Neuroserpin is expressed in the pituitary and adrenal glands and induces the extension of neurite-like processes in AtT-20 cells

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Two cDNAs encoding the serine protease inhibitor (serpin) neuroserpin were cloned from a rat pituitary cDNA library (rNS-1, 2922 bp; rNS-2, 1599 bp). *In situ* hybridization histochemistry showed neuroserpin transcripts in the intermediate, anterior and posterior lobes of the pituitary gland and medullary cells in the adrenal gland. Expression of rNS-1 mRNA was restricted to selected cells in the pituitary gland. Analysis of purified secretory-granule fractions from pituitary and adrenal tissues indicated that neuroserpin was found in dense-cored secretory granules. This result suggested that endocrine neuroserpin may regulate intragranular proteases or inhibit enzymes following regulated secretion. To investigate the function of neuroserpin in endocrine tissues we established stable anterior pituitary AtT-20 cell lines expressing neuroserpin. Cells with increased levels of neuroserpin responded by extending neurite-like processes. Extracellular proteolysis by serine protease plasminogen activators has been suggested to regulate neurite outgrowth. As neuroserpin inhibits tissue plasminogen activator (tPA) *in vitro*, we measured plasminogen-activator levels. Zymographic analysis indicated that AtT-20 cells synthesized and secreted a plasminogen activator identical in size to tPA. A higher-molecular-mass tPA–neuroserpin complex was also observed in AtT-20-cell conditioned culture medium. tPA levels were similar in parent AtT-20 cells and a stable cell line with increased levels of neuroserpin. There was no accumulation of a tPA–neuroserpin complex. Together these results identify endocrine cells as an important source of neuroserpin. Moreover they suggest that neuroserpin is released from dense-cored secretory granules to regulate cell–extracellular matrix interactions through a mechanism that may not directly involve tPA.

Key words: endocrine, serpin, tissue plasminogen activator.

INTRODUCTION

Tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are serine proteases which act extracellularly to cleave the inactive proenzyme plasminogen to form active plasmin. In the blood plasmin degrades fibrin, the major protein component of blood clots [1]. In addition to its role in fibrinolysis, the plasminogen activator/plasmin system has been implicated in biological processes involving tissue remodelling such as ovulation [2,3], metastasis [4,5] and neuronal plasticity [6,7]. Plasminogen activators are expressed in many tissues, including a number of endocrine tissues. Moderate levels of tPA enzymic activity have been measured in the adrenal glands, testis, thyroid and ovaries [8]. Islets of Langerhans isolated from rat pancreas have also been reported to contain and secrete plasminogen-activator activity [9]. More recent immunocytochemical studies have localized expression in the rat pancreas to a subpopulation of somatostatin cells [10]. tPA has also been reported in the rat adrenal medulla [10] and pituitary gland [11,12]. In the pituitary, cells containing growth hormone, prolactin, luteinizing hormone and adrenocorticotropin have been shown to express plasminogen activators. The function of tPA in these endocrine tissues is not known. Speculative roles range from release of biologically active peptides from inactive precursors to regulation of vascular permeability [9].

The enzymic activity of tPA is regulated by specific protease inhibitors belonging to the serpin superfamily. These include plasminogen-activator inhibitor-1 [13], plasminogen-activator inhibitor-2 [14] and protease nexin-1 [15]. More recently neuroserpin has been identified as an inhibitor of tPA. Neuroserpin was identified as an axonally secreted serpin of both central-nervous-system and peripheral-nervous-system neurons [16]. Sequence analysis suggested that neuroserpin was likely to inhibit trypsin-like serine proteases. Subsequent studies have shown that neuroserpin inhibits the serine proteases tPA, uPA, trypsin, nerve growth factor γ, plasmin and thrombin [17,18]. Kinetic analyses suggest tPA is the most likely target for neuroserpin.

The predicted role of neuroserpin as an inhibitor of tPA and the expression of tPA in a number of endocrine cell types prompted us to examine a possible role for neuroserpin in endocrine tissues. In this study we have cloned two neuroserpin cDNAs from a rat anterior pituitary cDNA library and used *in situ* hybridization histochemistry to localize neuroserpin transcripts in adult pituitary and adrenal glands. We have also found that neuroserpin is stored in neuroendocrine-derived dense-cored secretory vesicles. Furthermore, we show that overexpression of neuroserpin in AtT-20 cells leads to extension of neurite-like processes.

EXPERIMENTAL

Cloning, Northern-blot analysis and *in situ* hybridization histochemistry of rat neuroserpin

Two degenerate oligonucleotide primers based on conserved sequences that bracket the reactive-centre residue of chicken neuroserpin were used to amplify a $\approx$ 650-bp fragment from rat pituitary first-strand cDNA. The amplified DNA was subcloned into M13mp18 for sequence analysis and then used as a probe to isolate two neuroserpin cDNA clones (rNS-1, 2917 bp; rNS-2, 1599 bp) from a rat pituitary cDNA library. Both clones were sequenced using an ABI 377 DNA sequencer. For Northern

Abbreviations used: tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; GST, glutathione S-transferase.

