Research article

The role of Serine Proteases and Serine Protease Inhibitors in the migration of Gonadotropin-Releasing Hormone neurons

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Abstract

Background: Mechanisms regulating neuronal migration during development remain largely undefined. Extracellular matrix cues, target site released factors, and components of the migratory neurons themselves are likely all coordinated in time and space directing neurons to their appropriate locations. We have studied the effects of proteases and their inhibitors on the extracellular matrix and the consequences to the migration of gonadotropin releasing hormone (GnRH) neurons in the embryonic chick. Chick GnRH neurons differentiate in the olfactory epithelium, migrate along the olfactory nerve and enter the forebrain. The accessibility of this coherent cell group make it amenable for studying protease/inhibitor roles in migratory processes.

Results: Affigel blue beads were used to deliver a serine protease inhibitor, protease nexin-1 (PN-1), and a target protease, trypsin, to the olfactory epithelium coincident with initiation of GnRH neuronal migration. PN-1 inhibited neuronal migration while trypsin accelerated their transit into the CNS. Prior to initiation of migration, neither PN-1 nor trypsin altered the timing of neuronal exit. Trypsin did, however, accelerate the timing of neuronal crossing into the nerve-forebrain junction.

Conclusions: These data support the hypothesis that protease activity modulates neuronal movements across barriers. Moreover, the data suggest, for the first time, that aspects of GnRH neuronal migration may be cell autonomous but modulated by ECM alterations.

Introduction

A key component regulating neuronal migration is the appropriate spatio-temporal expression of extracellular matrix (ECM) molecules which contribute to the highway along which neurons travel. Proteins, such as serine proteases and their inhibitors, could alter the quality of this highway and thus play critical roles in migratory processes [1]. Members of the serine protease inhibitor superfamily, or serpins, act by binding to and permanently inactivating their target protease(s). One member of this family, protease nexin-1 (PN-1), was first described by Monard *et al.*[2]. Though initially characterized for its ability to stimulate neurite outgrowth [2–5], PN-1 also modulates neuronal migration as exemplified by Lindner *et al.*[6]. Their studies demonstrate that PN-1 slows granule cell movement from the external to internal layers in postnatal mouse cerebellar slice cultures.
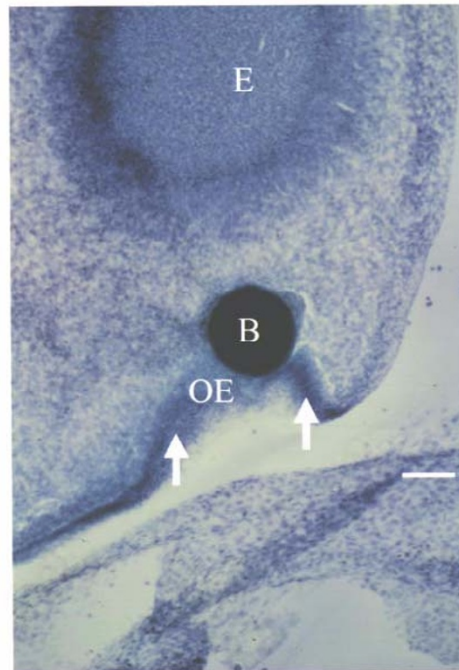
Figure 1
Sagittal section of a stage 21 chick embryo depicting the implanted bead (B) within the olfactory epithelium (OE). Tissue is counterstained with cresyl violet. Bead implantation did not disrupt the pseudostratified morphology of the placode epithelium (arrows). E=eye. Scale bar= 30 µm.

Interestingly, this inhibition of migration by protease inhibitors may be balanced by the stimulation of migration via proteases. For example *in vitro* addition of plasmin, a serine protease, accelerates the migration of neuroblastoma cells through a matrigel base by a factor of five [7]. The subsequent addition of aprotinin, a plasmin inhibitor, decreases the migratory population to the same degree [8,9]. Furthermore, Seeds *et al.*[10] demonstrated a retardation of granule cell migration in tissue plasminogen activator (tPA) knock-out mice. These data support the hypothesis that a balance exists between serine proteases and their inhibitors such that changes in this balance delay or promote neuronal migration. The present study was initiated to test this hypothesis *in vivo* by examining neuronal migration of chick gonadotropin releasing hormone (GnRH) neurons during embryogenesis. The site of origin of these cells in the olfactory placode, as well as the time course and migratory route along the olfactory nerve (ON) and into the forebrain are well documented [11–14]. Previous work in the laboratory has shown that olfactory axons emerge from the olfactory epithelium at stage 18 and are first joined by glia [15] and GnRH neurons [16] at stage 21.

In order to test whether proteolysis or its inhibition affect GnRH cellular migration, we performed experiments at two critical developmental time points. In both of these a protease or its inhibitor was applied by placing protein-coated beads at the olfactory placode. The first experiments tested whether application of either of these agents over the period of stage 21 to stage 29 affected the initial exit of GnRH neurons from the OE and/or their rate of migration along the ON and into the CNS. The second experiments tested whether GnRH neurons exited the OE irrespective of the effects of proteolysis on the maturation of the olfactory nerve. In this case protein-soaked beads were applied prior to GnRH exit (stage 17) and the effects were tested at succeeding stages up to and including their normal exit time (stages 18, 19, 20, 21). In the latter experiments the effects of proteolysis and its inhibition on the development of the olfactory nerve itself were tested using axonal, glial and neuronal outgrowth markers. These are the first experiments to demonstrate the critical roles of proteolysis and its inhibition on the regulation of GnRH cellular migration.

