The expression of tissue and urokinase-type plasminogen activators in neural development suggests different modes of proteolytic involvement in neuronal growth

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Summary

Tissue and urokinase-type plasminogen activators are serine proteases with highly restricted specificity, their best characterised role being to release the broad specificity protease plasmin from inactive plasminogen. It has frequently been suggested that these, and similar proteases, are involved in axonal growth and tissue remodelling associated with neural development. To help define what this role might be, we have studied the expression of the plasminogen activators in developing rat nervous tissue. Urokinase-type plasminogen activator mRNA is strongly expressed by many classes of neurons in peripheral and central nervous system. We have analysed its appearance in spinal cord and sensory ganglia, and found the mRNA is detectable by in situ hybridisation very early in neuronal development (by embryonic day 12.5), at a stage compatible with it playing a role in axonal or dendritic growth. Tissue plasminogen activator mRNA, on the other hand, is expressed only by cells of the floor plate in the developing nervous system, from embryonic day 10.5 and thereafter. Immunohistochemical and enzymatic analysis showed that active tissue plasminogen activator is produced by, and retained within, the floor plate. A mechanism is suggested by which high levels of tissue plasminogen activator produced by the stationary cells of the floor plate could influence the direction of growth of commissural axons as they pass through this midline structure.

Key words: floor plate, neuronal development, protease, urokinase, plasminogen, rat.

Introduction

Proteases are involved in developmental and metastatic events where cells implant into, or migrate through, other tissues (Valinsky et al., 1981; Saksela and Rifkin, 1988; Blasi et al., 1990). Migration of unparalleled complexity - by cell bodies and growth cones of axons and dendrites - is an essential and ubiquitous feature of neural development. The potential role of proteases in this is of considerable interest.

In the simplest of models, derived primarily from the behaviour of metastatic tumours (Moscatelli and Rifkin, 1988; Paganetti et al., 1988; Liotta and Stetler-Stevenson, 1989), secretion of proteases in advance of a migrating cell or growth cone would serve to cut a path through the surrounding tissue. In fact, plasminogen activators have been shown to be associated with migrating cephalic neural crest (Valinsky and LeDouarin, 1985), cerebellar granule cells (Krystosek and Seeds, 1981a; Seeds et al., 1990) and growth cones of cultured sympathetic and sensory neurons (Pittman, 1985; Pittman et al., 1989, 1990) and neuroblastoma cells (Krystosek and Seeds, 1981b); conversely, protease inhibitors restrict axonal penetration of three-dimensional astrocytic cultures (Fawcett and Housden, 1990) or collagen gels (Pittman and Williams, 1989) and the migration of cerebellar granule cells (Seeds et al., 1990).

If proteolysis is involved in the migration of neural cells or axonal growth cones, it must be closely regulated to prevent the destruction of the trophic factors and surface receptors that direct this movement. This could be partly achieved by restricted enzyme specificity, but in addition receptors - both for the proteases and their endogenous inhibitors - direct proteolytic action to highly specific sites within the tissue while protecting others (Blasi et al., 1990; Cunningham et al., 1990; Estreicher et al., 1990; Festoff et al., 1990; Grabham et al., 1991; Pittman et al., 1989, 1990; Schletche et al., 1990). Moreover, protease regulation of axonal growth is not mediated purely through extracellular proteolysis. Intracellular proteolytic degradation is thought to regulate axoplasmic traffic (Sahenk and Lasek, 1988), enabling the growth cone to switch from growth to terminal formation in response to physiological stimuli (Liuzzi and Lasek, 1987; Liuzzi, 1990). In addition, many proteases have domains homologous to regions of other non-prote-
olytic proteins, conferring a wide range of properties that can be independent of, or secondary to, proteolytic activity (Fenton, 1990).

