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Polysialic Acid Facilitates Migration of Luteinizing Hormone-Releasing Hormone Neurons on Vomeronasal Axons

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Luteinizing hormone-releasing hormone (LHRH) neurons migrate from the olfactory placode to the forebrain in association with vomeronasal nerves (VNN) that express the polysialic acid-rich form of the neural cell adhesion molecule (PSA-NCAM). Two approaches were used to investigate the role of PSA-NCAM: injection of mouse embryos with endoneuraminidase N, followed by the analysis of LHRH cell positions, and examination of LHRH cell positions in mutant mice deficient in the expression of NCAM or the NCAM-180 isoform, which carries nearly all PSA in the brain. The enzymatic removal of PSA at embryonic day 12 significantly inhibited the migration of nearly half of the LHRH neuron population, without affecting the VNN tract itself. Surprisingly, the absence of NCAM or NCAM-180 did not produce this effect. However, a shift in the route of migration, resulting in an excess number of LHRH cells in the accessory olfactory bulb, was observed in the NCAM-180 mutant. Furthermore, it was found that PSA expressed by the proximal VNN and its distal branch leading to the accessory bulb, but not the branch leading to the forebrain, was associated with the NCAM-140 isoform and thus was retained in the NCAM-180 mutant. These results provide two types of evidence that PSA-NCAM plays a role in LHRH cell migration: promotion of cell movement along the VNN tract that is sensitive to acute (enzymatic), but not chronic (genetic), removal of PSA-NCAM, and a preference of a subset of migrating LHRH cells for a PSA-positive axon branch over a PSA-negative branch in the NCAM-180 mutant.

Key words: polysialic acid; neuronal migration; LHRH neurons; olfactory system; NCAM; vomeronasal organ

During development, neurons expressing luteinizing hormone-releasing hormone (LHRH) arise from the olfactory placode, move medially and dorsally to the cribriform plate, migrate past the olfactory bulb (OB), and then proceed deeper into the developing forebrain (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). In mice, these LHRH neurons are first seen at embryonic day 11 (E11) in the medial wall of the developing olfactory epithelium (OE), the same position from which the vomeronasal organ (VNO) begins to bud (Halpern, 1987; Schwanzel-Fukuda and Pfaff, 1990; Garrosa et al., 1992). At this stage, olfactory axons begin to emerge from the VNO to form the vomeronasal nerve (VNN). One day later, the VNN splits into a large dorsal-oriented branch that extends toward the accessory OB (AOB) and a smaller caudal-oriented branch that extends into the forebrain (Yoshida et al., 1995). Beginning at E11 and continuing for several days, LHRH neurons migrate in association with the proximal portion of the VNN and then show a strong preference for the caudal branch of the VNN into the forebrain, suggesting that guidance cues operate in this pathway (Santacana et al., 1992; Wray et al., 1994; Norgren et al., 1995; Yoshida et al., 1995).

In looking for cell surface molecules associated with this migration, it has been noted that the polysialic acid-rich form of the neural cell adhesion molecule (PSA-NCAM) is expressed on olfactory axons in a variety of species, in addition to rodents (Key and Akeson, 1991; Murakami et al., 1991; Norgren and Brackenbury, 1993). Furthermore, the caudal VNN branch is rich in PSA and expresses the Ig superfamily glycopprotein TAG-1, whereas the dorsal branch is TAG-1-negative ($TAG\text{-}1^-$) and weakly PSA-positive (PSA^+) (Dodd et al., 1988; Wolfer et al., 1994).

PSA-NCAM is abundantly expressed in the embryo and plays a key role in both the regulation of axon tract formation and the migration of cells (for review, see Fryer and Hockfield, 1996; Rutishauser and Landmesser, 1996; O'Rourke, 1996). For example, the absence of PSA *in vivo*, produced by either removal with the PSA-specific endoneuraminidase N (endo N) or NCAM mutation, inhibits the migration of neurons from the subventricular zone of the lateral ventricle to the OB (Tomasiewicz et al., 1993; Ono et al., 1994). Although the exact mechanism of these effects is not fully defined, it has been proposed that the ability of PSA to promote cell migration involves the attenuation of cell–cell interactions to levels optimal for the making and breaking of cell contacts during locomotion (Hu et al., 1996).

In the present study, the role of PSA in the migration of LHRH neurons from the olfactory placode to the basal forebrain has been evaluated through both the use of injections of endo N into the E12 mouse embryo and the analysis of NCAM-deficient mutant mice. The results obtained are more complex than in the earlier studies on the subventricular zone, including differences between acute and chronic loss of PSA and indications that PSA can influence choices in the migration route. Both observations provide evidence that PSA can facilitate the migration of LHRH neurons along this axophilic pathway.

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MATERIALS AND METHODS

Animals. NCAM-180 mutant mice (CF-1 background) described previously (Tomasiewicz et al., 1993) and NCAM null mutant mice on a C57Bl/6 background (Cremer et al., 1994) were used for immunocytochemical and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) studies. E14 and E15 embryos were perfused with 4% paraformaldehyde in 0.01 M phosphate buffer (PB), pH 7.4, were post-fixed overnight at 4°C, and were processed for immunocytochemistry (E15) or DiI injection (E14). Timed pregnant C57Bl/6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were used in endo N experiments and were analyzed further with LHRH immunohistochemistry and DiI injections into the VNO.