Results
PN-1 and trypsin modulate GnRH neuronal migration in vivo during embryogenesis
All embryos analyzed were stage 21 at the time of bead implantation and stage 29 at the time of fixation. In control embryos (n = 10) receiving a PB coated bead, GnRH

Table 1: Per cent of GnRH neurons (+/- standard deviation) in each compartment in embryos implanted with bead at stage 21 and sacrificed at stage 29. An asterisk indicates that the bead implanted side was significantly different from the side contralateral to the bead. OE, olfactory epithelium; ON, olfactory nerve; TEL, telencephalon.

Experiment	BEAD SIDE			CONTRALATERAL SIDE		
	OE	ON	TEL	OE	ON	TEL
CONTROL N = 10	23+/-6.2	56+/-9.6	20+/-9.1	25+/-10.8	57+/-10.0	18+/-11.0
PN-1 N = 12	* 37+/-14.7	54+/-14.8	* 8+/-8.5	23+/-11.0	59+/-12.5	20+/-13.6
TRYPSIN N = 11	* 21+/-5.8	* 40+/-8.9	* 40+/-8.2	34+/-13.7	50+/-11.9	14+/-7.1

Table 2: Total GnRH neuronal population of embryos implanted with a bead at stage 21 and sacrificed at stage 29. There was no significant difference in the number of GnRH neurons across groups.

Experiment	Total cell #
CONTROL	1874+/-554.9
PN-1	1968+/-708.9
TRYPSIN	1461+/-551.6

neurons in each compartment were counted on the side ipsilateral and contralateral to the bead. There was no effect of the bead on GnRH neuronal distribution (Table 1) or total number (Table 2). Sections counterstained with cresyl violet revealed no morphological abnormalities of the epithelium caused by insertion of the bead (Figure 1).

In PN-1 treated embryos, the distribution of GnRH neurons within the olfactory epithelium, nerve and brain on the side contralateral to the bead and that of controls were not significantly different indicating that the contralateral side in experimental embryos served as an internal control. Therefore, statistical analysis was performed comparing values within embryos (i.e. GnRH neuronal distribution ipsilateral to the bead was compared to that contralateral to the bead). Significantly more neurons remained within the epithelium (P < 0.03) and fewer had crossed into the brain (P < 0.002, see Table 1) on the PN-1 treated side. The effects of PN-1 on GnRH neuronal distribution cannot be attributed to changes in total cell number as there was no difference in this parameter between groups (Table 2).

Other serine protease inhibitors tested included: soybean trypsin inhibitor and Type IV chicken ovoinhibitor (data

not shown). These protease inhibitors demonstrated a range of effects on the distribution of GnRH neurons across the three compartments. Both concentrations of soybean trypsin inhibitor tested (1 mg/ml, n = 6 and 10 mg/ml, n = 4) had no significant effect on the distribution of GnRH neurons. Application of type IV chicken ovoinhibitor led to a decrease in the percent of GnRH neurons remaining in the OE (P < 0.02) and a larger percent within the telencephalon (P < 0.03).

PN-1 has four known target proteases: urokinase plasminogen activator (uPA), tissue type plasminogen activator (tPA), thrombin and trypsin [17]. Evidence exists for a role of both trypsin [18,19] and uPA [20,21] in migratory processes. Therefore, both were tested in our system. All comparisons made were of the treated side (ipsilateral to the bead) and the untreated side (contralateral to the bead).

Trypsin resulted in fewer GnRH neurons within the epithelium (P < 0.008) and along the olfactory nerve (P < 0.04) and more within the brain (P < 0.0001, see Table 1). There was no significant difference in the total number of GnRH neurons (Table 2) compared to control. The effect of trypsin is specific since in embryos receiving a uPA bead, there was no effect of the protease on the distribution of GnRH neurons (data not shown).

GnRH neuronal migration is cell autonomous but modulated by composition of the ECM

As PN-1 can stimulate neurite outgrowth, [2–5] we tested its effects on outgrowth and maturation of the olfactory nerve. Maturation of the nerve was followed by immunoreactivity to axonal, glial and neuronal markers (summarized in Table 3). All embryos analyzed were stage 17 at the time of bead implantation and stages 18–21 at the time of fixation.

Unilateral insertion of PB coated beads into the olfactory placode of stage 17 embryos did not disrupt the development of either axonal or glial components of the olfactory nerve between stages 18–21 (Table 3). The time of axonal outgrowth and sequential acquisition of axonal and glial markers were identical to that observed previously [15]. The porous nature of the bead was compatible with axonal elongation through the mesenchyme towards the developing telencephalic vesicle. In fact, immunoreactive axons were found coursing through the bead (Figure 2). Likewise, there was no alteration in the timing of GnRH neuronal migration out of the epithelium. At stage 21, GnRH neurons were found within the olfactory epithelium and along the olfactory nerve (Table 3), a distribution appropriate for this stage.