Tissue and urokinase plasminogen activators (tPA and uPA, respectively) are synthesised as single polypeptide chains. These are proteolytically cleaved to yield the active, two chain enzyme, in which the smaller chain is a chymotrypsin-like serine protease. This cleaves plasminogen at a specific Arg-Val bond to release plasmin, which has much broader specificity (for lysyl- and arginyl-bonds; van Zonneveld et al., 1990). Plasminogen activators secreted by neurons can directly degrade fibronectin in extracellular matrix (Quigley et al., 1987; Seeds et al., 1990), and it is possible these proteases will have substrates other than plasminogen in developing nervous tissue (Pittman and Buetner, 1989). The heavy chains of both plasminogen activators contain domains homologous with regions found in other proteins. These domains, which differ between tPA and uPA (tPA has an N-terminal fibronectin type 1 domain, epidermal growth factor domain and two disulphide-bonded ‘kringle’ domains; uPA lacks the fibronectin, and one kringle, domain), confer binding specificities for cellular receptors and other co-factors, which accordingly differ for the two proteases (Saksela and Rifkin, 1988; van Zonneveld et al., 1990).

Most of our knowledge of the expression and action of plasminogen activators in nervous tissue comes from studies in culture (e.g. Pittman et al., 1990; Seeds et al., 1990; Kalderon, 1990). Both proteins are highly inducible, their rate of transcription (and subsequent translation) responding to a wide variety of stimuli (Schleuning and Medcalf, 1990; Botteri et al., 1990; Hantai et al., 1990; Clarke et al., 1991); hence their expression in culture need not reflect the in vivo situation. It is therefore important to establish their in vivo pattern of expression and activity during neural development.

Materials and methods

AS rats were used. Pregnancies were dated from the appearance of a vaginal plug (taken as embryonic day (E) 0.5 at noon). Rats gave birth at E21 which was taken as postnatal day (P) 0. Animals were examined at E10.5, 11.5, 12.5, 13.5, 16, 17 and 18, and P1, 3, 6, 14 and 56.

Oligonucleotide probes were 50-mers synthesised on an Applied Biosystems DNA synthesiser. Three antisense probes complementary to rat tPA cDNA (Ny et al., 1988) bases 207-158 (within the fibronectin type I domain), 1188-1139 (within the catalytic subunit) and 1778-1729 (in the 3′ untranslated region) were used, together with a control (sense) probe matching residues 1729-1778. For uPA, the antisense oligonucleotides were complementary to bases 606-557 (overlapping the site of cleavage to generate the active two-chain protease), 969-920 and 1185-1136 (both in the catalytic subunit), and the sense probe to bases 1136-1185 of mouse uPA (Belin et al., 1985). These were chosen as regions lacking homology between mouse tPA and uPA (Belin et al., 1985; Rickles et al., 1988). They were labelled with the 3′ tailing method using digoxigenin-11-deoxyuridine 5′ triphosphate (Dig-11-dUTP) (Baldino and Lewis, 1989). The reaction was carried out with 200 ng of probe in 2 mM CoCl2, 0.16 mM dATP, 0.02 mM Dig-11-dUTP (Boehringer Mannheim) and 25 units of terminal deoxynucleotidyl transferase (Boehringer Mannheim) in a final volume of 25 µl, for 1 hour at 37°C.

A 304-base riboprobe hybridising to uPA message encoding part of the catalytic domain of the protease was obtained as a HindIII-AccI fragment of mouse urokinase cDNA (kind gift of Dr D. Belin, Institut d'Histologie et d'Embryologie, Centre Medical Universitaire, Geneva) which we subcloned into a pGEM-4Z plasmid (Promega) and linearised with HindIII or EcoRI. A 515-base tPA riboprobe (alkaline hydrolysed to 150 base fragments for use) hybridising to the 3′ non-coding region of tPA message (bases 1891-2405) was produced by subcloning a SstI-Aval fragment of mouse cDNA (gift of Dr D. Belin) into pSP64. For each, sense and antisense riboprobes were labelled with Dig-11-dUTP by in vitro transcription as recommended by Boehringer Mannheim.