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blotting, RNA (10 μg) extracted from adult Sprague–Dawley rat pituitaries (TRIzol reagent; Life Technologies, Rockville, MD, U.S.A.) was probed with neuroserpin probes rpNS115, rpNS895 or rpNS1615 (see below)

In situ hybridization histochemistry

RNA probes were prepared to neuroserpin sequences 115–687 (rpNS115), 895–1487 (rpNS895) and 1615–2184 (rpNS1615) using T7 and T3 RNA polymerase (Ambion, Austin, TX, U.S.A.) and [^{35}S]UTP (Dupont NEN, Boston, MA, U.S.A.). Each probe was amplified using PCR and included T3 and T7 primer sequences to allow the preparation of sense and antisense probes. Details of the *in situ* hybridization histochemistry methodology are available at http://intramural.nimh.nih.gov/lcmr/snge/. Rat tissue sections were hybridized with sense and antisense riboprobes encoding either 5′ or 3′ probe sequences. Sections were opposed to Biomax MS film and then dipped in NTB3 nuclear track emulsion (Kodak). Sections were exposed to emulsion for 20 days at 4 °C before development with Kodak Dektol at 15 °C and counterstaining with cresylviolet. All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Preparation of neuroserpin antisera

Antibodies were prepared against a neuroserpin-glutathione S-transferase fusion protein (NS-GST, nucleotides 235–805 of rat NS-2 cDNA) and a synthetic peptide [NS-PEP, the C-terminus of rat neuroserpin, H-CGRVMHPETMNTSGHDFEEL-OH, coupled to diptheria toxoid by the manufacturer (Chiron Technologies, Clayton, Victoria, Australia)]. For analysis of neuroserpin expression in AtT-20 cells (see below) affinity-purified NS-PEP antiserum was used. The synthetic peptide was coupled to immobilized iodoacetyl on a cross-linked agarose (Pierce, Rockford, OR, U.S.A.) through the N-terminal cysteine residue and peptide-specific antibodies prepared as described by the manufacturer.

To test antibody specificity, recombinant neuroserpin was expressed in BSC-40 cells using the *Vaccinia* virus/T7 RNA polymerase hybrid system as described previously [19]. Equivalent amounts of protein (4 μg) from transfected or control cells were prepared for Western blotting and detected using both neuroserpin antisera (final dilutions: NS-GST, 1:2000; NS-PEP, 1:5000). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL®) as described by the manufacturer (Amersham Pharmacia Biotech, Uppsala, Sweden).

Analysis of immunoreactive neuroserpin in neuroendocrine secretory granules

Membrane and soluble fractions of bovine pituitary intermediate and neural-lobe secretory granules and bovine adrenal medullary chromaffin granules were prepared as described previously [20–23]. For Western-blot analysis 20 μg of soluble or membrane protein was used.

Preparation and characterization of stable mouse anterior pituitary AtT-20 cell lines expressing recombinant neuroserpin

AtT-20 cells were transfected with pcDNA3.1-NS using Lipofectamine (Life Technologies) and stable cell lines were identified following selection using the aminoglycoside antibiotic G418 (500 μg/ml). Neuroserpin expression levels in clonal cell lysates were determined by Western-blot analysis using affinity-purified NS-PEP antiserum.

The extension of neurite-like processes in parent AtT-20 cells and two stable cell lines overexpressing neuroserpin (NSAT-2 and NSAT-25) was quantified following staining of cells with the lipophilic tracer CM-DiI (Molecular Probes, Eugene, OR, U.S.A.). Cells were plated at a density of 1.4×10^4 cells/cm^2. Cells were fixed in 2% (w/v) paraformaldehyde/0.1% (v/v) glutaraldehyde 7 days after plating and stained with CM-DiI (1.5 μg/ml in PBS) at 4 °C for 60 min. Stained cells were photographed using a Zeiss Axiovert S100 inverted fluorescence microscope with a rhodamine filter set and neurite length was quantified using Analytical Imaging Station imaging software (AIS version 3.0, Imaging Research, Brock University, Ontario, Canada). A neurite was defined as a projection that was longer than half the longest length of the cell. Data were collected from between 56 and 67 cells for each cell line. For statistical analyses the total neurite lengths from individual cells were summed. To test the significance of differences observed in neurite lengths we performed a one-way analysis of variance (ANOVA).