Implantation of PN-1 beads did not stimulate premature olfactory nerve axogenesis. As in controls, very few MAP1b immunoreactive neurites were present at stage 18 (Table 3). However, PN-1 application did accelerate the biochemical differentiation of the axons. For instance, under normal conditions, olfactory axons acquire immunoreactivity to NCAM at stage 20. In PN-1 treated embryos, NCAM immunoreactivity was instead present at stage 19. The sequence of appearance of all markers, however, remained identical to controls. As the length of each developmental stage varies [22], it is difficult to determine precisely the amount of time by which biochemical development of the axons is accelerated. On average, however, biochemical differentiation is advanced by 7 hours. Just as PN-1 did not alter the timing of axogenesis, it also had no effect on the timing of glial entry into the nerve (stage 21). Under these conditions, that is, accelerated biochemical differentiation of olfactory axons, GnRH neuronal distribution was not altered. GnRH neurons were found within the epithelium and along the nerve and, most importantly, none had crossed into the brain.
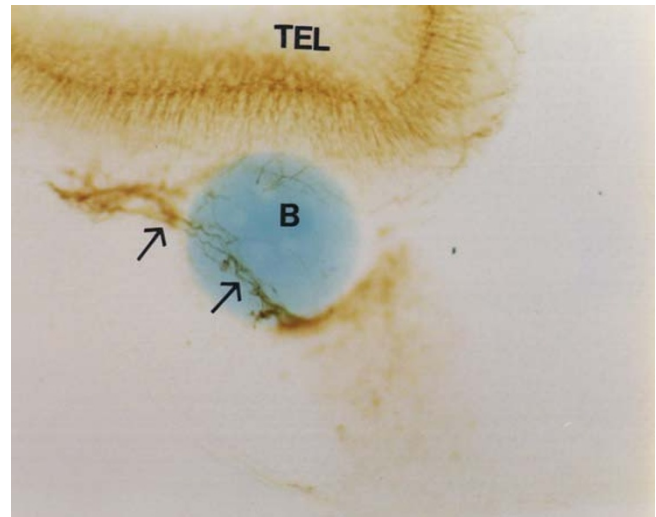

Figure 2
Sagittal section of a stage 21 chick embryo which received a PB-coated bead (B) at stage 17. Tissue is processed for immunocytochemistry using the axonal marker, MAP 1b. Immunoreactive axonal processes (arrows) extend out of the epithelium and course through the bead (arrows) towards the telencephalic vesicle (TEL). Scale bar= 10 μm.

Implantation of trypsin coated beads at stage 17 had no effect on either the timing of nerve development (axonal or glial) or the biochemical differentiation of the axons. The distribution of GnRH neurons, however, was altered (Table 3). Under normal conditions, large numbers of GnRH neurons are within the telencephalon by approximately stage 25. In the presence of trypsin, however, GnRH neurons crossed the nerve/brain junction at stage 21, representing a significant acceleration of this event. Even more significantly, these cells had continued along their normal migratory path into the presumptive septal and preoptic areas, their normal target sites.

Table 3: Time of appearance of immunoreactivity to axonal, glial and neuronal markers in embryos implanted with a bead at stage 17 and sacrificed at stages 18–21. The asterisk indicates the appearance of a marker earlier than in controls. OE, olfactory epithelium; ON, olfactory nerve; TEL, telencephalon.

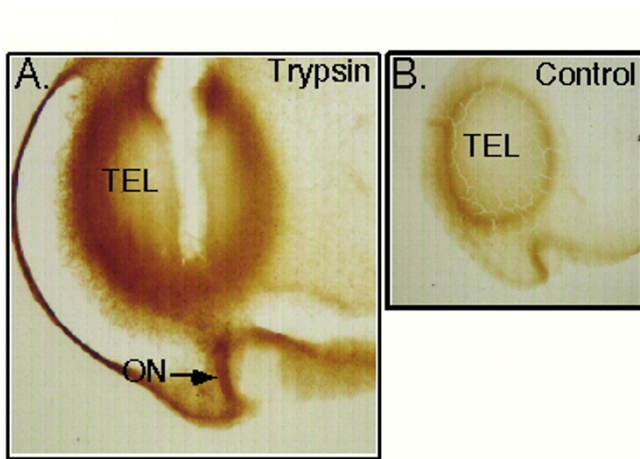|	MARKER:					
Experiment	MAP1b	NF	NCAM	PGP9.5	P_0	GnRH
CONTROL	stage 18	stage 19	stage 20	stage 21	stage 21	stage 21 OE/ON
PN-1	stage 18	* stage 18	* stage 19	* stage 20	stage 21	stage 21 OE/ON
TRYPSIN	stage 18	stage 19	stage 20	stage 21	stage 21	stage 21 OE/ON/ TEL

Figure 3
Sagittal section of stage 21 chick embryo immunostained with an anti-trypsin antibody. The olfactory nerve (ON, arrow) was immunoreactive for trypsin as were regions of the developing telencephalon (TEL). As a control, typsin protein was incubated with the anti-trypsin antibody for pre-absorption. This antigen/antibody complex was then used to immunostain sagittal sections of stage 21 chicks. Panel B demonstrates that pre-absorption eliminated all immunoreactivity in the head demonstrating the specificity of trypsin immunolocalization in experimental sections.

Figure 4
A 1 μm optical section of an El 5 mouse embryo cut in the sagittal plane at the level of the vomeronasal organ (VNO). MAP1b immunoreactive axonal bundles (A, arrows) of the vomeronasal nerve are identified by FITC fluorescence; PN-1 immunoreactivity (B, arrows) by Texas Red fluorescence. All fascicles were double labeled. Scale bar= 100 μm.

Application of uPA beads at stage 17 had no effect on axonogenesis or the nerve's biochemical differentiation or glial ensheathment. The distribution of GnRH neurons was likewise not altered (data not shown).