For in situ hybridisation, tissue was sectioned at 10 µm in the sagittal, horizontal and transverse (coronal) planes, fixed and, if necessary, dried and stored frozen as previously described (Xue et al., 1990). Slides at appropriate positions through the specimen were rehydrated, treated with proteinase K and acetic anhydride and hybridised overnight with the probe (at 37°C for oligonucleotide probes, 45°C for riboprobes) as before (Xue et al., 1990). For the oligonucleotide probes, a mixture of 5 ng of each of 3 antisense probes, or 15 ng of sense probe, in 30 µl of 50% formamide, 4× SSPE pH 7.4 (0.72 M NaCl, 0.04 M NaH2PO4, 4 mM EDTA, pH 7.4), 500 µg/ml ssDNA, 500 µg/ml yeast tRNA, 1× Denhardt’s and 10% dextran sulphate, was used; riboprobes were used at 125 ng in 25 µl of hybridisation buffer (Xue et al., 1990). After hybridisation with oligonucleotide probes, the sections were washed for 1 hour in 4× SSC at room temperature, and then four times in 1× SSC at 55°C each for 15 minutes before transferring to 100 mM Tris-HCl, pH 7.5 + 150 mM NaCl (buffer 1) for immunological detection of bound probe. Sections hybridised with riboprobes were washed in 4× SSC for 5, 15 and 30 minutes at room temperature, then treated with RNaseA and high stringency washes as before (Xue et al., 1990). Sections were then pre-incubated in buffer 1 containing 2% skimmed milk for 30 minutes at room temperature, and then incubated with anti-digoxigenin antibodies (use 2% skimmed milk as carrier protein) and alkaline phosphatase substrates as described by the manufacturers (Boehringer Mannheim). Nuclei were counterstained with the fluorescent Hoechst 33258 dye (bis-benzimide from Molecular Probes Inc, Eugene OR, used at 10 µg/ml) if required, and the sections coverslipped using Uvinert mountant (BDH).

For northern blot analysis, tissue was rapidly frozen in crushed dry ice and its poly(A)+ RNA extracted using FastTrack mRNA isolation kit (Invitron, San Diego, CA). Samples (4 µg/well) were electrophoresed in 1% agarose gels containing formaldehyde (Sambrook et al., 1989), along with RNA molecular weight markers (Marker II kit, Boehringer Mannheim), and blotted to nylon membrane (Hybond N+, Amersham) on which they were fixed by 2 minutes exposure to UV light. The membranes were hybridised with the same probes used for in situ hybridisation. For uPA, the membrane was prehybridised for 3 hours at 52°C in 50% formamide, 0.4 M NaCl, 2% skim milk powder, 400 µg/ml yeast tRNA, 200 µg/ml herring ssDNA, 4 mM EDTA, 1% SDS and 50 mM phosphate buffer pH 7.0. The uPA riboprobe (1 µg in 6 ml of the above buffer) was hybridised for 14 hours at 52°C, then the filter washed at 58°C, twice for 30 minutes with 2× SSC/0.1% SDS followed by 4 times with 0.1× SSC/0.1% SDS. For tPA, the prehybridisation buffer was 5× SSC, 10× Denhardt’s, 7% SDS, 100 µg/ml ssDNA and 20 mM phosphate buffer pH 7.0. The three oligonucleotide probes (100 ng of each in 5 ml of the above buffer) were hybridised at 53°C for 15 hours, and the membrane then washed at 48°C, twice for 30 minutes with 4× SSC/1% SDS, and then 4× 15 minutes with 1× SSC/1% SDS. Visualisation using anti-digoxigenin antibodies was as before.

tPA was visualised by immunohistochemistry using goat anti-
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human melanoma tPA IgG (American Diagnostica Inc; this cross-reacts with rat tPA) at a 1:500 dilution (overnight at room temperature), followed by rabbit anti-goat IgG (Dakopatts, 1:250, 7 hours at room temperature) and finally with FITC-conjugated affinity-purified sheep F(ab’)2 anti-rabbit F(ab’)2 (Morris et al., 1983) at 10 µg/ml. For control sections, the primary goat antibody was omitted. A variety of fixation and tissue processing procedures were assessed; freezing the tissue in powdered dry ice, followed by cryostating at 10 µm and immediate fixation of the sections in 2% paraformaldehyde for 20 minutes at room temperature, was both convenient and gave as good resolution as other procedures tried. Sections were pre-incubated in 15% skimmed milk in PBS for 30 minutes, incubations and washes were done in 5% skimmed milk in PBS.

tPA enzymatic activity was assayed using 80 µM methylsulphonyl-D-cyclohexyltyrosyl-glycyl-arginine paranitroaniline acetate (American Diagnostica Inc) in Tris-imidazole-NaCl buffer, pH 8.4 and ionic strength 0.3, as described by Magotti (1988). Spinal cord was rapidly dissected from E16 embryos, the meninges and sensory ganglia were stripped off and the spinal cord stretched straight onto aluminium foil. This was frozen in crushed dry ice, and the cord cut longitudinally to give a dorsal and ventral portion. This was homogenised in the Tris-imidazole-NaCl buffer containing 0.1% each of Triton X-100 and gelatin, membranes were pelleted by centrifugation (15 minutes in a microfuge at 4°C) and tPA activity in the supernatant was assayed immediately. Enzymatic activity was linear over a 5 hour period and assays were routinely read after 4 hours.