Enzymatic removal of PSA in vivo. Pregnant C57Bl/6 mice at E12 of gestation were anesthetized with intraperitoneal injections of Avertin (0.02 ml/gm body weight). Under aseptic conditions, an abdominal incision was made, and the embryos were exposed. Endo N (0.5 μl) or heat-inactivated endo N (Ono et al., 1994) was microinjected into two embryos from each litter. The wound was then closed with sterile sutures and staples, and the animal was allowed to recover. Three days later, the mice were anesthetized again, and the E15 embryos were perfused with 4% paraformaldehyde in 0.01 M PB, pH 7.4. The heads were post-fixed overnight and were placed into 0.1 M PB until processing for immunohistochemistry or DiI injection. Endo N injected into live embryos results in removal of PSA in <1 day, lasting up to 6 weeks (Landmesser et al., 1990; Shen et al., 1997). Endo N efficacy was assessed by immunostaining lateral sagittal sections through the head with antibodies to PSA-NCAM (see below).

Immunohistochemistry. Heads from 4% paraformaldehyde-perfused C57Bl/6 wild-type, NCAM-180 mutant, and NCAM null mutant mouse embryos were mounted in 5% agarose, and 70 μm sagittal sections were cut using a vibratome. The sections were placed in 25 mm net wells (Corning, Corning, NY), kept at 4°C, and pretreated in the following manner: 15 min in 0.01 M sodium-*m*-periodate (Sigma, St. Louis, MO) solution; three 10 min washes in 0.05 M PBS, pH 7.4; 15 min in 0.5% sodium borohydride; three 10 min washes in PBS; and 30 min in 3% NGS–1% H_2O2–PBS and 15 min in 5% NGS in PBS with 0.3% Triton X-100. The sections were incubated at 4°C for 18–22 hr on a rotating table in antibodies to LHRH, TAG-1, and PSA-NCAM and diluted in 0.1% Triton X-100–1% BSA–PBS. The sections were then washed for 1 hr with four changes of 0.02% Triton–1% NGS–PBS at room temperature. The sections were incubated with the appropriate biotinylated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) diluted at 1:200 in 1% NGS–0.32% Triton X-100–PBS for 2 hr and washed for 1 hr in four changes of 0.02% Triton–PBS. After incubation in ABC (Vector Laboratories, Burlingame, CA) for 1 hr, the sections were washed with Tris-buffered saline (TBS), pH 7.5, and developed with diaminobenzidine solution in TBS. The sections were then washed with PBS, placed on slides, dehydrated, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Slides were coded to ensure unbiased assessment of the LHRH cell population from the different groups of mice. LHRH cells were counted in three compartments (nose, brain, and OB) in sagittal sections at 400× magnification using an Axioplan (Zeiss, Oberkochen, Germany) microscope.

Antibodies. For the immunohistochemical analyses, LHRH neurons were labeled using the LR1 anti-LHRH antibody, a generous gift from Dr. Robert Benoit (Montreal General Hospital, Montreal, Canada). To detect PSA-NCAM, the 5A5 monoclonal antibody was used (Acheson et al., 1991). For the investigation concerning the TAG-1 adhesion molecule, the 4D7 monoclonal antibody was used (Yamamoto et al., 1986). RO25, the IgG fraction of a polyclonal rabbit antibody to NCAM, was used for Western blotting procedures. RO25 was made against rat NCAM purified from neonatal brain membranes by immunoaffinity isolation using the 3F4 monoclonal antibody (Shen et al., 1997). In immunoblots of SDS-PAGE fractionated mouse brain membrane proteins (NP-40 extract), it specifically recognizes the three major NCAM isoforms (180, 140, and 120).

DiI labeling of VNN. DiI (Molecular Probes, Eugene, OR), a lipophilic fluorescent dye, was used to label VNN fibers. To investigate the effect of endo N on VNN fibers, DiI crystals were placed in the VNO of endo N-treated, heat-inactivated endo N-treated, and littermate control E15 mice. DiI was also used to label the VNN in NCAM-180-deficient and NCAM null mutant mice at E14, as well as E14 C57/Bl6 mice for use as wild-type controls. The VNOs of 4% paraformaldehyde-perfused mice were exposed ventrally by dissecting through the soft palate. A crystal of DiI on the tip of a pulled capillary pipette was placed into each VNO. Each head was incubated in 5% sodium azide in PBS at 37°C for 3 d. For analysis, 120 μm sections in 5% agarose were cut sagittally with a vibratome. Sections were mounted in paraphenylenediamine in 0.1 M sodium bicarbonate buffer, pH 9.0, and visualized on a Zeiss microscope equipped with rhodamine filters.

Protein gel electrophoresis and Western blots. OBs and VNOs from E17 NCAM-180-deficient mice and E17 and postnatal day 0 C57Bl/6 (control) mice were homogenized in 0.05 M Tris–HCl buffer, pH 7.4, containing 0.2 mM PMSF, 0.25% aprotinin, and 1% Triton X-100. The homogenates were spun in a microcentrifuge for 30 min, and the supernatants were assayed for protein concentration with a BCA kit (Pierce, Rockford, IL). The supernatants were adjusted to 2.5 mg/ml, subjected to SDS-PAGE under reducing conditions, and blotted onto nitrocellulose paper (Bio-Rad, Hercules, CA). The paper was then blocked with 5% milk in TBS, pH 7.6, with 0.1% Tween (TBS-T) for 1 hr. After one 15 min and two 5 min washes with TBS-T, the blot was incubated overnight with the polyclonal anti-NCAM antibody, RO25, at a dilution of 1:2500 in TBS-T at 4°C. After three washes with TBS-T, the blot was incubated for 1 hr with peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch) at 1:10,000 in TBS-T. The blot was then washed four times with TBS-T and visualized by chemiluminescence using an ECL Western blotting kit (Amersham, Arlington Heights, IL).

