thapsigargin. An up-regulated and redistributed expression of STC was observed in neurons surrounding the core of acute infarcts in human and rat brains. Given that mobilization and influx of calcium is considered a main neurotoxic mechanism following ischemia, our results suggest that the altered expression of STC contributes to the protection of cerebral neurons against hypoxic/ischemic damage. Manipulation of the STC expression may therefore offer a therapeutic approach to limit the injury after ischemic brain insults.

Fish stanniocalcin (STC) is synthesized in a specialized organ adjacent to the kidney called the corpuscles of Stannius (1). An elevated level of calcium in plasma is a major stimulus for the secretion of STC (2, 3). It regulates calcium and phosphate homeostasis by acting on the gills to lower the calcium uptake (4, 5), on the kidney to increase phosphate reabsorption (6), and on the gut to inhibit intestinal calcium transport (7).

The cDNAs for human and mouse STC were recently cloned (8, 9). Human STC shares 60% identity and 80% similarity with fish STC. Infusion of recombinant human STC into rats reduced the renal excretion of phosphate (10). Addition of STC to the serosal surface of rat or pig duodenal mucosa reduced the net absorption of calcium and increased the uptake of phosphate (11).

We previously investigated gene expression during neural differentiation by using a human neural-crest-derived cell line, Paju, as an *in vitro* model. Induced terminal differentiation of Paju cells was found to strongly up-regulate the expression of STC. We also found an expression of STC restricted to mature neurons in human and mouse brain (12).

Cerebral neurons are highly vulnerable to tissue ischemia. Mobilization and influx of calcium has long been considered a major mechanism of ischemic cell death (13–16). Our histochemical stainings revealed the most prominent STC expression in the pyramidal cells of the cerebral cortex and hippocampus and in the Purkinje cells of the cerebellum (12), i.e., brain neurons known to be highly sensitive to ischemia (13). Given this, we hypothesized that STC may be involved in the protection against hypoxic damage.

Here, we show that treatment of cultivated neural cells with recombinant STC stimulated their uptake of phosphate. Expression of STC by transfection of STC cDNA conferred increased resistance to hypoxic stress and to mobilization of intracellular calcium induced by treatment with thapsigargin. An up-

regulated and intracellular redistribution of STC expression was seen in human and rat brain neurons in the “penumbra” of infarcted areas. Taken together, these findings suggest that STC plays an important role in maintaining and guarding the integrity of terminally differentiated neuronal cells challenged by ischemia and calcium-mediated cell death.

Materials and Methods

Cell Culture and Reagents. The Paju cell line (Paju/WT) (17) was established in our laboratory from the pleural fluid of a 16-yr-old girl who had a widespread metastatic neural-crest-derived tumor. The cells grow surface adherent in RPMI 1640 medium, supplemented with 10% FCS, penicillin G (50 mg/ml), streptomycin sulfate (50 mg/ml), and 1 mM glutamine. For subculturing, the cells were detached by treatment with 0.5 M EDTA. Human recombinant STC (hSTC) and rabbit antiserum against hSTC were prepared as described (18). Thapsigargin was purchased from Calbiochem.

Western Blotting. Cells were lysed for 10 min in an ice-cold buffer containing 20 mM Tris·HCl (pH 8.0), 0.2 mM EDTA, 3% Nonidet P-40, 2 mM orthovanadate, 50 mM NaF, 10 mM NaPPI, 100 mM NaCl, and 10 μ g/ml each of aprotinin and leupeptin. The samples were centrifuged at 14,000 \times g for 15 min, and the supernatants recovered. A total of 30 μ g of protein from each sample was separated by SDS/PAGE under reducing conditions and transferred electrophoretically to nitrocellulose filters. The filters were treated with 3% BSA in 20 mM Tris·HCl (pH 7.5)/150 mM NaCl/Triton X-100 for 2 h. Immunoblotting was done with 1:1,000 diluted rabbit antibodies to human STC antibody followed by peroxidase-conjugated secondary goat antibodies to anti-rabbit Ig. The blots were developed by enhanced chemiluminescence (Amersham Pharmacia).