PN-1 and trypsin expression along the GnRH migratory route
Endogenous trypsin immunoreactivity was present in the vast majority of cells within the head of the chick during the time period studied (stage 21–29). These cells included those within the olfactory epithelium and throughout the mesenchyme as well as axonal tracts within the developing face, including the olfactory nerve (figure 3, panel A). To ensure that the immunoreactivity we see in chick is specific, trypsin protein was pre-adsorbed with the anti-trypsin antibody. This complex was then used to immunostain chick sections (stages 21–23). No immunoreactivity was evident suggesting that the staining pattern seen in the chick using the anti-trypsin antibody was specific (figure 3, panel B). Unlike trypsin, no immunoreactive uPA was present within regions of the olfactory system or the developing telencephalic vesicle during this time frame (data not shown).

Antibodies that recognize chick PN-1 are not available and thus this protease inhibitor was localized using mouse embryonic tissue at developmental times corre-
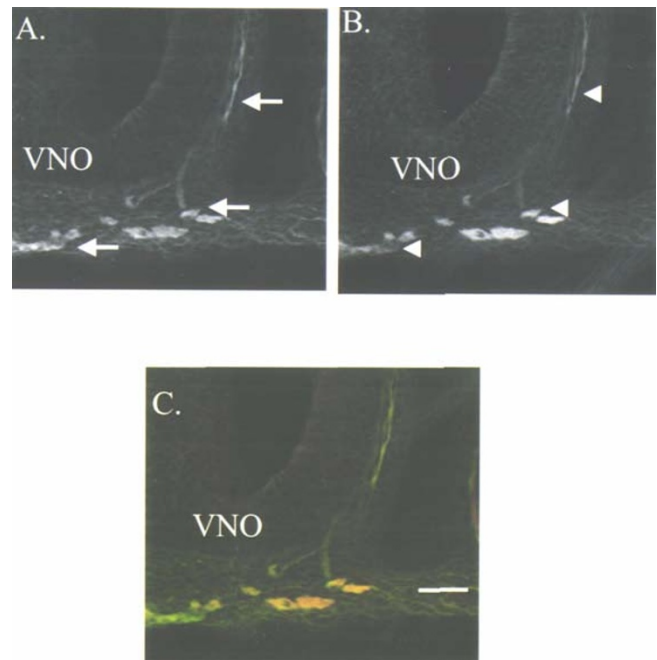
sponding to the chick stages 21–29. Double confocal immunofluoresence of mouse tissue (embryonic day 15, El 5) demonstrated that MAP1b immunoreactive axons of the vomeronasal nerve (VNN), the highway for GnRH neurons in the mouse [11,13] express PN-1 protein (Figure 4) but not trypsin (data not shown). Importantly, PN-1 immunoreactivity was not limited to MAP1b immunoreactive axons, but was also present within the basal laminae of the vomeronasal organ (VNO) as well as within the adjacent mesenchyme (Figure 4). In both El 5 and adult mouse brains, PN-1 immunoreactivity within the olfactory bulb was diffuse but appeared to be more concentrated within the glomerular layer (data not shown). S100 positive glia of the VNN express trypsin (Figure 5) within the cell bodies and along major processes; the diffuse staining suggested it was also in the matrix of the VNN (Figure 5, 5B). It is clear that PN-1 protein was not present in the same structures as those immunoreactive for trypsin (Figure 6) or GnRH (data not shown).

Discussion
This is the first report in which the *in vivo* application of a serine protease or its inhibitor have been demonstrated to affect embryonic neuronal migration. Our data demonstrate stage specific effects of PN-1 and trypsin on the dis-
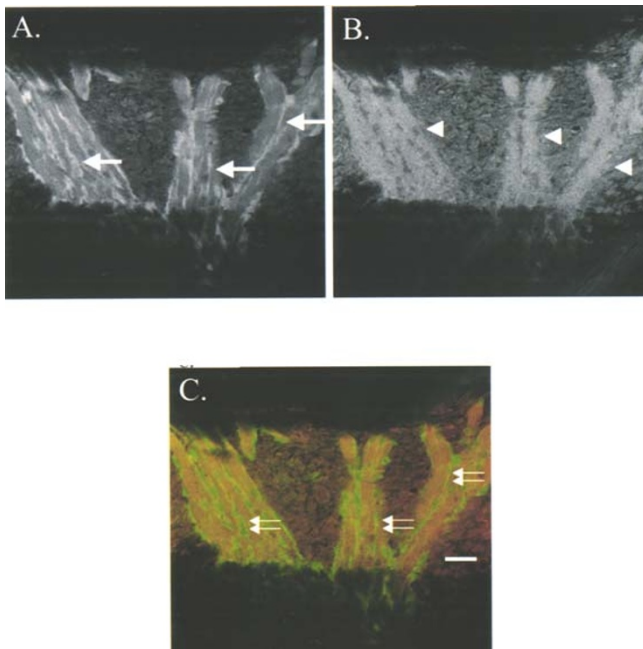
Figure 5
A confocal image of fascicles of the El 5 mouse embryo vomeronasal nerve prior to its penetration into the telencephalon. S100 immunoreactive glial cells which ensheath the nerve (FITC, A) colocalize with trypsin immunoreactivity (Texas Red, arrowheads delineating staining) of the vomeronasal nerve. Arrows point to glial cell bodies or their processes. C. This co-localization is best seen in an overlay where the double labeled glial cell bodies and their processes are yellow (double arrows). There is also red fluorescence external to the glia, most likely in the extracellular matrix. Scale bar= 100 μm.