RESULTS

At E12, the VNO is fully formed and segregated from the main OE. The majority of LHRH neurons at this stage were visible within the VNO or in association with TAG-1-positive (TAG-1$^+$) vomeronasal axons along the nasal septum. Immunocytochemical analysis at E12 revealed the colocalization of LHRH neurons with TAG-1$^+$ vomeronasal axons leading from the VNO across the cribriform plate and projecting a short distance into the forebrain (Fig. 1*A*,*B*). At E14, after crossing the cribriform plate, TAG-1$^+$ vomeronasal axons were observed to deviate from the main TAG-1$^-$ VNN, to extend caudally toward the forebrain, and then to defasciculate within their target tissue (Fig. 1*C*). LHRH neurons (Fig. 1*D*) followed this course closely, with the vast majority of these cells migrating along the caudal branch into the forebrain. Double-label immunofluorescence studies demonstrated that LHRH neurons are almost always seen in direct association with TAG-1$^+$ fibers (data not shown).

PSA is expressed along the LHRH neuron migration pathway

Intense PSA immunoreactivity was found on vomeronasal axons that originate in the VNO and project into the forebrain (Fig. 2*A*). The axons of OE neurons, which extend to the OB, were PSA$^+$ as well. A few cell soma along the VNN also appeared to be PSA$^+$ (Tobet et al., 1993); however, these cells were too infrequent to represent a significant population of migrating LHRH cells. Endo N treatment (see Materials and Methods) of E12 mouse embryos completely abolished PSA expression throughout the entire brain and nose (Fig. 2*B*), as seen at E15 by immunocytochemistry using antibodies to PSA-NCAM. These findings indicate that PSA is associated with the LHRH migration pathway but not with the majority of LHRH cells and that endo N can be used to test the possible function of PSA in the migration process.

Enzymatic removal of PSA *in vivo* inhibits LHRH cell migration

The most direct test of PSA function in a particular developmental event is through the use of endo N injection at the appropriate time and site. The effectiveness of this approach is indicated by the fact that endo N has an absolute specificity for PSA, produces a potent and long-lasting effect, even *in vivo*, and does not otherwise alter the NCAM polypeptide (Ono et al., 1994). The latter property makes this approach more definitive than even an

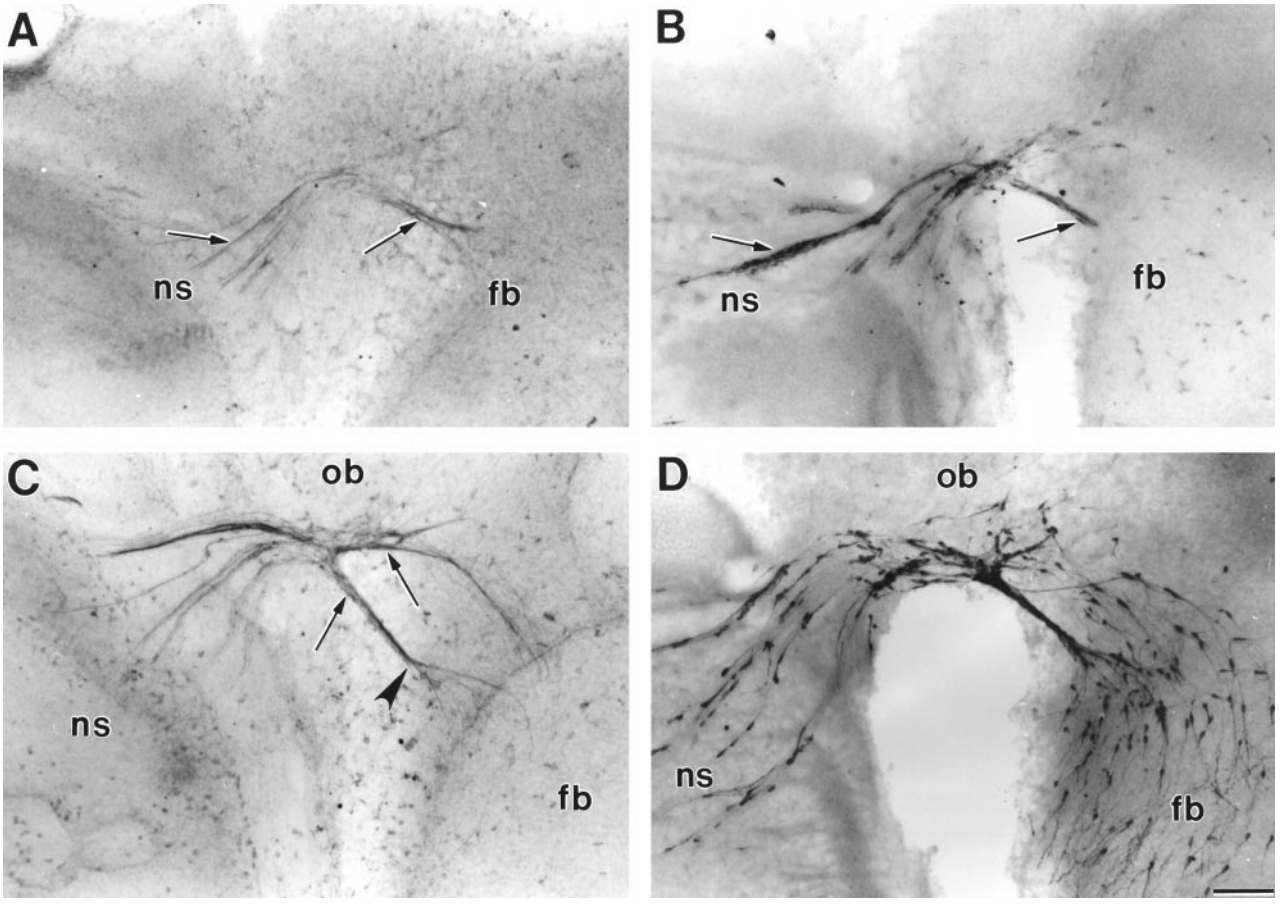

Figure 1. LHRH neuron migration is associated with TAG-1$^+$ pathways. *A*, At E12, antibodies to TAG-1 react with vomeronasal axons (*arrows*) that extend along the nasal septum (*ns*) to the forebrain (*fb*). *B*, At E12, antibodies to LHRH react with neurons migrating (*arrows*) from the VNO to the forebrain. *C*, At E14, TAG-1 immunoreactive axons converge on the ventromedial surface of the olfactory bulb (*ob*) and turn caudally into the forebrain, where they branch (*arrows*) and defasciculate (*arrowhead*). *D*, Migrating LHRH immunoreactive neurons also converge on the ventromedial surface of the OB and then disperse caudally and ventrally in the forebrain. Rostral is to the *left*. Scale bar, 100 μm.