Results

Specificity of probes used for the individual plasminogen activator mRNAs

Given the two plasminogen activators are 40% homologous (at the amino acid level) to each other, and homologues of their individual domains are found widely in other proteins, it was essential first to verify the specificity of the probes used to detect and distinguish these two proteases. Kidney was examined since it is one of the few tissues with high levels of tPA and uPA message and enzymatic activity (Danglot et al., 1986; Rickles and Strickland, 1988; Kristensen et al., 1991). For in situ hybridisation, we have used three different oligonucleotide probes (for each enzyme) directed to sequences that are not shared between tPA or uPA, and riboprobes directed to regions of minimal homology.

Rat kidney showed distinctively different patterns of expression of message for the two plasminogen activators. This was particularly evident in the cortex, where tPA message (Fig. 1A-C) and protein (Fig. 5A) were present in cells within the glomeruli, which lacked uPA message; the latter was found on epithelial cells lining some tubules traversing the cortex (Fig. 1D,E), and more generally in the medulla (not shown). For each protease, the three different oligonucleotides used separately, and the riboprobe, all gave the same pattern of labelling. This different expression of tPA and uPA in kidney cortex is in agreement with the immunohistochemical study of human kidney (Saksela and Holthofer, 1987) and in situ hybridisation study of mouse kidney (Kristensen et al., 1991). We therefore proceeded with the analysis of developing nervous system. For this, we routinely used (and show below) labelling of tPA mRNA with the three oligonucleotide probes (applied together), although the main findings were confirmed with each oligonucleotide probe used separately, and with the riboprobe. The riboprobes gave stronger labelling, and for uPA we show labelling with the riboprobe (compare Fig. 1E with D), although again its labelling pattern was identical to that obtained with the oligonucleotides.

Fig. 1. Different patterns of labelling of rat kidney cortex by in situ hybridisation with digoxigenin probes for tPA (A-C) and uPA (D,E). For tPA, oligonucleotide probes label cells within the glomeruli (e.g. arrows in A; C is high power view of a single glomerulus) with no labelling of adjacent tubules; B is a negative control with phase-contrast optics to enable the glomerulus (arrow) to be seen. For uPA, oligonucleotide probes label some of the tubular epithelia in the cortex (D, small arrows) but not the glomerulus (larger arrow); labelling by the riboprobe (E) is much stronger, (arrows point to glomeruli which are just visible by their background labelling). Scale bars are 20 µm (C), 50 µm (B) and 100 µm (A,D,E).
uPA is expressed by developing neurons: analysis of spinal cord and sensory ganglia

The developmental pattern of uPA expression in spinal cord and associated sensory ganglia typifies that found more generally in nervous tissue, and moreover enables direct comparison with the expression of tPA. In early postnatal life, very strong labelling of uPA mRNA is seen by in situ hybridisation in large cells in the ventral horns of the spinal cord (Fig. 2A, arrows). Their morphology and position identify these as motor neurons. A control section (Fig. 2B), labelled with the sense oligonucleotide, shows no significant signal.

Are the motor neurons the only cells labelled in ventral horn? A more complete picture of the cellular composition of this area is provided by a nuclear counterstain. Given the deep purple of the primary alkaline phosphatase stain for message, we have used Hoechst dye seen under UV optics to display nuclei. This gives relatively weak labelling of the large neuronal nuclei, and much intenser labelling of glia and other cells with small, compact nuclei (e.g. neuroblasts). Fig. 2C,D shows the same field of the ventral horn under bright-field (Fig. 2C) and UV (Fig. 2D) optics. The exposure of the fluorescence picture has been adjusted so that the bright glial nuclei are well defined, although only the stronger of the neuronal nuclei can be seen. Asterisks in Fig. 2C correspond to those marking some glial nuclei in Fig. 2D. The majority of nuclei in this field are both intensely fluorescent with the Hoechst dye, and are totally unlabelled with the uPA riboprobe; the large motor neurons, on the other hand, have intense uPA reactivity filling their somatic cytoplasm.