Figure 6
A lμm optical section of an El 5 mouse embryo cut in the sagittal plane at the level of the vomeronasal organ (VNO). A. PN-1 was localized using Texas Red fluorescence. Immunoreactive sensory cells (arrowheads) were found occassionally which supports the co-localization of this protein in vomeronasal nerve axon bundles (Figure 1). PN-1 is also concentrated in the basal laminae (arrow) and displays a meshwork pattern in the nerve. B. Trypsin was identified by FITC fluorescence. Trypsin staining forms a fine meshwork within the VNO. Note that sensory cell in A. (arrowhead) is not positive for trypsin. The staining pattern for trypsin suggests it also has an extracellular localization. There is no co-localization of PN-1 and trypsin proteins. Scale bar= 100 μm.

tribution of chick GnRH neurons along their migratory route, without an alteration of their total population.

Stage specific introduction of these proteins was via unilateral insertion (into either the olfactory epithelium or placode) of protein coated Affigel Blue beads. The use of these beads is an established method widely utilized to deliver exogenous proteins during development. For example, in their study of limb development, Fallon *et al.*[23] demonstrated depletion of iodinated FGF protein from Affigel Blue beads indicating protein does indeed diffuse out of the bead. In fact, the authors demonstrated that approximately half the iodinated protein diffused off the bead within the first 24 hours and 70% diffused by approximately 60 hours. These data indicate that protein is present and diffusing into the surrounding area up to 60 hours following implantation of the bead. Our experiments were carried out within this window of time (there are 24 hours between stage 17 to 21 and 60 hours between stage 21 and 29). In addition, subsequent studies examin-
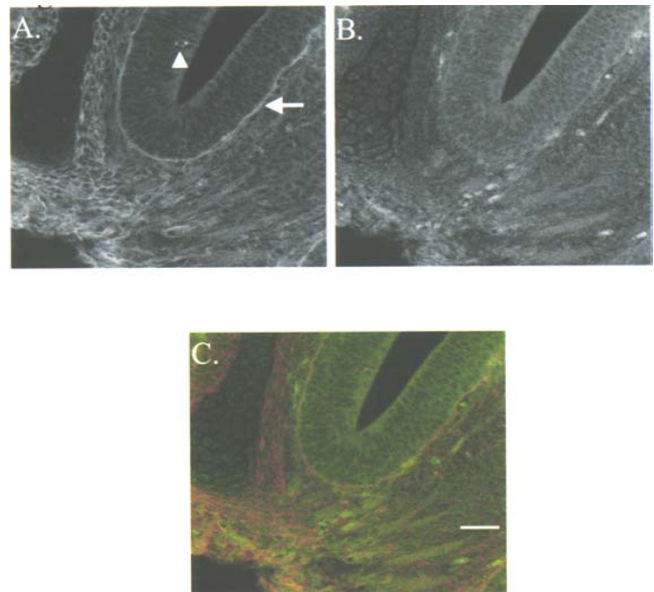
ing the roles of BMP, FGF's and Shh have also utilized Affigel Blue beads to exogenously introduce various protein components [24,25]. The results of these studies have been consistent with the known temporal and spatial distributions of these proteins.

Modification of the extracellular milieu via local shifts in the protease/inhibitor balance may regulate neuronal migration. This hypothesis is supported by our findings on stage 21–29 embryos. In these experiments, inhibition of endogenous trypsin-like activity by PN-1 resulted in fewer GnRH neurons exiting the olfactory epithelium and a corresponding decrease in the number entering the brain. Conversely, increasing trypsin availability promoted neuronal crossing of these two boundary zones. We hypothesize that the serine protease/inhibitor pair act by altering the ECM through which GnRH neurons migrate. Matrix involvement in migration is supported by the identification of matrix components throughout the GnRH migratory route. At the epithelium, GnRH neurons cross the

basal laminae to initiate their migratory phase [15]. The olfactory nerve expresses several matrix molecules [heparan and keratan sulfate proteoglycans [26], laminin [27], phosphocan [28], lectin-binding domains [29]]. Any or all of these may serve as substrates for proteolysis. The three domains, the olfactory epithelium, olfactory nerve and the brain, represent regulatory road blocks to the GnRH cell. By inhibiting local proteolysis, PN-1 produces a non-conducive environment at these points. Likewise, promotion of local proteolysis by trypsin, results in a permissive migratory environment.

Though modification of the ECM composition may indeed explain the data, another explanation may lie in the activation/inhibition of protease activated receptors (PAR's). These two hypotheses are not mutually exclusive but rather may work in concert to regulate neuronal migration. PAR'S are G-coupled receptors activated by proteolytic cleavage of their exo-domains. This cleavage exposes an amino acid sequence which then serves as a tethered ligand activating transmembrane signaling. Four PAR'S have been identified (PAR 1–4); PAR'S 1,3 and 4 are activated by thrombin while PAR2 is activated by trypsin [30]. Activation of PAR'S may alter ECM composition. For example, Hattori *et al.*[31] demonstrated that thrombin activation of PAR1 led to deposition of P-selectin, a leukocyte adhesion molecule, on the surfaces of endothelial cells. This change in matrix composition allows for leukocyte attachment and subsequent infiltration of the endothelium. PAR'S are expressed in the developing rat CNS [32] as well as in the embryonic chick [33]. Though we did not localize PAR expression in our model system, it remains a possibility that their activation or inhibition may modulate the ECM composition and thus GnRH neuronal migration.