NCAM gene mutation, which can reflect the function of the polypeptide itself, as well as PSA, and causes a deficiency of NCAM in all tissues throughout the life of the mouse.

To test whether PSA is required for LHRH cell migration, either endo N or heat-inactivated endo N was injected into the heads of E12 mice. After 3 d, the mice were killed, and LHRH cell migration and PSA-NCAM expression was evaluated by immunocytochemistry. LHRH cells were counted in three regions (nose, bulb, and forebrain) on tissue sections of untreated ($n = 3$), endo N-injected ($n = 7$), and heat-inactivated endo N-injected ($n = 5$) animals (Fig. 3). There was no significant difference in LHRH cell migration between untreated mice and mice treated with heat-inactivated enzyme ($F = 0.72$; $p > 0.5$). For the two groups of injected animals, the total numbers of LHRH cells at E15 were not significantly different (endo N-injected embryos, 1258 ± 89 cells; inactivated enzyme-injected embryos, 1123 ± 47 cells; mean ± SEM). In controls injected with inactivated enzyme, 50.7 ± 5.3% of LHRH cells had migrated into the forebrain, 21.7 ± 3.4% remained in the nasal cavity, and 27.5% ± 4.2% were in the bulb. In contrast, in endo N-treated animals, only 29.5 ± 9.0% of LHRH neurons migrated into the forebrain and 45.4 ± 9.2% remained in the nose, with 25.2 ± 9.0% in the bulb. Two-way ANOVA revealed a highly significant interaction between compartment and treatment ($F = 18.79$; $p < 0.001$). Thus, the overall effect of the removal of PSA at E12 was to reduce by nearly half the number of LHRH neurons that successfully migrate across the cribriform plate compared with controls.

Examples of the positions of LHRH cells at E15 in the nose and brain in endo N-treated mice compared with mice injected with heat-inactivated enzyme are shown in Figure 4. In control animals, the small number of LHRH neurons that remained in the nose were more evenly distributed along the VNN (Fig. 4*A*). In endo N-treated animals, numerous LHRH neurons were found along the VNN on the nasal septum, often forming large clusters (Fig. 4*B*). Although most LHRH neurons in control animals migrated into the forebrain (Fig. 4*C*), there were fewer neurons typically seen in the forebrain of endo N-treated animals (Fig. 4*D*). However, it is important to note that the smaller number that were seen in the forebrain of control animals were found along the normal migratory pathway.

NCAM mutant mice exhibit LHRH cell migration defects that are distinct from those produced by endo N

Because PSA is attached to the NCAM polypeptide and is almost entirely associated with the NCAM-180 isoform in the brain, mutant mice deficient in these polypeptides (Tomasiewicz et al., 1993; Cremer et al., 1994) can, in principle, provide additional evidence for the role of PSA-NCAM in the migration of neuronal

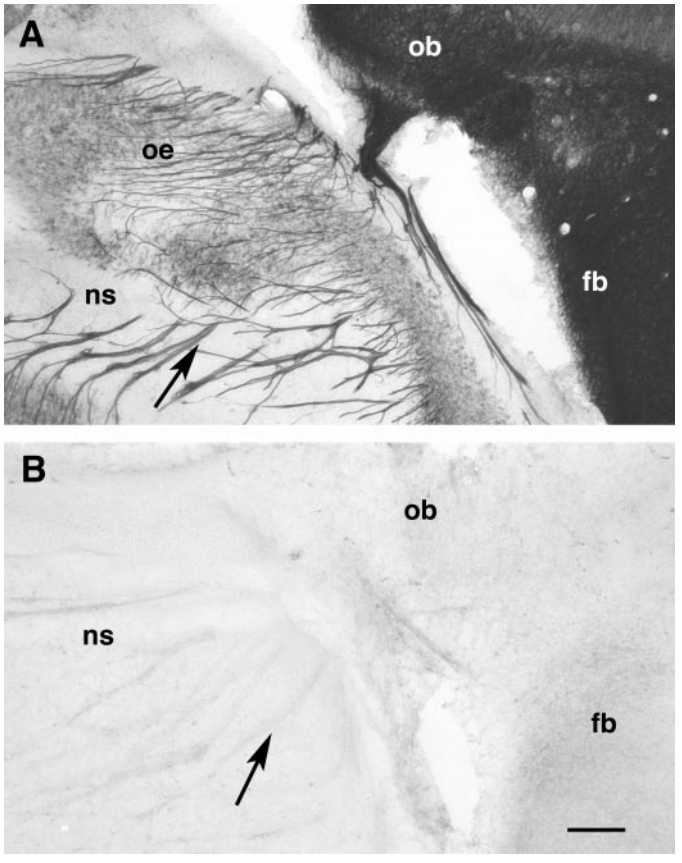

Figure 2. Endo N treatment abolishes PSA expression *in vivo*. *A*, At E15, PSA-NCAM is expressed along the nasal septum (*ns*), on the VNN (*arrow*), in the olfactory epithelium (*oe*), and is also heavily expressed in the forebrain (*fb*). *B*, In wild-type mice injected with endo N at E12 and analyzed at E15, PSA-NCAM immunoreactivity is nearly undetectable on VNN fibers (*arrow*) or in the forebrain. Scale bar, 100 μm.