Phosphate Uptake. Phosphate (32 Pi) uptake was measured as described (19) at 37°C in Locke’s buffer (20) (pH 7.2–7.4) consisting of 5.5 mM KCl, 1.0 mM MgCl₂, 2.5 mM CaCl₂, 5.5 mM glucose, 8.5 mM Hepes, and 160 mM NaCl. After preincubation in the assay medium for 10 min, the uptake was initiated by addition of 200 ng/ml of recombinant STC together with 125 μ M KH₂³²PO₄ (200 μ Ci/ μ mol). At indicated time points, 32 Pi uptake was terminated by washing with cold stop solution [10 mM Tris·HCl (pH 7.2)]. The cells were lysed in 0.1% SDS in water, and the 32 Pi activity was measured by liquid scintillation.

Expression Vector Constructs and Transfection. Human *stc* cDNA containing the full-length ORF was cloned into the *Bam*HI site

Abbreviations: STC, stanniocalcin; hSTC, human recombinant STC; Pi, inorganic phosphate.

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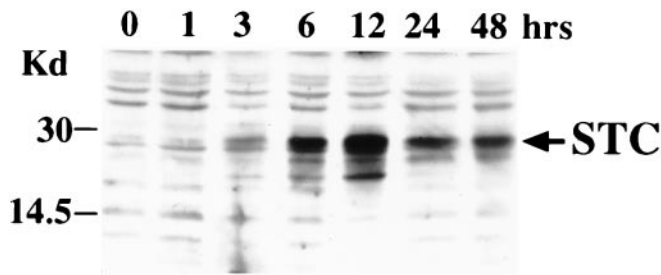


Fig. 1. Elevated extracellular calcium induces STC expression in the neural cell line Paju. The cells were cultivated in 5.4 mM CaCl_2 and lysed at indicated time points (1–48 h). Western blotting with rabbit antibodies revealed accumulation of STC. Time point 0 shows Western blotting of lysate from Paju cells cultivated in normocalcemic (0.7 mM) medium (control).

of a pcDNA3 expression vector (Invitrogen). Paju cells were transfected with 5 μg of the vector construct by using Lipofectamine Reagent according to the instructions of the manufacturer (GIBCO/BRL). Transfected cells were selected for resistance to G418 (700 $\mu\text{g}/\text{ml}$) for 3 wk and single-cell cloned (Paju/STC). Three Paju/STC clones were analyzed in this study with identical results. Control cells were transfected with the empty vector (Paju/C).

Cell Viability Assay and Luciferase Assay for ATP Monitoring. Cell viability was assessed by trypan blue exclusion (BDH). ATP was quantitated by an ATP monitoring kit (BioOrbit, Tampere, Finland) according to the manufacturer's instructions. In brief, cells were lysed in luciferase buffer with 1% Triton X-100, and the ATP-dependent activity was monitored by a luminometer (BioOrbit).

In Situ Hybridization. Single-stranded RNA probes were generated *in vitro* by T7 RNA polymerase from the pcDNA3 vector (Invitrogen) in which a cDNA for human stc was cloned in either sense or anti-sense orientation. The product was labeled with digoxigenin (Roche Molecular Biochemicals). A sense probe was used as control. The hybridization was carried out as described by the manufacturer (Roche Molecular Biochemicals).

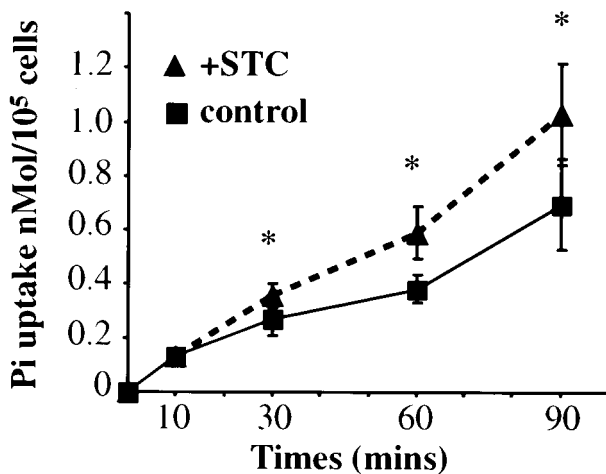


Fig. 2. Effects of extracellular STC on Pi uptake in Paju cells. The cells were incubated in Lockes' buffer containing 160 mM NaCl. The phosphate uptake was initiated by addition of 200 ng/ml recombinant STC together with 125 μM $\text{KH}_2^{32}\text{PO}_4$, and the radioactivity was measured at indicated time points. No STC was added to the control samples. The data are presented as mean \pm SD. *, $P < 0.05$ compared with control samples (Student's *t* test, $n = 6$).