The fact that mammalian PN-1 alters neuronal migration in an avian species suggests the presence of endogenous serine protease activity in the region. Such a protease, with trypsin-like immunoreactivity, was detected by immunocytochemistry. To biochemically characterize this endogenous activity, non-neurally derived trypsin inhibitors were tested. At the concentrations tested, soybean trypsin inhibitor had no effect on GnRH migration. These results could be due to differences in binding affinity between soybean trypsin inhibitor and PN-1 for the endogenous trypsin-like molecule. The inhibition of trypsin-like proteases by different inhibitors (including PN-1 and soybean trypsin inhibitor) varies widely and a lack of an effect is not uncommon (e.g. 34).

Contrary to the effect seen with PN-1, type IV chick ovoinhibitor accelerated neuronal migration with fewer GnRH neurons in the epithelium and correspondingly more within the brain. At least two mechanisms can explain this

result. First, type IV chick ovoinhibitor may not be acting as an inhibitor in our system as it has been demonstrated to be ineffective in other systems [34–36]. Alternatively, if it is serving as a protease inhibitor, it may inactivate a molecule different from that affected by PN-1 and, in doing so, generate an environment permissive for neuronal migration.

Both non-neuronally derived trypsin inhibitors tested yielded results different from that of PN-1 suggesting they possess different protease specificities, affinities, or functions. Regardless of their mechanisms of action, these data support the hypothesis that changes in enzymatic activities can influence neuronal migration.

In contrast to PN-1, trypsin leads to a permissive environment during this developmental period (stages 21–29) resulting in a global acceleration of GnRH neuronal migration out of the olfactory epithelium and into the brain. Trypsin cleaves peptide bonds at the carboxyl side of either arginine or lysine residues. This rather non-specific cleavage suggests trypsin may alter many different components of the ECM which themselves vary in each compartment. Interestingly, in the chick, trypsin immunoreactivity was present throughout the head as well as in the olfactory nerve. This ubiquitous distribution suggests that the specificity of the protease/inhibitor system may be imparted by an avian equivalent of PN-1.

We found no effect on GnRH neuronal distribution in uPA-treated embryos and no uPA immunoreactivity. This result is not surprising in light of a study by Del Bigio *et al.*[37] in which the expression pattern of uPA was analyzed in the postnatal mouse. Based on its spatio-temporal expression pattern, the authors concluded that uPA may function in processes of synaptogenesis and remodeling rather than in cell migration. In addition, our data indicate that simply coating beads with any serine protease is not sufficient to influence migration. Thus, not only is our experimental approach unbiased, but the antagonistic relationship of PN-1 and trypsin on GnRH neuronal migration is specific.

A PN-1 specific antibody that cross reacts with chick tissue is not available, thus we were unable to follow its expression in the chick. Although avian and mammalian serpins are members of the same superfamily, they are evolutionarily distinct [38]. This divergence likely accounts for the species specificity of the PN-1 antibody. To determine whether PN-1 is expressed by the olfactory system during the time of active GnRH neuronal migration, we examined its expression in the embryonic mouse where its mRNA is known to be expressed [39]. At E15, PN-1 protein is present within MAP1b immunoreactive axons of the vomeronasal nerve (the migratory path used by GnRH

neurons in mammals). PN-1 immunoreactivity was also present within the basal laminae of the vomeronasal organ, a barrier crossed by GnRH neurons when they initiate their migration. This staining pattern, along with our data on PN-1's effect on migration, strengthens our hypothesis that this serine protease inhibitor plays a role in neuronal migration. In addition, expression of other serine protease inhibitors has been described in other migrating neurons. For example, Krueger *et al.*[40] demonstrated increased expression of neuroserpin within migrating granule cells of the cerebellum in the post natal mouse.

In the mouse, trypsin immunoreactivity is present within S 100 immunoreactive glia which ensheath axons of the vomeronasal nerve. As with PN-1, it is also present in the extracellular matrix where it is likely that the protease/inhibitor pair function to regulate, at least in part, the environment through which GnRH neurons migrate.

Due to its ability to stimulate neurite outgrowth, we asked whether PN-1 could alter the timing of formation of the olfactory nerve and if so, what effect this might have on GnRH neuronal migration. For these experiments, bead implantation into the olfactory placode was performed at stage 17 just prior to olfactory nerve axonogenesis. PN-1 did not accelerate neurite outgrowth but did accelerate detectable levels of biochemical differentiation markers of the olfactory nerve. These data suggest that the olfactory neurons can respond to PN-1 directly or to changes in the local environment caused by PN-1.

The distribution of GnRH neurons was not altered by PN-1 at stages 17–21 in spite of the accelerated maturation of the olfactory nerve. Likewise, PN-1 did not alter the timing of the nerve's ensheathment by glia. This suggests that glial migration is independent of any matrix changes induced by PN-1. The fact that GnRH neurons and the glial ensheathing cells exit at the same time (stage 21) may be coincidental or may be due to an interaction between these two populations. Alternatively, the timing of exit of GnRH neurons and glia may be cell-autonomous events.

Implantation of a trypsin bead at stage 17 had no effect on the biochemical maturation of the olfactory nerve nor on the timing of glial or GnRH neuronal migration. At this early stage, at least some of the barriers to GnRH neuronal exit from the epithelium are in place [15]. Therefore, the lack of an effect on migration by trypsin suggests that the initiation of migration is an inherent property of the GnRH neuron (and glial cell). At stage 21, however, trypsin did significantly alter the distribution of GnRH neurons such that a large population was already in the brain. These neurons not only entered the brain early but also traveled caudalward within the brain along their normal arching trajectory.