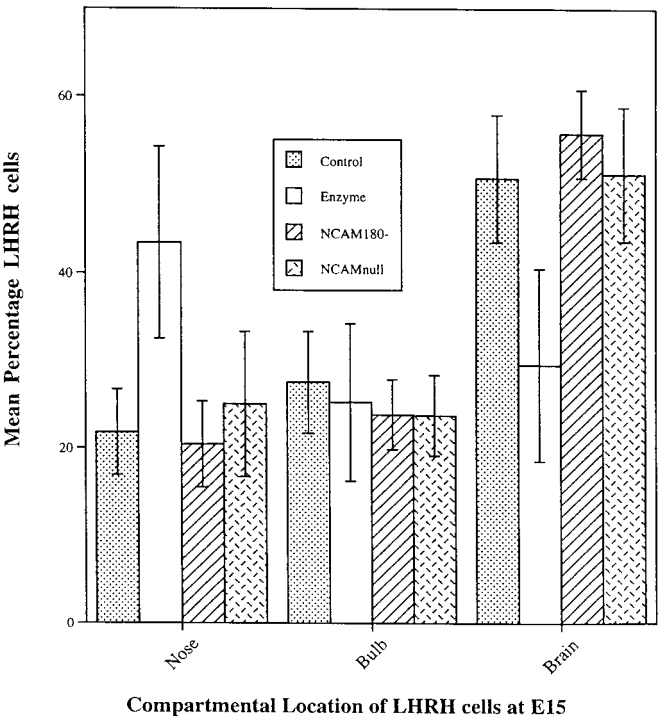

Figure 3. Distribution of LHRH cells in nose, OB, and forebrain compartments. LHRH cells were counted in three compartments (nose, bulb, and forebrain) on tissue sections of endo N-injected animals ($n = 7$), boiled endo N-injected animals ($n = 5$), NCAM-180 mutant animals ($n = 8$), and NCAM null animals ($n = 6$). Two-way ANOVA revealed a highly significant difference between the neuronal position and endo-N treatment ($F = 18.79$; $p < 0.001$).

precursors in the subventricular zone, as described previously (Ono et al., 1994). However, as indicated above, the two approaches are fundamentally different with respect to the molecular target of the perturbation (i.e., carbohydrate vs polypeptide), as well as its site and duration. In the present study, the analysis used to describe migration at E15 in the animals treated at E12 with endo N was also performed on E15 NCAM mutant embryos (Fig. 3). For the NCAM-180 mutants, 20.4 ± 4.9% of the LHRH neurons were found in the nose, 23.8 ± 4.0% were in the OB, and 55.8 ± 5.0% were in the forebrain. For NCAM null mice, 25.0 ± 8.3% of the LHRH neurons were in the nose, 23.7 ± 4.6% were in the OB, and 51.2 ± 7.6% were detected in the forebrain. The total number of LHRH neurons in NCAM-180 and NCAM null mice was 1553 ± 79.3 and 1282.0 ± 76.3, respectively. The number of LHRH neurons in NCAM-180 mice was significantly higher than the number in wild-type or NCAM null mice, which may be related to the different background strain of these transgenic mice. In contrast to the acute removal of PSA by endo N treatment, the locations of LHRH neurons at E15 in either NCAM mutant mouse were not significantly different from the positions of LHRH neurons in wild-type mice.

In considering the difference between the NCAM-180 and NCAM null mutant mouse studies, it is significant that in the NCAM-180 mutant, other NCAM isoforms, particularly that of NCAM-140, are still expressed (Tomasiewicz et al., 1993). In fact, after obtaining the above results, it was discovered that this residual NCAM expresses PSA in portions of the VNN. In wild-type E14 mice (Fig. 5*A*,*C*), PSA-NCAM was abundant in the forebrain, OB, VNO, VNN, a subpopulation of migrating LHRH cells (Fig. 5*C*, *inset*), and on olfactory nerves emerging from the OE. In particular, PSA-NCAM expression was seen along the VNN projecting dorsally into the bulb, as well as caudally into the forebrain. In the NCAM null mutant, no staining for PSA was obtained (data not shown; Cremer et al., 1994), as would be expected from the fact that PSA is uniquely attached to this polypeptide. With NCAM-180 mutant mice, PSA expression was again absent from the OB, forebrain, and LHRH cells along the migration route and was significantly decreased along the main olfactory nerves (Fig. 5*B*,*D*). However, high levels of PSA were still detected in the NCAM-180 mutant VNO and on the main VNN extending dorsally across the OB to the AOB but remarkably not on those vomeronasal axons that extend caudally into the forebrain. These differences allowed us to examine the effect of selective PSA expression on the direction of LHRH cell migration, because a small number of neurons migrate along the main VNN to the AOB in all animals studied. In wild-type, endo-N treated, and NCAM null mutant mice, which express PSA on both branches of the VNN, 53.4 ± 5.2 neurons were observed migrating along the main VNN toward the AOB. In NCAM-180 mutant mice, however, nearly twice as many (104.4 ± 16.0) LHRH neurons followed the main VNN to the AOB. In sum, the NCAM-180 mutant animals presented a situation in which PSA expression was selectively spared on a subset of VNNs