Returning to the cross-section of entire cord (Fig. 2A), the axonal tracts (marked, on the right side of the section, with asterisks), where the cell bodies are of oligodendrocytes and astrocytes but not neurons, do not contain labelled cells. Labelling (at this stage, notably weaker than that of the motor neurons) of cells is seen in those regions of the cord containing neuronal nuclei. Again the size and position of these uPA-expressing cells suggests they are neurons, although identification is not as unambiguous as in the ventral horn; at high power they also can be seen to be the cells with Hoechst-pale nuclei, surrounded by more numerous, uPA-negative cells with compact, intensely fluorescent nuclei (not shown).

A similar analysis of the dorsal root ganglion (Fig. 2E,F) is even more informative since the cellular composition in the peripheral nervous system is simpler. The section shown is of a thoracic ganglion, the neurons of which lie astride the main nerve (dorsal root). The large size of the cells containing heavy uPA-labelling in their cytoplasm unambiguously identifies them as neurons (Fig. 2E). The exposure shown of the Hoechst nuclear stain allows their pale nuclei to be seen (curved arrows point to identical cells in Fig. 2E and F). They are surrounded by the intensely fluorescent nuclei of their satellite (glial) cells, which are devoid of uPA staining. The nerve running through the centre of the ganglion, which contains a high density of nuclei (blurred at this level of exposure) of Schwann cells and connective tissue (endoneurial and perineurial fibroblasts), is also completely negative for uPA (Fig. 2E).

This pattern of expression of uPA message was found at all embryonic ages at which motor and dorsal root ganglion neurons could be discerned. Thus essentially the same picture was seen at E18 (Fig. 3A) and E12.5 (Fig. 3C; at this younger age, the more dorsally located neurons of the intermediate grey and dorsal horn have not yet differentiated). At E12.5, motor neurons were already intensely labelled within the spinal cord, as were the dorsal root ganglion neurons. At E10.5, before either group of cells is formed, there is essentially no staining within or beside the spinal cord (Fig. 3D). Cells of the allantoic membrane on the same section (Fig. 3E) provided a strong positive control. (Curiously, there was a weak alkaline phosphatase reaction lining the ventricle (Fig. 3D) which was not seen on control sections (Fig. 3F). This labelling appeared to be extracellular (protruding into the ventricle) rather than cytoplasmic). At no stage was any uPA message found associated with the floor plate cells.

tPA expression by the floor plate

High levels of tPA message were detectable at only one site in the developing embryo, namely the cluster of floor plate cells lining the ventral midline of the ventricular cavity (Fig. 4A), from its most caudal extent in the spinal cord up to the caudal end of the third ventricle. This was first detectable at E10.5 as a weak signal confined strictly to the floor plate, which increased in intensity up to E13.5 and remained confined to the floor plate until E17. At high power, the hybridisation signal could be seen to be concentrated at the pole of the cell facing the ventricle (Fig. 4B). Sense oligonucleotides did not label any structure in the tissue (Fig. 4C,D). The protease was also detectable immunohistochemically, restricted to the floor plate (Fig. 5C,D) from which it could be extracted in an enzymatically active form. When the spinal cord of E16 embryos was bisected longitudinally, tPA enzymatic activity was found to be enriched in ventral, as compared to dorsal, spinal cord (Fig. 6). The floor plate would be only a small component of the ventral section of cord used, and inevitably some cords twisted during the cutting, so contaminating the dorsal sections with floor plate. The concentration of tPA in the floor plate is therefore likely to be considerably more than 10-fold above its level in the surrounding tissue.

Over the embryonic period E10.5 until E17, no tPA was detectable in the ventricular floor rostral to the floor plate (i.e. rostral to the notocord). We particularly examined the
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optic chiasm, where the floor of the ventricle widens so that the commissural optic fibres pass through a much extended ‘floor plate’-like array of radial glia, and could detect there neither tPA mRNA (Fig. 4F) nor antigenic activity (not shown), even though both (Fig. 4G for in situ hybridisation) were readily demonstrated in the floor plate of the spinal cord on the same sections.