Taken together, these data suggest that in the presence of either trypsin or PN-1 (at stage 17), GnRH neurons exit the olfactory epithelium at stage 21, as in controls. Therefore, the ability to initiate migration resides within the neuron itself such that alterations of ECM composition during this time (stage 17–21) are irrelevant to the timing of this initial event. The finding that the neurons enter the CNS early in trypsin-treated embryos suggests that continued movement may be a cell autonomous event with trypsin-induced proteolysis generating a permissive environment. Finally, these data demonstrate that the GnRH neuron's ability to transit across the glia limitans and through the brain to its target sites is independent of additional maturation of the neuron itself, the olfactory nerve or the brain.

Our data support a role of proteases and their inhibitors in neuronal migration. The complex interactions between a neuron and its environment are likely not regulated by proteolysis alone. The characteristic directed migratory pattern taken by the GnRH neuron and the cohesiveness of the population suggests a more complex system is in place to ensure their appropriate translocation into the brain. In fact, Kramer and Wray [42] have identified a novel gene, nasal embryonic LHRH (GnRH) factor or NELF, which is expressed by GnRH neurons during their migratory phase and olfactory sensory neurons as they initiate axonogenesis. Antisense depletion of NELF led to a decrease in both olfactory axonal outgrowth and the number of GnRH neurons exiting the epithelium. The authors suggest that NEFL may function as a migratory factor. It seems likely therefore that several components interact to orchestrate the proper migration of neurons.

Materials and Methods
Animal and tissue preparation
Chicks
Fertilized eggs (white leghorn, SPAFAS, Preston, CT) were placed in an incubator (Humidaire Incubator, Co. New Madison, OH) at 99.5°C and 85% relative humidity. A window was cut in each shell to determine the developmental stage of each embryo [22]. To visualize the extraembryonic membranes, Fast Green (1:10,000 Molecular Probes, Eugene, OR) was applied to the surface of the embryo and membranes were teased apart using forceps. Appropriately sized Affigel Blue beads (BioRad, Hercules, CA) were unilaterally inserted either into the olfactory placode (stage 17) or the olfactory epithelium (stage 21) and the egg was returned to the incubator until time of fixation. Control animals were implanted with beads coated with 0.1 M phosphate buffer, pH 7.3 (PB, N = 10). Experimental embryos were implanted with beads coated with PN-1 bead (N = 12) or trypsin (N = 11). Embryos receiving a bead at stage 17 were fixed at stage 18 (N = 9), stage 19 (N = 16), stage 20 (N = 17) or stage 21 (N = 9). Embry-

os receiving a bead at stage 21, were fixed at stage 29 (N = 10).

Embryos were fixed by immersion in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for 1–7 days depending on the stage (younger embryos required shorter fixation times). Fixed embryos were embedded in 8% gelatin and fixed in 4% paraformaldehyde. 100 µm sagittal sections were cut on a vibratome (DSK Microslicer 1500E, Ted Pella, Inc., Redding, CA) and immunocytochemistry was performed on free-floating sections. Tissue was then mounted onto slides, dehydrated through graded alcohols, cleared with HemoD (Fisher) and coverslipped with Permount (Fisher).

Mice
As reagents for the localization of PN-1 in the chick are not available, we examined its localization in the embryonic mouse at the time of GnRH migration. Immunolocalization of PN-1 protein was performed on embryonic day 15 (El 5, day of plug was considered E0.5). Swiss Webster mice were used (Charles River, Kingston, MA). Mouse embryos (N = 6) and an adult female (N = 1) were processed for double label immunofluorescence to determine the cellular localization of PN-1 and trypsin. The mothers were euthenized with CO_2 and embryos placed in PB and decapitated rapidly. The adult was anesthetized with nembutal (70 mg/kg) and perfused transcardially. Tissue was treated as described above.

Anesthesia, Surgery, and Perfusion
All procedures for the mice met with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Columbia University.

Affigel-Blue bead preparation
Affigel Blue beads (BioRad, Hercules, CA) were sized using a dissecting microscope and forceps as described by Vogel *et al.*[41]. Beads 30–50 µm in diameter were used for stage 21 embryos and beads 20 µm in diameter were used for stage 17 embryos to accommodate the smaller target area. Beads were placed in one of the following solutions: 3.1 mg/ml of recombinant rat PN-1; 2.3 mg/ml of trypsin (type III bovine pancreas, Sigma, St. Louis, MO); 1 mg/ml urokinase plasminogen activator (uPA, Sigma, St Louis, MO); 1 or 10 mg/ml soybean trypsin inhibitor (Sigma, St Louis, MO); 10 mg/ml type IV chick egg white purified ovoinhibitor (Sigma, St Louis, MO) at 4°C overnight and used the next day. Control beads were soaked in PB overnight at 4°C and used the next day.

Free floating immunocytochemistry
Single label
Tissue was washed and treated with 0.5% hydrogen peroxide to remove endogenous peroxidase activity. Following

an overnight incubation in 0.1% Triton X-100 (Tx100, Sigma, St Louis, MO) containing 3% normal goat serum in PB at 4°C, sections were incubated in rabbit polyclonal anti-GnRH antibody (SW-1, 1:5000 in 0.1% Tx100 in PB, generously provided by Dr. S. Wray, NIH) for at least 4 days at 4°C. Following three PB washes, tissue was then incubated in biotinylated goat-anti-rabbit-IgG (1:200, Vector Labs, Burlingame, CA) in 0.1% Tx100 in PB overnight at 4°C. The following day, sections were washed in PB and incubated in avidin-biotin-HRP complex (1:200, Vector Labs, Burlingane, CA) for at least 3 hours at room temperature. After PB washes, bound antibody was detected using 3,3'-diaminobenzidine (DAB, Sigma, St Louis, MO) as the chromogen.