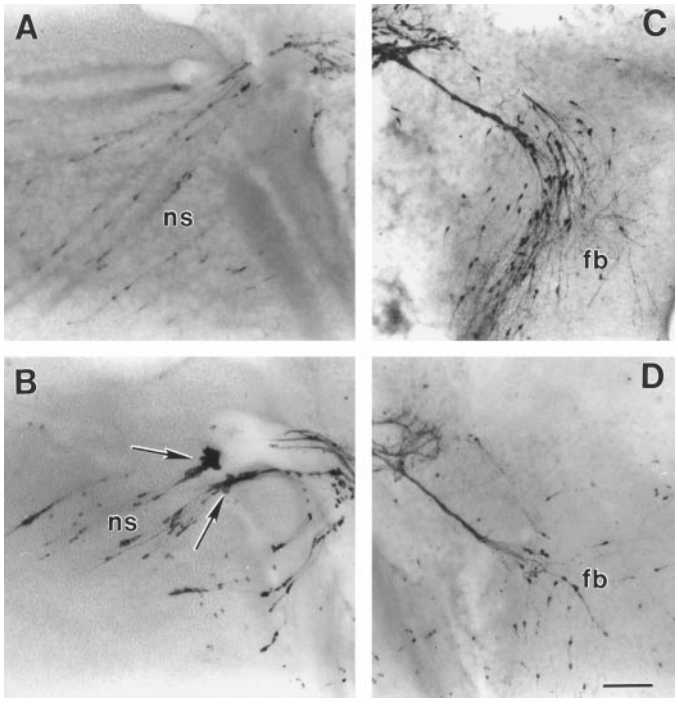

Figure 4. PSA removal alters the pattern of LHRH neuron migration. In contrast to the controls (*A*), LHRH cells were concentrated in the nasal septum (*ns*) of endo N-treated animals (*B*) at E15. Large clusters of LHRH neurons (*arrows*) are visible along the VNNs. *C*, At E15, most of the LHRH neurons in controls are found farther along the migratory pathway in the OB and forebrain (*fb*). *D*, Significantly fewer LHRH neurons are present in the forebrain of endo N-treated mice. The remaining neurons continued along the normal migratory pathway. Rostral is to the *left*. Scale bar, 100 μm.

whose axons extend into the AOB and not into the forebrain. In fact, LHRH neurons in the NCAM-180 mutant appeared to initially migrate normally; however, an excessive number of cells chose to follow the PSA^+ VNN into the AOB rather than to the forebrain.

To confirm the molecular basis for this remarkable expression pattern, we determined which proteins express PSA in this region of the NCAM-180 mutant mice. VNOs were dissected from E17 mutants, homogenized in buffer containing anionic detergent, treated with endo N, and analyzed by SDS-PAGE, followed by immunoblotting with polyclonal antibodies to NCAM (Fig. 6). This analysis revealed that the NCAM-140 polypeptide isoform was significantly increased in homogenates of VNOs and OBs after endo N treatment, suggesting that the persistent PSA is associated with NCAM-140 in the VNO and VNN of NCAM-180 mutant mice.

Effects of endo N treatment and NCAM mutation on the VNN

It is possible that the loss of PSA might affect the structure of the VNN itself and thus indirectly alter cell migration. From previous studies on PSA (Rutishauser and Landmesser, 1996), alterations of the VNN would probably involve axon growth and branching. To examine such effects, axon growth and branching patterns of the VNN were compared by DiI tracing in endo N-treated and untreated animals. In control E14 mice (Fig. 7*A*), DiI placed in the VNO labeled axons that projected to both the AOB and the forebrain. DiI-labeled axons in the forebrain appeared very similar to the pattern of forebrain axons that express the TAG-1 glycoprotein (Fig. 1*C*). As shown in Figure 7*B*, endo N treatment at E12 did not significantly change either the growth or fasciculation of VNO axons projecting into the forebrain at E14. Although it was difficult to photograph the VNN nearer to the VNO because of the DiI crystals, no change in appearance with endo N treatment was detected. From these results, it would appear unlikely that the affect of endo N on cell migration in the proximal part of the pathway reflected an effect on formation of the VNN.

In contrast, the increase in the number of cells that migrate into the OB of the NCAM-180 mutant may be related to a change in the morphology of the branch of the VNN that extends into the forebrain. Whereas this tract was similar in appearance for the control and null mutant mice (Fig. 7*A*,*D*), there was a marked increase in the fasciculation of these fibers in NCAM-180-deficient mice (Fig. 7*C*).

DISCUSSION

The results obtained in this study provide two types of evidence that PSA-NCAM plays a role in LHRH cell migration: a promotion of cell movement along the VNN that is sensitive to enzymatic but not genetic removal of PSA-NCAM, and a preference of the migrating LHRH cell for a PSA^+ axon branch over a PSA^- branch when presented with this choice in the NCAM-180 mutant.

The enzymatic removal of PSA at E12 significantly inhibited the migration of nearly half of the LHRH neuron population without detectably affecting the total number of LHRH cells, the migration route, or the formation of the VNN itself. Considering the experimental paradigm, this effect *in vivo* is highly significant. However, there remains the possibility that residual PSA may be expressed in some treated animals, particularly during the first day after the injection. Second, LHRH cells are known to be heterogeneous, not only for PSA expression, but for other molecules that could modulate cell migration. For example, GABA is only expressed in a subset of the total LHRH cell population (Tobet et al., 1996). Thus, the migration of some populations of LHRH neurons is likely to be more susceptible to such perturbations than are others. Third, a significant number of LHRH cells have already reached the cribriform plate at E12 when the endo N treatment begins. These factors may contribute to the incomplete stoppage of LHRH neuron migration into the forebrain in our experiments. Nonetheless, our data do indicate the efficacy of endo N in cleaving PSA and in retarding the migration of these cells in embryonic mice.

Surprisingly, the absence of NCAM (and thus of PSA) in the null mutant did not produce a major alteration in LHRH neuron migration. The reason for the discrepancy between the enzymatic and genetic perturbations has not been defined. However, the two experimental approaches are fundamentally different with respect to the timing and duration of the PSA removal (acute at E12–E15 vs a germ line defect) and the fact that the mutations affect NCAM polypeptide expression, as well as that of PSA. Thus, either a developmental compensation to the chronic defect or a molecular compensation via simultaneous loss of NCAM could contribute to the normal initial migration behavior observed in the NCAM null mutant.