Determination of tPA and neuroserpin levels in AtT-20 cells

tPA levels were measured in medium samples from AtT-20 and NSAT-2 cells by zymography [24]. For analysis of secreted tPA, cells were cultured in Opti-MEM I medium (Life Technologies). On the fourth day cells were extracted in 1 × lysis buffer (50 mM

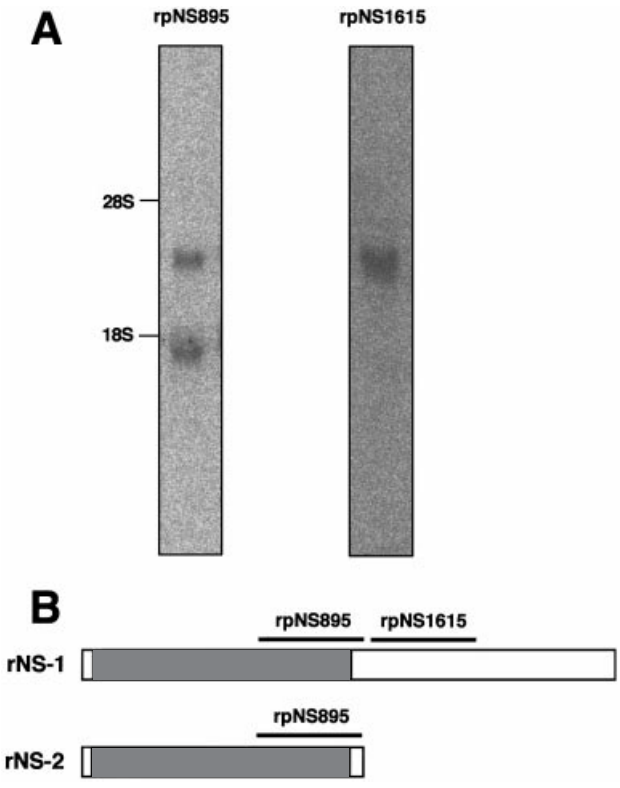

Figure 1 Identification of neuroserpin mRNAs in the rat pituitary gland by Northern-blot analysis

(**A**) Total RNA (10 μg) from the rat pituitary gland was loaded on to lanes of an agarose/formaldehyde denaturing gel and transferred to a nylon membrane for Northern-blot analysis. Membranes were hybridized with a cDNA probe recognizing both rNS-1 and rNS-2 cDNAs (rpNS895) or a cDNA probe specific for rNS-1 (rpNS1615). The migration positions of 28 S and 18 S rRNA are indicated. (**B**) Schematic of rNS-1 and rNS-2 cDNAs showing the relative positions of the rpNS895 and rpNS1615 probes. The coding sequence is shaded.

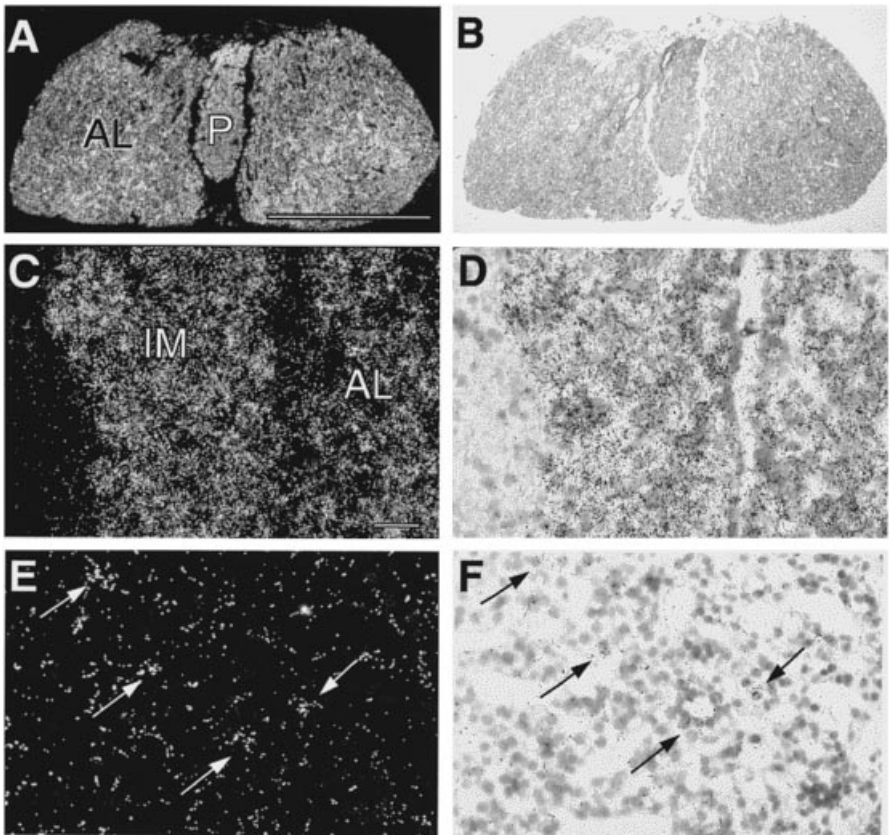

Figure 2 Analysis of neuroserpin expression in adult rat pituitary by *in situ* hybridization histochemistry

Coronal sections of the adult rat pituitary gland were hybridized with a riboprobe recognizing both rNS-1 and rNS-2 cDNAs (**A**–**D**) or a riboprobe specific for rNS-1 (**E**, **F**). Dark-field (**A**, **C**, **E**) and bright-field (**B**, **D**, **F**) images of the same field are shown. The anterior (AL), intermediate (IM) and posterior (P) lobes are indicated. Arrows indicate selected cells in the anterior lobe that are selectively labelled. The scale bar represents 1 mm in **A** and **B** and 200 μm in **C**–**F**.