Brains processed for immunocytochemical localization of protease, PN-1 or axonal/glial markers were treated as described above, however the primary antibodies used were specific for each protein (see list below). Sections were viewed and photographed with an Olympus Vanox microscope (New York/New Jersey Scientific Inc., Middlebush, NJ).

Double label
Brains processed for double label immunofluorescence were treated as described above. The first antibody was detected using the appropriate biotinylated IgG followed by avidin-fluorophore (either Texas Red or FITC, Sigma, St Louis, MO), while the second antibody was detected by a directly conjugated IgG-fluorphore. All fluorescent images were acquired with a Zeiss confocal microscope using LSM 410. Images were acquired at 40 or 100X using 16 averaged scans at 1028×1028 pixel resolution and printed using Adobe Photoshop IV.

Control
To test the specificity of the method, tissue was processed as described above for the first antibody. In the second set of reactions, 3% normal mouse serum (Vector Labs, Burlingane, CA) in 0.1% Tx100 in PB was substituted for the primary antibody. This was followed by horse anti-mouse IgG-FITC. Under these conditions, no FITC immunofluorescence was detected. As an additional control for trypsin immunoreactivity in the CNS, trypsin protein was incubated with the anti-trypsin antibody for 4 hours at 4°C for antibody adsorption. This antigen/antibody complex was then utilized to immunostain chick tissue sections; no immunoreactivity was detected (see figure 3).

Antibodies/antisera and dilutions
Axonal markers
NCAM: A mouse monoclonal antibody raised against the highly sialylated embryonic forms of the neural cell adhesion molecule was used (1:1, Developmental Studies Hybridoma Bank).

NF: Two mouse monoclonal antibodies raised against the heavy (200 kD) and light (68 kD) forms of neurofilament were used (1:200 each, Sigma, St Louis, MO). Both antibodies recognize phosphorylated variants of neurofilament.

MAP1b: A mouse monoclonal antibody to the microtubule associated protein 1b was generously provided by Dr. Lester Binder, University of Alabama at Birmingham. The antibody was used at 1:1000 and was selected because MAP1b is the earliest expressed MAP both *in vivo* and *in vitro*[43].

PGP9.5: A rabbit polyclonal antibody raised against human protein gene product 9.5 was used at 1:1000 (Biogenesis, Ltd,. Poole, England). This is a neuron-specific ubiquitin carboxy terminal hydrolase.

Glial markers
P$_0$: A mouse monoclonal antibody, 1E8, raised against the mammalian myelin protein, P$_0$, was used at 1:4000 and was generously provided by Dr. R. Brackenbury (University of Cincinnati, Cincinnati, OH). This antibody recognizes Schwann and ensheathing cells of the olfactory nerve but not astrocytes.

MAP4: A mouse monoclonal antibody raised against microtubule associated protein 4, MAP4, was generously provided by Dr. Chloe Bulinski (Columbia University, NY, NY) and used at 1:1000. MAP4 is the major MAP expressed by non-neuronal tissue.

S100: A rabbit polyclonal anti-S100 antibody was used at 1:1000 (Biogenesis, Ltd, Poole, England). S 100 is a central and peripheral glial cell marker.

GnRHmarker
GnRH: a polyclonal antibody raised against GnRH conjugated with glutaraldehyde to ovalbumin was generously provided by Dr. Susan Wray (NIH). This antibody recognizes amino acids: 3,4,7,8,9, and 10 of the GnRH peptide and was used at 1:5000.

Protease antibodies
Trypsin: An anti-human cationic trypsin antibody was used at 1:1000 (Biodesign, Kennebunk, ME).

uPA: An anti-uPA antibody was used at 1:1000 (Accurate Chemical, Westbury, NY).

Protease inhibitor antibody
PN-1: An anti-rat PN-1 antibody was used at 1:500 and was generously provided by Dr. D. Monard (Friedrich Miescher Institut, Basel, Switzerland).

Coagulation Assay
To ensure that the proteases and inhibitors used were active, a coagulation assay was performed. Blood alone (from PD) coagulated in approximately 2 minutes. Incubation of blood with 1 mg/ml uPA delayed coagulation significantly (by several minutes). Incubation with combined PN-1 and uPA resulted in rapid coagulation as in control.

Cell counts and statistical analysis
Cell counts for PN-1 and serine proteases were performed by PD who was unaware of the experimental status of the embryos. Those for the other serine protease inhibitors were carried out by a second observer (XLZ). Both observers carried out independent counts on separately prepared sets of PB controls. An equal number of immunoreactive tissue sections was counted for each side of the face (i.e. the side containing the bead and the contralateral side). Selected sections were recounted by AJS to check for accuracy. Cell number was determined via a modified optical dissector method [44–46]. Examination of tissue reveals three focal planes; the nucleus being in crisp focus in only one of these planes. This profile allows cells (nuclei) to be counted in each of the three planes. Double counting is avoided for those cells in the top and bottom planes by counting alternate sections.

Immunoreactive GnRH neurons separated into categories depending on location: olfactory epithelium (OE), olfactory nerve (ON) and telencephalon (CNS). Numbers in each category were then expressed as a percent of total number of GnRH neurons on that side of the face. The contralateral side served as a within animal control. To analyze for significant differences in percentage of GnRH neurons in each category, an unpaired students t-test ($p < 0.05$) was performed.

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