How might PSA promote cell migration in this system? In previous studies, PSA has been proposed to facilitate cell translocation, probably as a result of reduced cell interactions, through two quite different cellular mechanisms: (1) the initial separation of the cell from its tissue of origin, as in the separation of

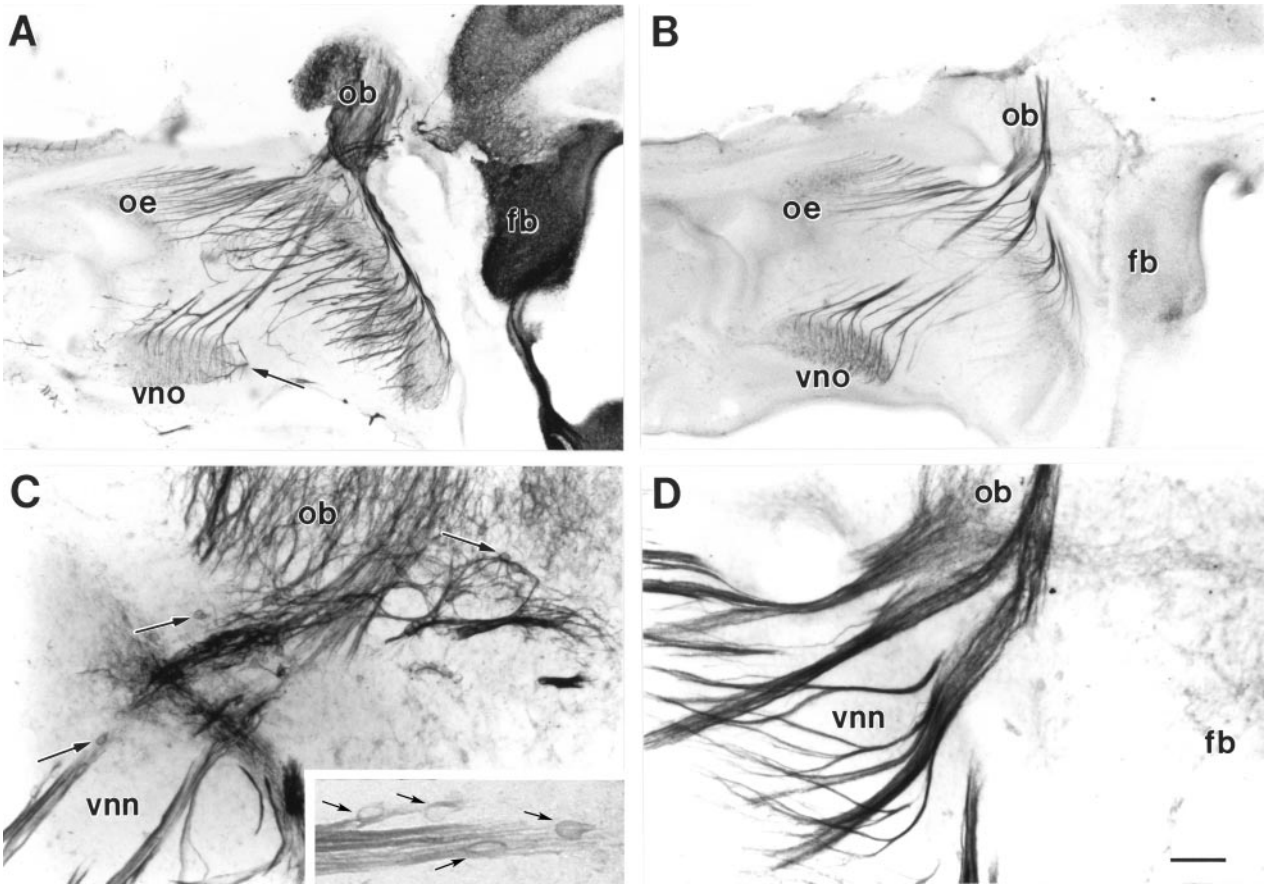

Figure 5. PSA-NCAM expression in wild-type and NCAM-180-deficient mice at E14. *A*, PSA-NCAM expression in the wild-type mouse is visible in the vomeronasal organ (*vno*) and olfactory epithelium (*oe*). There is also widespread immunoreactivity in the olfactory bulb (*ob*) and forebrain (*fb*). Trigeminal nerves (*arrows*) are also PSA-NCAM-positive, because they project into the caudal VNO. *B*, In the NCAM-180 mutant, PSA-NCAM is expressed along the main VNNs as they emerge from the VNO, along the nasal septum, and dorsally into the OB. PSA-NCAM is significantly decreased in the OE, whereas there is little to no reactivity in the forebrain, OB, and trigeminal nerves. *C*, At higher magnification, PSA-NCAM-positive cells are visible (*arrows*) along the LHRH neuron migration pathway. PSA-NCAM expression is also seen on the vomeronasal nerve (*vnn*) as it extends into the forebrain (*fb*). The *inset* in *C* is a high magnification of PSA^+ cell bodies (*arrows*) in association with VNN fibers along the nasal septum. *D*, The NCAM-180-deficient mouse exhibits none of these immunoreactive PSA^+ cell bodies and shows very little expression of PSA-NCAM on the caudal branch of the VNN that extends into the forebrain. Rostral is to the *left*. Scale bar (in *D*): *A*, *B*, 200 μm; *C*, *D*, 50 μm; *inset*, 20 μm.