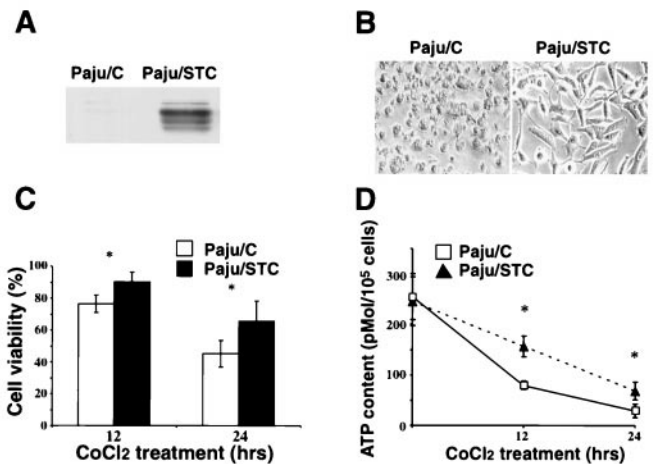


Fig. 3. Overexpression of STC increases cell resistance to hypoxic insult. (A) Paju cells transfected with stc cDNA (Paju/STC) expressed STC as shown by Western blotting. (B) Morphology of control Paju/C (Paju cells transfected with empty vector) and Paju/STC cells after 24 h in the presence of 300 μM CoCl_2 . (C) Cell viability assay of Paju/C and Paju/STC cells treated with CoCl_2 for indicated time periods. (D) ATP contents of Paju/C and Paju/STC cells treated with 300 μM CoCl_2 for indicated times. The data are presented as mean \pm SD. *, $P < 0.05$ compared with control samples (Student's *t* test, $n = 5-6$).

The bound label was detected by a peroxidase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals).

Immunohistochemistry. The tissues were fixed in 4% buffered formaldehyde, routinely processed, and embedded in paraffin. Sections of 4 μm thickness were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma) and dried for 12 h at 37°C.

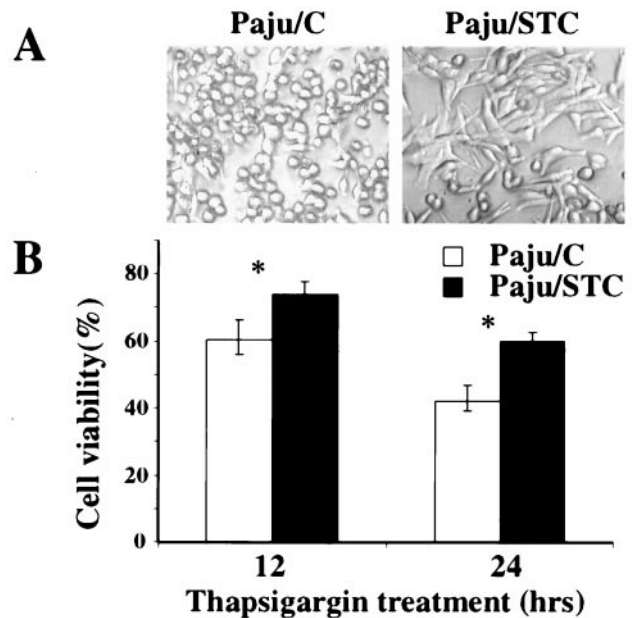


Fig. 4. Overexpression of STC increases cell resistance to mobilization of intracellular calcium induced by treatment with thapsigargin. (A) Morphology of Paju/STC and Paju/C after treatment for 12 h with 10 μM thapsigargin in serum-free culture medium. (B) Cell viability assay of Paju/C and Paju/STC cells treated with thapsigargin for indicated time periods. The data are presented as mean \pm SD. *, $P < 0.05$ compared with control samples (Student's *t* test, $n = 5$).