By E17, tPA mRNA was detectable not only on the floor plate, but also on the adjacent ependymal cells lining the more ventral regions of the ventricle (Fig. 4G). During the
Fig. 4. Labelling for tPA message in rat brain using combination of three oligonucleotides. (A) E13 spinal cord; labelling is in the floor plate, between arrows. (B) E16, higher power view of labelling of floor plate cells which is clustered at the pole of the cell (e.g. areaheads) pointing towards the ventricle. (C, D) Control labelling in normal (C) and phase (D) optics; asterisk is just below the floor plate in each. (E) Adult rat brain, caudal end of the III ventricle where it narrows to form aqueduct; tPA labelling is of the ependymal cells lining the entire circumference of the ventricle. (F) E17 coronal section of the optic chiasm; clear area between arrows is where the optic nerve axons are crossing (the weak background labelling of the tissue has been brought out photographically to allow the anatomy to be seen; G allows comparison between a positive tPA signal and the background over nervous tissue on the same section). (G) Spinal cord on same section as F, showing labelling of floor plate plus some of the ependymal cells located higher up the sides of the ventricle. Scale bars are 100 µm (A,E), 10 µm (B), 50 µm (C,D) and 20 µm (F,G).
first postnatal week, this spread to ependymal cells lining the full extent of the ventricular surface in spinal cord and hind brain (i.e. where the floor plate had been), and additionally on ependymal cells lining the caudal regions of the third ventricle (Fig. 4E), including the lateral recess under the hippocampus. This pattern persisted as the dominant tPA labelling in the adult CNS.

Northern analysis of plasminogen activator expression

To confirm the pattern of expression of the plasminogen activators in developing nervous tissue, mRNA was analysed by northern blots (Fig. 7). The uPA riboprobe detected a single mRNA species, of 2.3 kb, present in developing spinal cord at E16 and more strongly at P1, but could not detect message in cerebral cortex at this age. However, the same band was detectable in young adult (P28) cortex. A single, relatively weak band of 2.7 kb was detected by the tPA oligonucleotides in mRNA from E16 spinal cord, but not in E16 cortex. We could not detect tPA message in cortex or spinal cord at later ages with this method (not shown). The reported sizes of uPA and tPA mRNA in the mouse are approximately 2.4 and 2.8 kb (Rickles and Strickland, 1988; rat tPA message is of similar size (Ny et al., 1988)).

Discussion

uPA expression is compatible with suggested roles for proteolytic involvement in axonal growth

In considering the role of proteases in neural development, attention has focused primarily on their potential for regulating the migration of the cell or growth cone producing the protease, either by cutting a path through tissue in advance of a growth cone or by regulating adhesion of the migrating cell. The expression of uPA message by both motor and dorsal root ganglion cells is compatible with such an ‘autocrine’ role in the movement of axonal growth cones. Both sets of neurons are born in a rostral-to-caudal gradient spanning a couple of days, with axonal growth starting in the more mature branchial regions at E11.5-12.5 (Altman and Bayer, 1984; we have converted their dates to the E0 convention we use). The major period of axonal growth by both sets of neurons extends throughout embryonic, and into early postnatal, life (Reynolds et al., 1991; Wentworth, 1984a,b; Altman and Bayer, 1984). Motor neurons grow their dendrites over the same period (Wentworth, 1984a; Altman and Bayer, 1984); dorsal root ganglion neurons have no dendrites. The onset of expression of uPA message coincides with the onset of process outgrowth by these cells. This protease may therefore be involved in enabling such movement, as suggested by studies in tissue culture. Both sympathetic and sensory neurons in culture release uPA (but not tPA) (Pittman, 1985), predominantly through their growth cones rather than via the cell body or proximal region of their neurites (Pittman, 1985; Pittman...
Plasminogen activators in nerve (et al., 1990). Released uPA is bound to cell surface receptors restricted to sites of focal adhesion (Pittman et al., 1989), where it is thought to degrade adhesive contacts, so promoting movement of the leading edge of the cell (Pittman et al., 1989, 1990; see also Estreicher et al., 1990). In addition, uPA may promote Schwann cell mitosis behind growing axons (Baron-van Evercooren et al., 1987).