Hepes, 150 mM NaCl, 10 mM EDTA, 1 % Triton X-100, 2 mM sodium pyrophosphate, 10 mM sodium fluoride; four freeze–thaw cycles) and medium samples were concentrated 10-fold by ultrafiltration (Centricon-10, Amicon, Danvers, MA, U.S.A.). Cell lysate (20 μg), equal volumes of concentrated medium (25–30 μg of protein), recombinant tPA (20 pg) and recombinant tPA complexed with neuroserpin in conditioned medium from *Drosophila* S2 cells overexpressing neuroserpin (R. M. Hill and N. P. Birch, unpublished work) were processed for zymography. No lytic zones were seen in zymography experiments when plasminogen was omitted, indicating that casein hydrolysis was plasminogen-dependent. Levels of tPA in AtT-20- and NSAT-2-cell-conditioned medium were estimated by comparison with lysis zones generated by known amounts of recombinant tPA (Actilyse, Boehringer Ingelheim, Auckland, New Zealand). Immunoreactive neuroserpin levels were determined in AtT-20- and NSAT-2-conditioned medium by quantitative Western blotting. Concentrated media samples and dilutions of a recombinant histidine-tagged neuroserpin standard (purified from a rNS-2 recombinant *Drosophila* S2 cell line; L. C. Coates and N. P. Birch, unpublished work) were fractionated on SDS/polyacrylamide (10 %) gels and immunoreactive neuroserpin detected by chemiluminescence using a Fuji LAS1000 imaging system. Levels of immunoreactive neuroserpin were determined by quantification of the light output using Image Gauge software (version 3.0, Fuji Photo Film, Tokyo, Japan).

RESULTS

Rat pituitary first-strand cDNA was used as a template to amplify a rat neuroserpin-specific probe using degenerate primers based on the published chicken neuroserpin sequence [16]. This probe was used to screen a rat pituitary cDNA library. Two neuroserpin cDNAs were cloned and sequenced. rNS-1 was 2917 bp and rNS-2 was 1599 bp (GenBank accession numbers AF193014 and AF193015 respectively). Both clones encoded 410-amino-acid proteins and differed at only two amino acids. Leu^{12} and His^{177} in rNS-1 were substituted for Met^{12} and Gln^{117} in rNS-2. Both substitutions were the result of single base changes. The principal difference between the two clones was the length of the 3′ untranslated region reflecting alternative use of polyadenylation signals. rNS-2 showed 93 % and 92 % similarity to mouse and human neuroserpin, respectively. The reactive-centre loop region of our rat clones differed from the published chicken [16] and mouse sequences [25] only by the conservative substitution of valine for alanine at residue 372 [26].

To investigate the distribution of both rat neuroserpin mRNA species, probes able to recognize both neuroserpin cDNAs or specific for rNS-1 were amplified by PCR. Rat pituitary RNA hybridized with a probe encompassing a 3′ region of predominantly coding sequence common to both rNS-1 and rNS-2 (rpNS895) recognized two mRNA species with sizes of $\approx$ 3 and $\approx$ 1.6 kb (Figure 1A). A second 5′ probe (rpNS115), also encoding sequence common to rNS-1 and rNS-2, gave identical