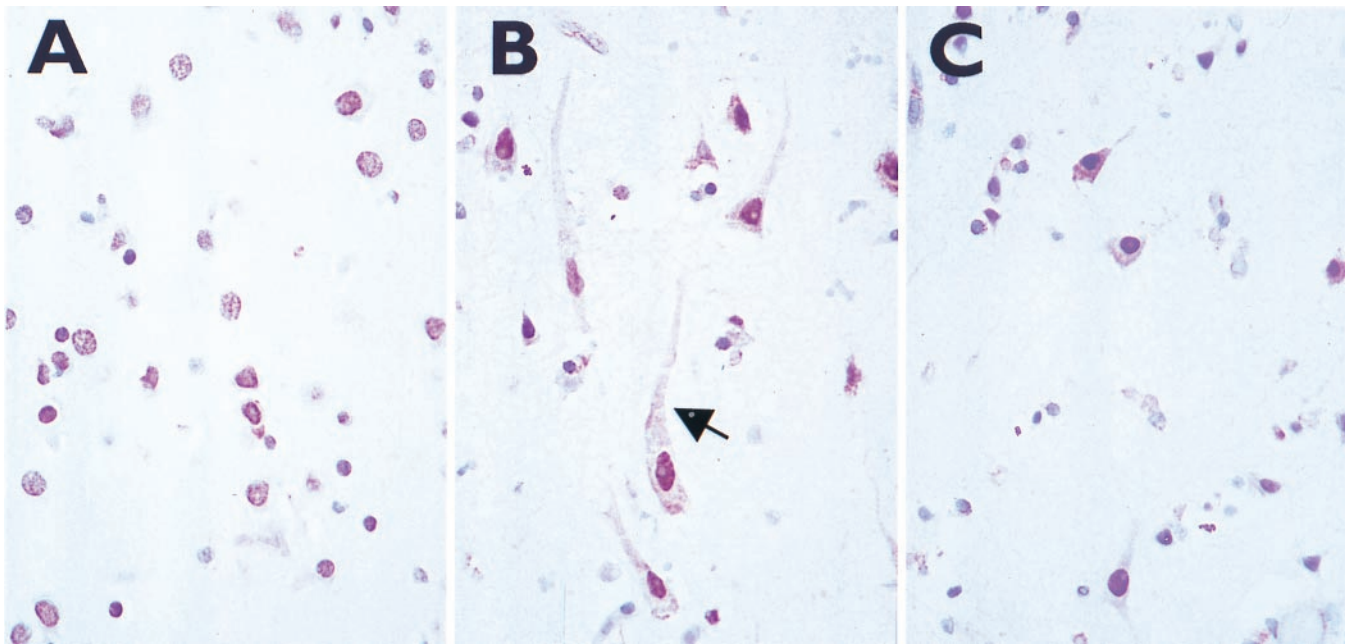


Fig. 5. Immunohistochemical demonstration of STC in infarcted human parietal brain cortex. (A) Staining of a corresponding area from the contralateral hemisphere of a brain with a 15-h-old infarct (control). (B) Staining of the "penumbra" of the damaged area in the infarcted hemisphere of the same brain. (C) Staining of an ischemic area from another brain with a 3-day-old infarct. Arrows indicate staining of neuronal processes.

The deparaffinized and rehydrated sections were processed in a microwave oven (21) and treated with a methanol-perhydrol solution (0.5% hydrogen peroxide in absolute methanol) for 30 min at room temperature to block endogenous peroxidase activity. Immunohistochemical stainings were performed as described (12). Staining with STC antibodies preabsorbed with recombinant STC protein and with normal rabbit serum served as controls.

Samples of Infarcted Human Brains. Specimens were collected at autopsy from the infarcted hemisphere and the corresponding contralateral brain area of patients who had died at different times after the onset of ischemic stroke symptoms. The samples were dissected, processed, and histologically analyzed as described in the Helsinki Stroke Study (22).