Having started to express uPA message from early in their development, neurons continue to express this mRNA even in adult brain (Dent et al., in preparation; see northern blot, Fig. 7). The protease clearly cannot be involved in neuronal migration at this stage, although tissue plasticity in adult brain, such as synaptic rearrangement associated with learning (Lynch and Baudry, 1984), has also been suggested to involve proteolysis. Alternatively, changing the location to which the uPA is sent, from extracellularly at the growth cone to retention intracellularly in the axon, could change its action to one of growth inhibition (Liuzzi, 1990). The appearance of uPA mRNA, which we have described for motor and sensory neurons, occurred at the same stage of development for all other neurons that we could identify in central and peripheral nervous system (including the sympathetic ganglion neurons). In spinal cord, the later maturing, more dorsally located neurons of the intermediate zone and dorsal horn expressed uPA message at progressively later periods (Figs 2A, 3A and C). This resulted in more intense labelling, at P1 compared to E16, of the uPA message in northern blots of spinal cord (Fig. 7). Neural development proceeds later in the cerebral cortex than the spinal cord, and here the proportion of cells expressing uPA is too low even at P1 for its signal to be detected in northern blots (Fig. 7).

tPA expression by stationary floor plate cells: a role in redirecting axonal growth?

Given the expectation that proteolysis will be associated with cell movement, the finding that tPA is expressed, and active enzyme retained locally, by the cells of the floor plate was unexpected since these cells are among the few sessile structures in developing nervous system. However, the high level of tPA retained here could play a critical role in controlling the direction of growth of axons passing through the floor plate.

Floor plate cells act as guide posts for growing commissural fibres in the spinal cord (and probably in hind brain (Bourrat and Sotelo, 1990)). Commisural axons in the spinal cord grow from their medially located cell bodies towards the floor plate on the ventral midline (Fig. 8A; Wentworth, 1984b; Bovolenta and Dodd, 1990), responding to a diffusible chemotropic signal (Tessier-Lavigne et al., 1988; Yaginuma and Oppenheim, 1991). On passing through the floor plate, they immediately make a right-angled turn to head anteriorly towards the brainstem (Fig. 8A), initially growing beside the floor plate but never re-entering it; rather, as they advance they move progressively more laterally (i.e. away from the floor plate) (Bovolenta and Dodd, 1990). At the same time, the contralateral axons grow to the floor plate, ignore the emerging ipsilateral axons which are turning anteriorly, and enter the floor plate; on emerging, they turn anteriorly towards a signal which the ipsilateral axons are ignoring. Thus passage of the axons through the 50-100 µm width of the floor plate induces a change in the growth cones, so they ignore one set of signals that they had previously followed, and respond to signals that they had previously ignored.

It is difficult to envisage a mechanism reliant solely on a changing pattern of gene expression or protein synthesis at the neuronal cell body, followed by protein transport to the growth cone, which could produce such precise re-orientation of axonal growth. On the other hand, the presence within the floor plate of high levels of a protease with very limited substrate specificity provides a mechanism whereby a latent set of receptors could be activated, and a redundant set removed, very efficiently. The change in growth characteristics would be entirely conditional upon passage of the axons through the floor plate, and so apply equally to the first axons, which grow only a short way to the midline, and later ones, which travel many times this distance.
It is a mechanism that would, over an extended developmental period, allow the migration of a set of axons to respond rapidly and precisely to a spatial cue. Although tPA (and uPA) are not present at the midline in the forebrain, i.e. rostral to the floor plate (Kingsbury, 1930), a similar proteolytic regulation of axonal growth by a different selective protease could be involved in commissural growth there, since the guidance of any set of axons that opt for a contralateral, rather than ipsilateral, route poses similar problems at the molecular level.

Could tPA cleave known axonal molecules?

For the commissural axons of the spinal cord, two surface molecules are known which are selectively distributed in development: one (TAG-1) to the pre-floor plate surface of the axons, the other (L1) to their anteriorly directed, post-floor plate surface (Kingsbury, 1930), a similar proteolytic regulation of axonal growth by a different selective protease could be involved in commissural growth there, since the guidance of any set of axons that opt for a contralateral, rather than ipsilateral, route poses similar problems at the molecular level.