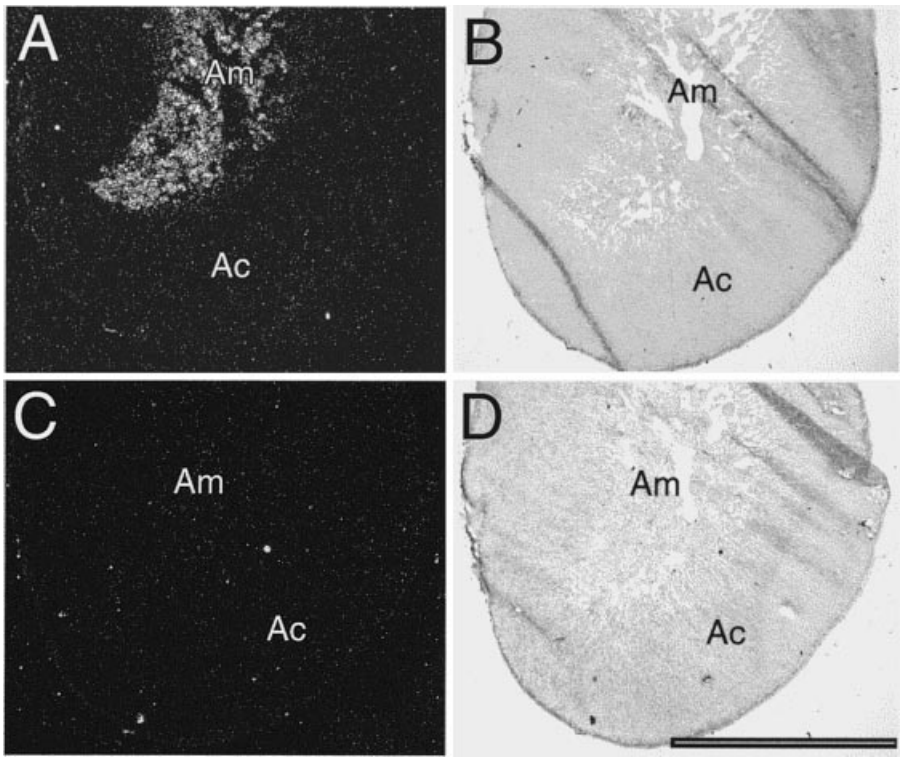

Figure 3 Identification of neuroserpin mRNA in the adult adrenal gland by *in situ* hybridization

Coronal sections of the adult rat adrenal gland were hybridized with a riboprobe recognizing both rNS-1 and rNS-2 cDNAs (**A**, **B**) or a riboprobe specific for rNS-1 (**C**, **D**). Dark-field (**A**, **C**) and bright-field (**B**, **D**) images of the same field are shown. The adrenal cortex (Ac) and adrenal medulla (Am) are indicated. The scale bar represents 1 mm.

results (results not shown). A third probe specific for 3′-untranslated sequence unique to rNS-1 (rpNS1615) recognized only the 3-kb mRNA species (Figure 1B).

The cellular distributions of rNS-1 and rNS-2 were examined in the pituitary and adrenal glands by *in situ* hybridization histochemistry. Identical distributions were seen with rpNS115 and rpNS895 antisense probes, which hybridize to both neuroserpin transcripts. Sections probed with the control sense rpNS895 riboprobe showed no labelling (results not shown). Hybridization with the rpNS895 probe, which detects both neuroserpin transcripts, revealed high levels of neuroserpin mRNA in the intermediate and anterior lobes of the pituitary (Figures 2A–2D). Melanotropes in the intermediate lobe showed strong labelling. Most cells in the anterior pituitary were also strongly labelled. Interestingly, neuroserpin transcripts were also detected in the posterior lobe. In contrast, the rNS-1-specific probe (rpNS1615) labelled selected cells in the anterior lobe of the pituitary (Figures 2E and 2F). The peptidergic phenotype of these cells has not been identified. In the adrenal gland, labelling with the generic neuroserpin probe (rpNS895) was restricted to medullary tissue (Figures 3A and 3B). No transcripts were seen in the adrenal cortex. No labelling was seen with the rNS-1-specific probe (Figures 3C and 3D).

To probe the molecular forms and subcellular distribution of rat neuroserpin in neuroendocrine tissues we prepared two polyclonal antisera, one to residues 42–231 (as a GST-fusion protein, NS-GST) and another to residues 392–411 (as a synthetic peptide coupled to diptheria toxoid, NS-PEP). The specificity of these antisera were tested using recombinant neuroserpin expressed in BSC-40 cells. Immunoreactive neuroserpin was detected with both antibodies following expression of the rNS-2 cDNA (Figure 4A). The major immunoreactive form of neuroserpin had a molecular mass of $\approx$ 52 kDa. Minor bands of $\approx$ 48 and $\approx$ 46 kDa were also observed. The NS-PEP antiserum was significantly more sensitive than the NS-GST antiserum. No neuroserpin-immunoreactive bands were detected in untransfected cell lines, demonstrating the specificity of these antisera for analyses of endogenous forms of neuroserpin.

To determine whether neuroserpin is present in dense-cored secretory granules in neuroendocrine tissues we prepared highly enriched secretory granules from bovine pituitary glands using iso-osmotic sucrose/metrizamide gradients. Chromaffin granules were also isolated from the bovine adrenal medulla. Secretory-granule membrane and lysate fractions were prepared and immunoreactive neuroserpin detected by Western blotting using the NS-PEP antiserum. Immunoreactive neuroserpin was detected in both membrane and soluble fractions (Figure 4B). In pituitary intermediate-lobe-derived secretory granules a single immunoreactive form of neuroserpin with a molecular mass of $\approx$ 55 kDa was seen. A slightly smaller $\approx$ 53-kDa form of neuroserpin was detected in the soluble lysate from secretory granules isolated from the neural lobe of the pituitary. No membrane-associated immunoreactive neuroserpin was detected in neural-lobe secretory-granule membranes. Both the $\approx$ 55- and $\approx$ 53-kDa forms of neuroserpin were detected in chromaffin granule membrane and lysate fractions. Similar results were obtained with the NS-GST antiserum (results not shown).