Experimental Animals and Induction of Focal Brain Ischemia. All animal experiments and surgical procedures were approved by The Animal Research Committee of the Helsinki University Central Hospital and the governmental animal health authorities. A total of 37 male Wistar rats weighing 310–380 g were used. Focal cerebral ischemia was induced by the suture occlusion of the medial cerebral artery of anesthetized animals (23). Reperfusion was established by withdrawing the suture occluder after 90 min. The control animals underwent the same procedure, but the suture occluder was inserted only 10 mm above the carotid bifurcation and withdrawn 1 min later. After postischemia periods of 2, 6, 24, 72 h, and 7 days, the experimental animals were anesthetized and subjected to transcardial perfusion with 200 ml of 0.9% saline for 5 min or until the perfusion fluid was clear. The controls underwent transcardial perfusion 24 h after the sham occlusion. The brains were immediately removed, 2-mm-thick coronal slices were dissected at the level of the optic chiasm, fixed in phosphate-buffered (pH 7) 4% formaldehyde, and embedded in paraffin.

Results

Elevated Extracellular Ca^{2+} Induces STC Expression in Cultivated Neural Cells. Elevated environmental calcium is a major trigger of STC synthesis in fish (2). Given this, we cultivated Paju cells in

RPMI 1640 medium containing 5.4 mM CaCl_2 or MgCl_2 . The expression of STC protein increased strongly after 6 h of culture in hypercalcemic medium and reached plateau levels at 12 h (Fig. 1). Addition of equimolar concentrations of Mg^{2+} to cultures of Paju cells had no effect on the STC expression (data not shown).

STC Stimulates Pi Uptake in Cultured Neural Cells. STC has been reported to stimulate tubular phosphate reabsorption in rat kidney by acting on the renal Na-phosphate cotransporter (10). Addition of 200 ng/ml hSTC to Paju/WT cells significantly increased their rate of Pi uptake (Fig. 2).

STC Confers Increased Resistance to Hypoxia and Hypercalcemia. Treatment with CoCl_2 is commonly used to mimic hypoxic insults both *in vitro* and *in vivo* (24, 25). Paju cells overexpressing STC after transfection with stc cDNA (Paju/STC, clone-27) and control cells transfected with the empty vector (Paju/C) (Fig. 3A) were cultivated for 12 and 24 h in the presence of 300 μM CoCl_2 . Only the Paju/STC cells retained high viability, whereas the control Paju/C cells had rounded up and mostly detached (Fig. 3B and C). The protective role of STC was further substantiated by the fact that, in the presence of CoCl_2 , Paju/STC cells maintained an efficient ATP synthesis in comparison to Paju/C cells (Fig. 3D).

Thapsigargin, an inhibitor of Ca^{2+} ATPases in the endoplasmic reticulum, mobilizes intracellular calcium stores and leads to increased levels of intracellular free calcium (26). Paju/STC cells displayed clearly elevated resistance to treatment with thapsigargin at concentrations toxic to Paju/C cells (Fig. 4).

Transiently Up-Regulated and Redistributed Neuronal Expression of STC in Human Parietal Cortex Surrounding Brain Infarcts. We reported that the constitutive expression of STC in mammalian brain was restricted to neurons. Immunohistochemistry revealed STC expression in the cytoplasm and the nuclei, except for the nucleoli. The cytoplasm of larger neurons, like the pyramidal cells of the parietal cortex, hippocampus, and the Purkinje cells