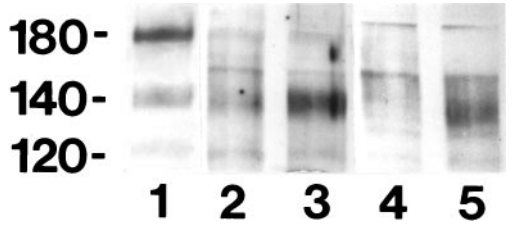

Figure 6. SDS gel electrophoresis and immunoblot analysis of NCAM polypeptides in VNO and OB tissue from NCAM-180-deficient mice. *Lane 1* shows NCAM bands at 180 and 140 kDa from a wild-type E17 OB extract, reacted with the polyclonal anti-NCAM antibody RO25. No NCAM-120 was visible. E17 VNOs from NCAM-180-deficient mice were homogenized and run in *lanes 2* and *3*. E17 OB proteins from the same animals were run in *lanes 4* and *5*. Protein samples in *lanes 3* and *5* were treated with endo N before electrophoresis. In both the VNO and OB homogenates, there was a visible increase in NCAM-140 after endo N treatment.

secondary myotubes from primary myotubes in the chick hindlimb (Fredette et al., 1993), and (2) the facilitation of make or break interactions between the migrating cell and its substrate, as in the cooperative streaming of olfactory interneuron precursors in the mouse subventricular zone (Ono et al., 1994; Hu et al., 1996). In the nasal compartment, PSA is present in the tissue of origin, the VNO, and along the VNN, a pattern consistent with either mechanism. However, ventricular zone and LHRH cell migration are clearly distinct in that the subventricular zone does not contain axons and that only a small subpopulation of LHRH cells are themselves PSA^+. Furthermore, the observation that after PSA removal LHRH cells accumulated in clusters along the VNN in the nasal septum suggests that the defect occurs along the pathway and thus is more likely to involve the axon substrate-associated PSA. These differences may explain why the NCAM mutants were not found to phenocopy the enzymatic removal of PSA in LHRH cell migration.

In contrast to the null mutant or endo N-treated animals, the NCAM-180 mutation had a clear effect on the route of migration of a subpopulation of LHRH cells, resulting in an excess of LHRH cells within the AOB. A possible explanation for this finding may be that in the NCAM-180 mutant there is PSA (associated with the remaining NCAM-140 isoform) expressed on the main VNN that extends into the bulb, whereas there is no PSA along the normal migration route to the forebrain. Thus, cells are presented with a choice between a PSA^+ or a PSA^-

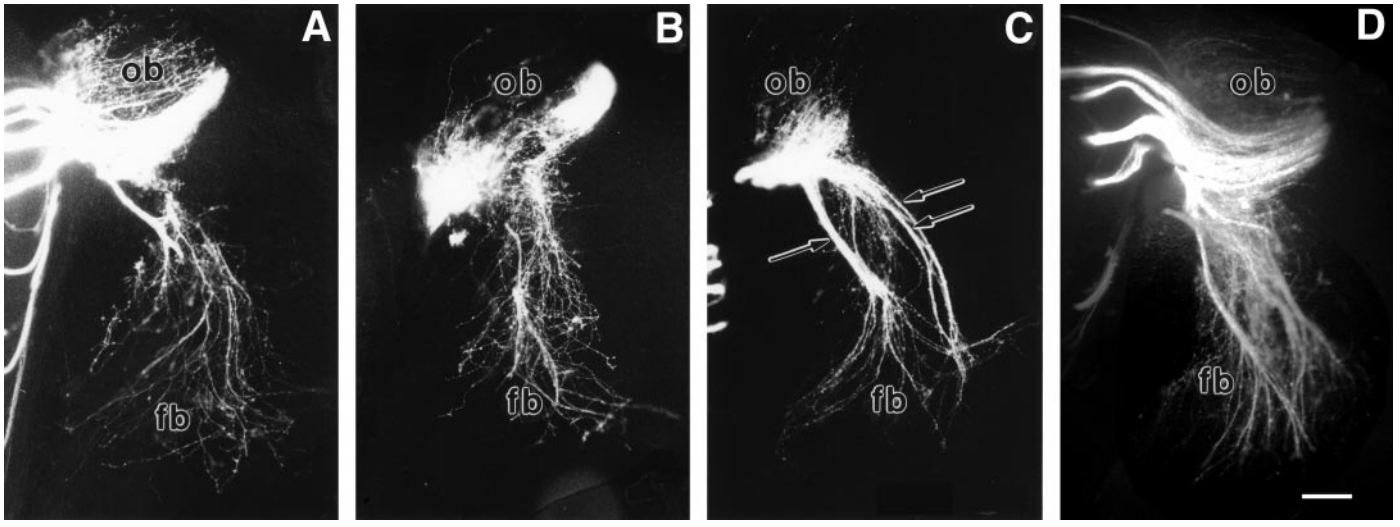

Figure 7. DiI labeling of VNNs in normal, endo N-treated, NCAM-180-deficient, and NCAM null mice at E14. *A*, VNNs in a control mouse at E14 extend along the medial surface of the olfactory bulb (*ob*) or caudally into the forebrain (*fb*). *B*, Cleavage of PSA by treatment with endo N did not seem to drastically alter the course or fasciculation patterns of these nerves. *C*, The caudal VNN in NCAM-180 mutant mice fasciculated to a higher degree (*arrows*), but their overall trajectories remained unchanged. *D*, In contrast, caudal VNNs of NCAM null mutants were more similar to those found in the wild-type and endo N-treated animals. Rostral is to the *left*. Scale bar, 100 μm.

pathway, and although most cells still prefer the forebrain route, an increased number follow the PSA^+ route to the bulb. This aberrant behavior, however, is not apparent in either the null mutant or endo N-treated animals, suggesting that an absence of PSA on both routes does not alter pathway choice.

As noted in Results, differences in the fasciculation of the VNN may also provide a mechanism for the observed misrouting of cells in the NCAM-180 mutant. In these animals, but not the wild-type, null, or endo N-treated mice, there was a pronounced increase in the size of the fascicles that entered the forebrain. It is possible that axophilic cell migration is facilitated by a looser bundling of the axonal substrate and that with tighter bundles the migrating cells were unable to interact as efficiently with the forebrain-bound tract.

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