To investigate the function of neuroserpin in endocrine cell lines we established a number of stable mouse anterior pituitary AtT-20 cell lines expressing recombinant neuroserpin. Parent

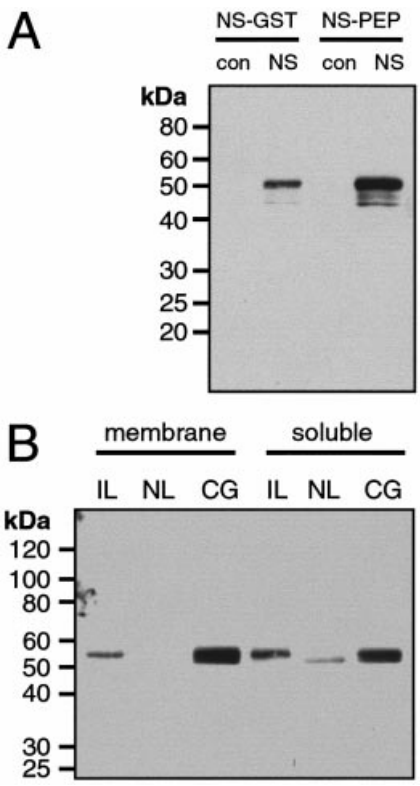

Figure 4 Western-blot analysis of neuroserpin in neuroendocrine secretory vesicles

(**A**) Media from BSC-40 cells expressing recombinant neuroserpin (NS) or from untransfected cells (con) was applied to a SDS/polyacrylamide (10%) gel under reducing conditions, blotted on to nitrocellulose membrane and probed with antisera raised against a neuroserpin-GST fusion protein (NS-GST) or a synthetic peptide corresponding to the C-terminus of neuroserpin (NS-PEP). (**B**) Purified secretory vesicles from the intermediate lobe (IL) or neural lobe (NL) of the pituitary or chromaffin granules from the adrenal medulla (CG) were lysed and separated into membrane and soluble fractions. Equivalent amounts of protein (20 μg) were applied to a SDS/polyacrylamide (10%) gel under reducing conditions, blotted on to nitrocellulose membrane and probed with the NS-PEP antiserum. For both **A** and **B**, immunoreactive neuroserpin was detected using a goat anti-rabbit secondary antibody coupled to peroxidase and enhanced chemiluminescence.

AtT-20 cells synthesize low levels of neuroserpin (Figure 5A). We characterized two cell lines expressing increased levels of neuroserpin. Neuroserpin expression resulted in a major morphological change to the cells. Parent AtT-20 cells develop neurite-like processes (Figure 5B). Both cell lines overexpressing neuroserpin showed increases in the lengths of these neurite-like processes (or neurites), with processes terminating in growth-cone-like structures. The lengths of the neurites were compared between the different cell lines. An ANOVA was performed on the total neurite lengths. This showed a highly significant ($P < 10^{-8}$) difference between the mean total neurite lengths of the different cell lines. Estimates for the mean total neurite lengths were 79 ± 19 μm for AtT-20 cells, 191 ± 20 μm for NSAT-2 cells and 128 ± 18 μm for NSAT-25 cells. Figure 5(B) shows dot plots for the total neurite length for each cell line. For each cell line a confidence interval for the mean is shown, with overall confidence of 95%. These intervals show clearly that there are significant differences between the mean neurite lengths. Estimates of the differences between each pair of cell lines, which are all significant with $P \leqslant 0.05$ and have a simultaneous confidence level of 95%, are that the mean neurite length for NSAT-25 cells is between 12 and 87 μm more than that for AtT-20 cells. The mean total neurite length for NSAT-2 cells is between about 24 and 102 μm more than that for the NSAT-25 cells.

As tPA has been suggested to be an inhibitory target for neuroserpin we measured tPA activity in the medium from parent AtT-20 and NSAT-2 cells by zymography (Figure 6). The major lysis zone in AtT-20 cell-lysate and culture medium samples from both cell lines had the same apparent molecular mass as the recombinant tPA standard, supporting its identity as tPA. The NSAT-2 cell line had higher levels of enzymically active tPA in cell lysates and slightly lower levels in culture medium in comparison with parent AtT-20 cells. A faint higher-molecular-mass band was also seen in AtT-20-cell conditioned medium. This lysis zone migrated to the same position as a neuroserpin–tPA complex and probably represented tPA that had dissociated from neuroserpin during processing of the zymograms [27]. There was no accumulation of this complex in NSAT-2-cell conditioned medium. Quantification of immunoreactive neuroserpin and enzymically active tPA indicated a $\approx$ 2900-fold molar excess of neuroserpin over tPA in NSAT-2-cell conditioned medium ($\approx$ 20 pmol immunoreactive neuroserpin; $\approx$ 7 fmol enzymically active tPA).

DISCUSSION

This is the first report of neuroserpin expression in the pituitary and adrenal glands. These results, and those of others who reported low-level expression of neuroserpin mRNA in the heart and pancreas [17,25], indicate that neuroserpin has functional roles in the endocrine system beyond synaptic-restructuring functions postulated in the nervous system [16–18]. We have cloned two neuroserpin cDNAs from a rat anterior pituitary cDNA library. Using riboprobes which detected both neuroserpin clones or a riboprobe specific for rNS-1 we have shown that the two mRNAs expressed in the pituitary reflect alternative usage of polyadenylation sites. Similar usage of alternative polyadenylation signals appears to occur in chicken [16] and probably mouse neuroserpin transcripts [18]. However, in humans, only a single 1.8-kb transcript has been detected [25]. We have found differential expression of rNS-1 and rNS-2 mRNAs in both the rat pituitary and adrenal glands. However, the implications this may have for neuroserpin protein levels remain to be determined.