PERIPHERAL PENUMBRA CORE

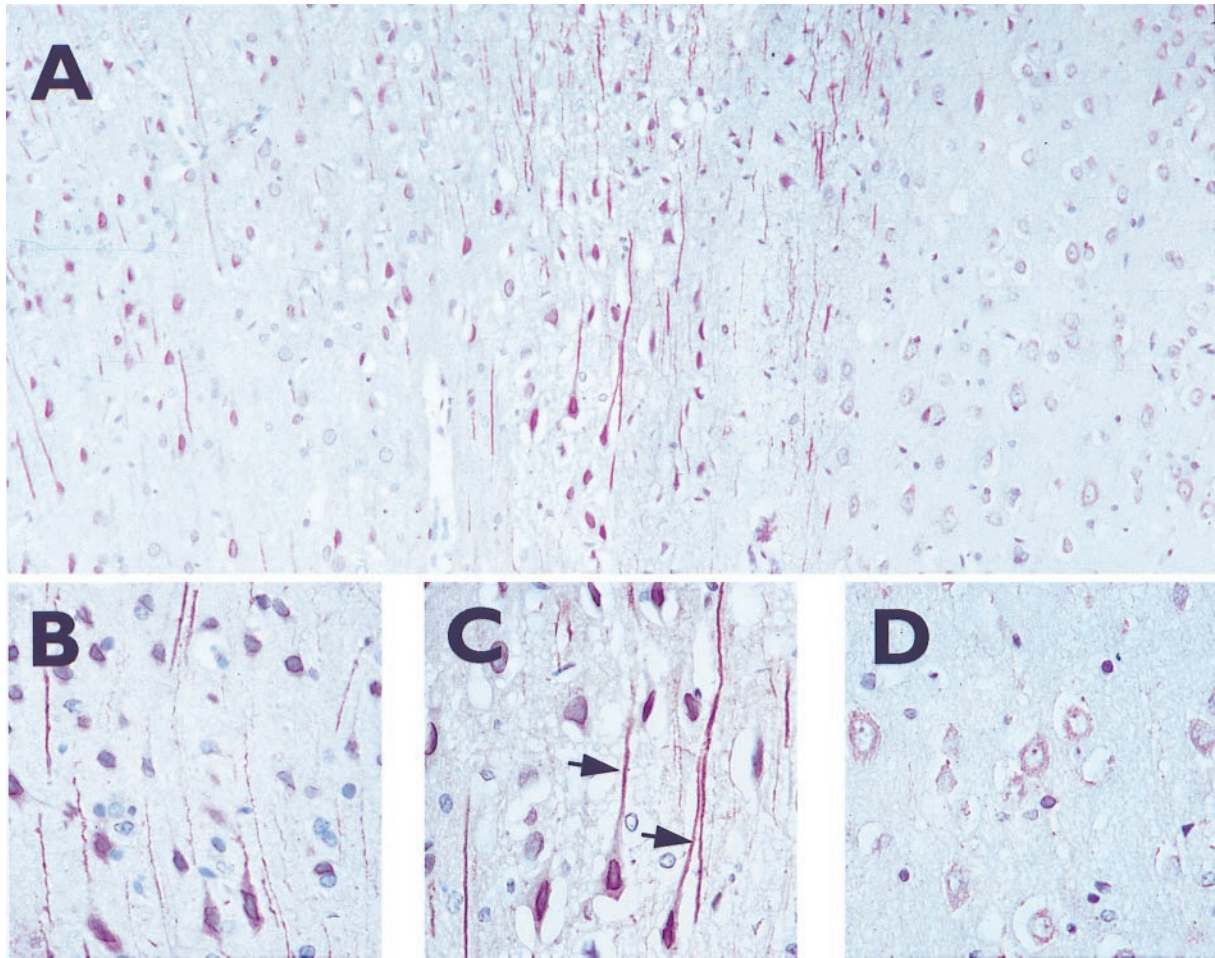


Fig. 6. Immunohistochemical staining of STC in brains of rats subjected to experimental ischemia. (A) Six hours after induced focal brain ischemia covering the infarct core, “penumbra” and peripheral area. (B–D) Greater magnifications of the corresponding areas shown in A. Arrows indicate staining of neuronal processes.

of cerebellum and the large neurons in basal nuclei also stained for STC (12).

Immunohistochemical stainings of sections from the brain of a patient who died within 15 h after onset of ischemic stroke revealed a clearly altered distribution of STC in the neurons close to the infarcted area. When compared with neurons in corresponding areas of the contralateral hemisphere, there was an overall increased intensity of staining with a prominent reactivity in the cytoplasm of larger cortical neurons and in the neuronal processes (Fig. 5A and B). This increased and redistributed neuronal staining of STC was less notable in brain sections obtained from a similar location of the ipsilateral hemisphere harboring a 3-day old ischemic infarct (Fig. 5C). Control stainings with normal rabbit serum or with STC antibodies preabsorbed with recombinant STC protein gave no neuronal staining (data not shown).

Altered STC Expression in Experimental Ischemic Brain Insults in Rat.