However, it is pertinent to point out that TAG-1, and not other related neuronal surface molecules, has one site near its carboxy terminus that is a candidate for tPA cleavage. The established site of tPA cleavage of plasminogen is the RV bond (using single letter code) in a tight, disulphide-bonded loop, CPGRVVGC (Forsgren et al., 1987; Higgins and Bennett, 1990). A very similar sequence, Y. Sumi and others

Fig. 8. (A) Diagram showing the course of growth of commissural axons, ventrally to the floor plate (shaded with lines), through it and then turning immediately rostrally to grow towards the brain, initially beside the floor plate but then growing more laterally, away from the midline. (B) Diagram representing the structure of TAG-1 (left; the disulphide-bonded oval represents the immunoglobulin-type domains, the rectangles the fibronectin type III domains, the arrow points to the interdomain region containing the PRVAP sequence shown in Table 1), expressed on the pre-floor plate surface of a commissural axon, and L1 (right, immunoglobulin and fibronectin domains as for TAG-1) on the post-floor plate surface.

Table 1. Site of cleavage of plasminogen by tPA (arrow), the putative cleavage site in TAG-1, and the corresponding residues between the second and third fibronectin type III repeats of other neural surface molecules

<table>
<thead>
<tr>
<th>Species/molecule</th>
<th>Residues</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Human plasminogen</td>
<td>558-566</td>
<td>CPGRVVGC</td>
</tr>
<tr>
<td>Rat TAG-1</td>
<td>784-788</td>
<td>PRVAP</td>
</tr>
<tr>
<td>Mouse L1</td>
<td>792-796</td>
<td>PQVS</td>
</tr>
<tr>
<td>Mouse/rat NCAM</td>
<td>687-691</td>
<td>PTAPI</td>
</tr>
<tr>
<td>Chick NCAM</td>
<td>677-681</td>
<td>PTVIP</td>
</tr>
<tr>
<td>Human NCAM</td>
<td>810-814</td>
<td>PSEAP</td>
</tr>
<tr>
<td>Mouse F3</td>
<td>780-784</td>
<td>PTEVP</td>
</tr>
<tr>
<td>Chick F11/contactin</td>
<td>796-800</td>
<td>PMVP</td>
</tr>
<tr>
<td>Chick NgCAM</td>
<td>785-789</td>
<td>PLVP</td>
</tr>
<tr>
<td>Drosophila fasciculin II</td>
<td>745-749</td>
<td>PSQM</td>
</tr>
<tr>
<td>Drosophila neuroglian</td>
<td>815-819</td>
<td>PLDA</td>
</tr>
</tbody>
</table>

Forsgren et al., 1987; 
Furley et al., 1990; 
Moos et al., 1988; 
Barthels et al., 1987; 
Small et al., 1987; 
Cunningham et al., 1987; 
Dickson et al., 1987 (residues not assigned numbers in paper); 
Gennarini et al., 1989; 
Brummendorf et al., 1989; 
Ranscht, 1988; 
Grumet et al., 1991; 
Burgoon et al., 1991; 
Harrelson and Goodman, 1988; 
Bieber et al., 1989.
fibronecrtin type III domains on other neuronal surface glycoproteins (Table 1) or exposed elsewhere in the extracellular regions of these proteins (not shown).

If this site on TAG-1 is used by tPA in the floor plate, the larger N-terminal fragment of TAG-1 released from the membrane would contain all the immunoglobulin domains, and the RGD-containing second fibronectin domain. Two fibronecrtin domains would be left on the membrane, which may themselves be inactive. However, two similar domains of β-pleated sheets form the structure of the extensive cytokine receptor superfamily (Bazan, 1990). The two domains so exposed are in theory capable of functioning as a receptor for a trophic factor (e.g. directing growth anteriorly) by interacting with another transmembrane signalling molecule, for instance in the way that the IL-6 (Hibi et al., 1990) and CNTF (Davis et al., 1991) receptors do.

This mechanism that we have suggested for tPA redirecting the growth of commissural axons emerging from the floor plate provides a specific and apposite example of how a proteolytic mechanism might work. However, none of the surface receptors involved in directing the growth of these axons, or any substrates for tPA in the floor plate, have yet been identified, and both remain the focus of future work.

Y. S. is a Senior Scientist of Teijin Ltd (Tokyo), and M. A. R. D. was supported by a fellowship from the International Spinal Research Trust. We thank Dr D. Belin for the gifts of the mouse tPA and uPA cDNAs.

References


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(Accepted 18 August 1992)