Our results also show that neuroserpin is found in dense-cored secretory vesicles. The molecular sizes of 55 and 53 kDa are similar to the 52- and 47-kDa immunoreactive forms of neuroserpin reported in extracts of mouse [17]. The basis for the different-molecular-mass forms is not known but may represent different neuroserpin glycoforms. Whereas axonal secretion of neuroserpin from dorsal root ganglion neurons was one of the selection criteria for further characterization of this protein, early investigations of neuroserpin secretion suggested neuroserpin was more likely to be secreted by the constitutive secretory pathway [28]. Our results show that neuroserpin is found in dense-cored secretory granules in at least some neuronal and endocrine tissues. Therefore, neuroserpin could inhibit intravesicular serine proteases with trypsin-like specificity. Neuroserpin has also been proposed to regulate extracellular proteases and play a role in the regulation of synaptic connectivity [16,18]. Proteins that fulfil such roles are believed to be secreted following neuronal stimulation and function pericellularly [29,30]. Our results indicate that neuroserpin would show a stimulation-dependent release. Interestingly, over half of the immunoreactive neuroserpin was associated with the secretory-vesicle membrane, suggesting that neuroserpin could also act at the cell surface.

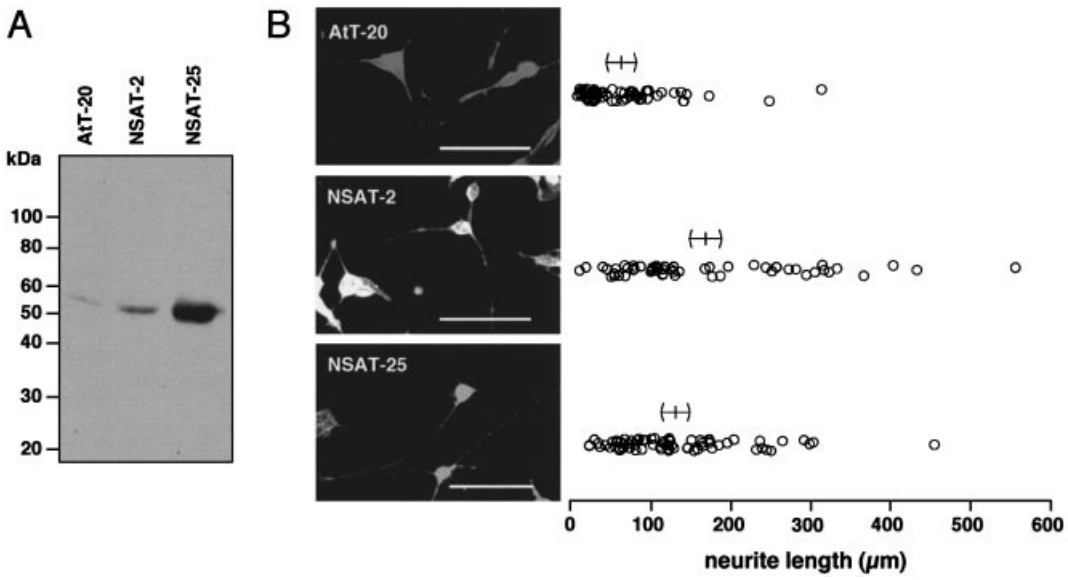

Figure 5 Neuroserpin expression in AtT-20 cells induces the extension of neurite-like processes

(**A**) Neuroserpin expression was measured in AtT-20 cells and two AtT-20 stable cell lines transfected with the rNS-2 cDNA. Equivalent amounts of protein (20 μg) were applied to a SDS/polyacrylamide (10%) gel under reducing conditions, blotted on to nitrocellulose membrane and probed with affinity-purified NS-PEP antiserum. Immunoreactive neuroserpin was detected using a goat anti-rabbit secondary antibody coupled to peroxidase and enhanced chemiluminescence. (**B**) AtT-20 cells and two stable cell lines expressing neuroserpin were stained with CM-DiI and photographed using a fluorescence microscope (scale bars, 100 μm). The total neurite lengths from each individual cell were summed and the data presented alongside as a dot plot. For each cell line a confidence interval for the mean is shown, with overall confidence of 95% (+).