To further investigate the changes in STC expression in cerebral neurons in response to ischemia, we studied brains from rats subjected to experimental transient focal ischemia. In sections from the ischemic core, a slight decrease in STC staining in neurons was seen already after 2 h. After 6 h, only a faint

cytoplasmic STC staining remained in the swollen neurons (Fig. 6), and it decreased further on the third day in parallel with the maturation of the infarct. A redistributed and up-regulated expression of STC, corresponding to that observed 15 h after an infarct in the human brain, was seen in the “penumbra” zone surrounding the infarct core. The neurons displayed a strong cytoplasmic immunoreactivity for STC, which was also translocated to the neuronal processes (Fig. 6). This accentuated and redistributed pattern of STC in the neurons of the “penumbra” area was most prominent at 2 and 6 h. *In situ* hybridization using an *stc* anti-sense RNA probe revealed an up-regulated *stc* gene expression in the neurons of the “penumbra” zone (Fig. 7). Hybridization with a control, sense-*stc* RNA probe did not give any specific reaction (data not shown). The expression of STC declined gradually, and, by day 7, returned to the pattern observed in sections of brains from sham-operated animals or from the noninfarcted, contralateral hemisphere (Fig. 8). The neurons did not stain with normal rabbit serum or antibodies preabsorbed with recombinant STC (data not shown).

Discussion

We originally reported that expression of STC is induced in mammalian neurons during terminal differentiation both *in vitro*

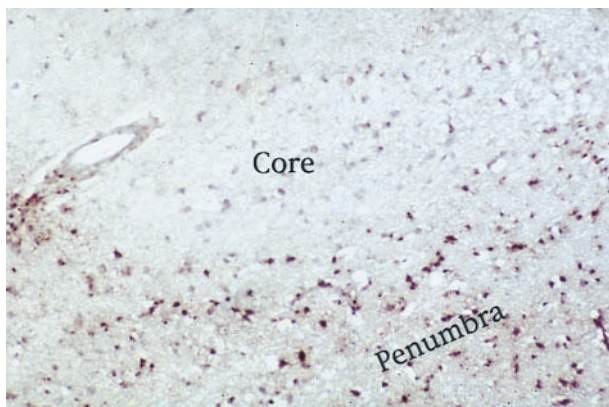


Fig. 7. *In situ* hybridization with an *stc* anti-sense RNA probe showing an up-regulated neuronal expression of *stc* mRNA in the "penumbra" zone surrounding the core of a 6-h-old experimental infarct in the rat brain.

and *in vivo* (12). It raised the question why this evolutionarily highly conserved glycoprotein hormone, which regulates calcium, and phosphate homeostasis in fish (3) is constitutively expressed in the human nervous system? Here, we show a redistributed and up-regulated expression of *STC* in brain neurons in response to cerebral ischemia in rats and humans. We also present data demonstrating that neural cells expressing *STC* display increased resistance against hypoxic challenge *in vitro*.

High concentrations of environmental calcium are known to trigger increased *STC* synthesis in fish (2). Analogously, we found that cultivation of the human neural-crest-derived Paju cells in hypercalcemic medium induced *STC* expression independently of neural differentiation. It is therefore conceivable that calcium may