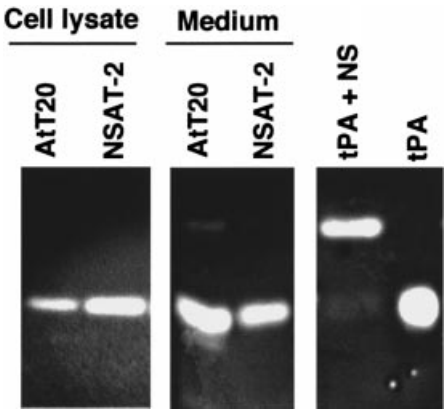

Figure 6 Measurement of tPA levels in AtT-20 and NSAT-2 cell lines by zymography

Samples of cell lysate and conditioned medium were electrophoresed on 10% SDS gels and subject to zymography for 40 h. Recombinant tPA and recombinant tPA that had been preincubated with conditioned medium containing rat neuroserpin (tPA + NS) were included as controls. Similar results were obtained from three independent experiments.

In light of the expression of neuroserpin in the anterior pituitary gland we investigated the function of neuroserpin in AtT-20 cells. This neuroendocrine cell line derived from the mouse anterior pituitary gland is widely used as a model cell line for studies of endocrine cell biology. Our results demonstrate that AtT-20 cells express low levels of neuroserpin and that increased levels of neuroserpin expression lead to extension of neurite-like processes. Although NSAT-25 cells expressed higher levels of neuroserpin than NSAT-2 cells their total median neurite length was slightly lower. This suggests that there is a threshold level of neuroserpin that mediates extension of neurite-like processes in AtT-20 cells.

How might neuroserpin regulate the extension of neurite-like processes from AtT-20 cells? Presumably it involves either direct or indirect modulation of the interaction between the extending growth cone and the extracellular matrix. Laminin and other extracellular-matrix proteins have been shown to be involved directly in neurite outgrowth [29–32]. tPA, a serine protease that is inhibited by neuroserpin *in vitro* [17,33], has been shown to degrade extracellular-matrix proteins [34,35]. The effects of tPA and plasminogen-activator inhibitors on neurite outgrowth have been investigated using the phaeochromocytoma (PC12) cell line. PC12 cells can be induced to differentiate into a sympathetic neuron phenotype by nerve growth factor. In this model system excess proteolytic activity has been suggested to be detrimental to neurite outgrowth [36] and inhibition of plasminogen activators has been linked to increased neurite outgrowth [37]. However, PC12 cells overexpressing tPA also showed increased neurite outgrowth [38]. Zymography and enzymic assays using a tPA peptide substrate [Chromozyme tPA (Roche Molecular Biochemicals, Auckland, New Zealand), results not shown] suggest strongly that AtT-20 cells synthesize tPA. In AtT-20 cells we found only a small difference in the levels of tPA between the parent and the neuroserpin-overexpressing cell lines and no accumulation of tPA–neuroserpin complexes. We also found that neuroserpin levels were much higher than tPA levels in NSAT-25 cells. These results do not implicate tPA as a key regulator of neurite outgrowth in AtT-20 cells. Neuroserpin may instead stimulate neurite outgrowth by modulating cell adhesion and cell migration independent of its function as a protease inhibitor. There is accumulating evidence that the serpin type-1 plasminogen-activator inhibitor (PAI-1) can regulate these processes. PAI-1 regulates adhesion of cells to the adhesive gly-

coprotein vitronectin through a mechanism likely to involve integrins, uPA and the uPA receptor [39–41].

Whereas AtT-20 cells are widely considered to be an endocrine cell line, Huttner and colleagues [42] have identified several properties in these cells characteristic of peptidergic neuronal cells. These include the fine structure of cytoplasmic neurite-like processes extended by AtT-20 cells, which are essentially identical to neurons and neuronal growth cones, and the expression of neurofilament proteins, microtubule-associated protein 2, tau protein and synapsin I. The insulin-secreting pancreatic β cell line RINm5F also extends neurite-like processes when exposed to the neurotrophic factor, nerve growth factor [43]. Our data suggest that neuroserpin may be an important factor mediating the neurotypic neurite-outgrowth response of endocrine cells and is consistent with the suggestion that neurons and endocrine cells share a common developmental programme. The expression of neuroserpin in embryonic tissues including endocrine tissues ([18], and E. Mezey and N. P. Birch, unpublished work) supports a role for neuroserpin during endocrine cell development. The extension of cellular processes by endocrine cells may aid in the migration of cells to their final locations in the developing gland. These data raise the possibility of links between neuronal growth factors, neuroserpin and extension of neurite-like processes that warrant further investigation. The expression of neuronal-like properties by an anterior pituitary cell line [42] suggests a common programme of differentiation with neurons and could indicate a similar neurite-extension role for neuroserpin in the nervous system. Neuroserpin has been identified in the peripheral and central nervous systems [16]. The expression pattern of neuroserpin during embryogenesis has led to the suggestion that it plays an important developmental role, particularly in later developmental processes such as synaptogenesis [18]. It has also been implicated in long-term potentiation and excitotoxin-induced neural degeneration through a mechanism assumed to involve regulation of tPA proteolytic activity [17,18]. Our results suggest that neuroserpin may also regulate synaptic growth through a mechanism independent of its function as a protease inhibitor.

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