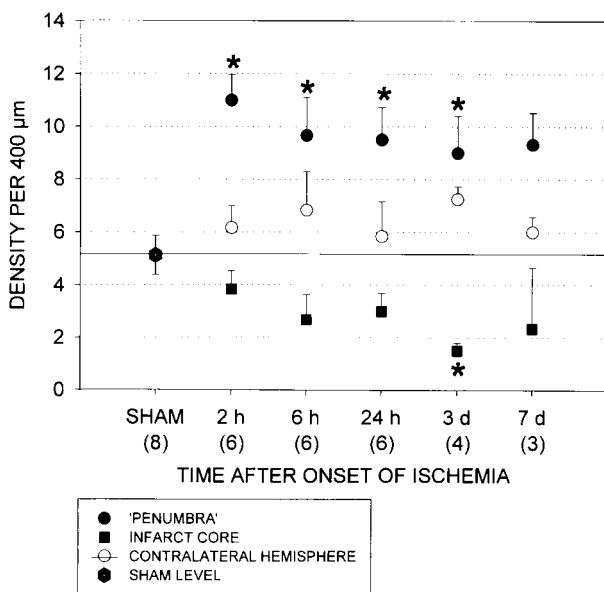


Fig. 8 The density of *STC*-stained neuronal processes was histologically quantitated in the cerebral cortex in three locations of the infarcted rat brains: the ischemic core, the narrow rim surrounding the infarct ("penumbra"), and the homologous area in the contralateral, nonischemic hemisphere. Corresponding cortical areas were examined in sham-operated animals. An investigator blinded to the source of the samples counted the numbers of vertically oriented *STC*-stained neuronal processes cut by a 400- μ m bar placed horizontally on the cerebral cortex. The data are expressed as mean \pm SE. Statistical tests were performed according to one-way ANOVA followed by Dunn's post hoc test. *, $P < 0.05$ for the given number of animals in each group.

also trigger the up-regulated and redistributed expression of *STC* that we observed in the neurons surrounding the ischemic core.

An elevated intracellular concentration of free calcium is considered a primary neurotoxic mechanism in brain damage induced by different means, including ischemia (13, 14). The increased concentrations of cytoplasmic free calcium after hypoxia are derived both from an influx and a release from intracellular stores, such as mitochondria and endoplasmic reticulum (27). Recent evidence indicates that mobilization of the intracellular calcium stores is in itself sufficient to activate neuronal apoptosis (28).

The pyramidal neurons of the cerebral cortex and hippocampus are known to carry the highest density of calcium channels, especially agonist-gated channels such as NMDA-channels. This seems to correlate with their selective vulnerability to ischemic, epileptic, and hypoglycemic cell death through calcium-mediated necrosis (13).

Our present results indicate that the elevated *STC* expression protects neurons against potentially harmful calcium levels after hypoxia. This notion is supported by our finding that neural cells constitutively overexpressing transfected *STC* display elevated resistance to treatment with CoCl_2 , which mimics hypoxic stress and leads to influx of calcium (29). *STC*-expressing cells also displayed increased resistance to mobilization of intracellular calcium accomplished by treatment with thapsigargin. It is tempting to speculate that, during phylogenesis, terminally differentiated cerebral neurons have retained *STC* as a means to guard the cellular integrity.

Although the molecular details of the mode of action of *STC* in neurons still need to be elucidated, *in vivo* studies have demonstrated that transfusion of rats with h*STC* increases the reabsorption of Pi in renal tubular epithelium (10). *STC* itself does not bind or sequester calcium, but recent findings indicate that the sodium-dependent phosphate cotransporter (NaPi) is a primary target of *STC* activity (10). Here, we show that addition of *STC* *in vitro* to Paju cells stimulated uptake of Pi. Moreover, we have observed that Paju cells overexpressing *STC* display a higher steady-state rate of Pi uptake (our unpublished observations). Pi has been shown to buffer intracellular free Ca^{2+} by increasing its sequestration to organelles (30). These findings are interesting in view of a recent study demonstrating that addition of inorganic phosphate increases neuronal survival *in vitro* during the acute phase after oxidative and excitotoxic insults (31). Glinn *et al.* (32, 33) reported that Pi influx stimulates ATP synthesis and enhances the energy charge of neurons cultivated from fetal rat brain. They also found that neurons preexposed to Pi had higher steady state levels of ATP than Pi-starved cells. Elevated stores of high-energy phosphate have been found to improve neuronal survival under excitotoxic conditions (31, 33).

There were, however, no measurably increased levels of ATP in *stc*-transfected or *STC*-treated cells (data not shown), whereas Paju/*STC* cells showed a significantly increased ability to maintain the ATP synthesis in a hypoxic environment.

Taken together, our findings demonstrate a previously uncharacterized neurochemical control mechanism by which *STC*, known to regulate calcium and phosphate homeostasis in fish, contributes to the protection of neurons challenged by hypoxia or ischemia. Similar patterns of *STC* expression were found in rat and human stroke. Manipulation of the *STC* system may therefore offer an approach to therapeutic interventions aimed at limiting the damage after brain insults.

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