Kavita Singh

Neurotrophin Expression in the Developing Olfactory System

Key Terms:

- Neurotrophin
- Olfactory
- Anterograde
- Brain-Derived Neurotrophic Factor (BDNF)
- Neurotrophin-3 (NT3)
- Tyrosine Kinase B (trkB)
- Tyrosine Kinase C (trkC)

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For Kavita Singh, maximizing her undergraduate college experience meant involving herself in biomedical research at UC Irvine. During her second year, Kavita began her investigation of olfactory development. The excitement of learning and fine-tuning lab and analytical skills was coupled with participating in dynamic research with findings that suggest a novel understanding of anterograde transport in the CNS. Kavita plans to continue her commitment to research as a medical school student.

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Abstract

During development, neurons compete for neurotrophic molecules from the targets they innervate and can degenerate without this support. Although this retrograde mode of support is well-documented, recent evidence suggests that anterograde support is also important. In the developing rat olfactory system, the olfactory nerve contacts the forebrain just before the olfactory bulbs form at embryonic day 13 to 14. If contact between the nerve and forebrain does not occur, the bulbs do not develop, suggesting that anterograde trophic cues are supplied by the nerve. Embryonic rat tissue was processed for *in situ* hybridization to localize mRNAs for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) along with their receptors, tyrosine kinase B (trkB) and C (trkC). Neurotrophin-3 and BDNF mRNAs were found to be expressed in olfactory epithelium by embryonic day 14, at a time when mRNAs for trkB and trkC were also expressed in developing forebrain. These findings suggest that olfactory bulb development may be influenced by neurotrophins anterogradely supplied by the olfactory nerve.

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Faculty Mentor

Christine Gall

Charles Shivers set out to demonstrate that the conflict in Northern Ireland has not been due to religious differences, but to tensions between politically motivated interest groups. He wanted to explain how the politics and economics of dominance have played a key role in perpetuating the conflict, and how this situation was changing, providing some hope for a breakthrough. Not long after he finished his thesis the world watched in

Kathleen Guthrie

College of Medicine awe as the Good Friday Agreement was College of Medicine signed that brought peace to the long suffering region. Charles persistently sharpened his thinking on the subject and revised the structure and style of his composition. He can be congratulated for his insightful analysis and original writing.◇

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Introduction

Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) belong to a family of trophic proteins called neurotrophins, which promote the differentiation and survival of neurons during development.[1] They act by binding to a family of closely related glycoprotein tyrosine receptor kinases.[1,2] Brain-derived neurotrophic factor preferentially binds tyrosine receptor kinase B (trkB); NT3 preferentially binds tyrosine receptor kinase C (trkC), but also binds trkB with a lower affinity.[1] In the peripheral nervous system, developing neurons send their processes into their target tissue and compete with each other for neurotrophic factors provided by the target cells.[1] Trophic factors bind to receptors on the axon terminals and form a complex. This receptor complex is internalized and transported from the target to the neuron cell body (Figure 1). Neurons that do not receive adequate trophic support die. Neurotrophic factors were previously thought to promote neuronal survival in this retrograde manner. However, recent evidence suggests that anterograde transport of neurotrophins may also occur in some parts of the central nervous system.[3,4,5,6,7]

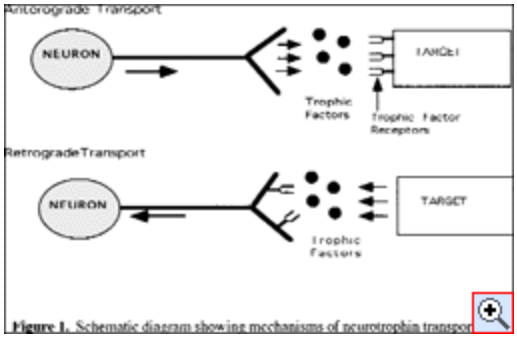

Figure 1
Schematic diagram mechanisms of neurotrophin transport.

In the developing rat olfactory system, olfactory axons have an organizing effect on the developing forebrain. At embryonic day 12 to 13, olfactory nerve axons arising from the sensory neurons in the olfactory epithelium contact and encircle the rostral telencephalon.[8,9] At about embryonic day 14, the olfactory bulbs begin to evaginate. By embryonic day 16, additional olfactory axons have innervated the forebrain, and the olfactory bulbs are distinct.[9] This sequence of events suggests that olfactory nerve axons may trigger development and differentiation of olfactory bulb neurons.

were examined in embryos ranging from embryonic day 14 to 20. In addition, NT3 and trkB immunoreactivity were examined in the olfactory epithelium and bulbs of neonatal rats. The observed patterns of neurotrophin and trk expression suggest that trophic factors may be anterogradely transported to the developing olfactory bulbs.

Materials and Methods

Animals:
Timed pregnant, female Sprague Dawley rats were deeply anesthetized using sodium pentobarbital (100 mg/kg body weight), and embryos at day 14 to 20 (mate date=embryonic day 0) were surgically removed. Embryos older than embryonic day 16 were decapitated. The embryos/heads were frozen in 2-methylbutane at -40°C. Frozen sections were cut sagittally in a cryostat at 20 mm and collected onto Vectabond-coated slides (Vector Laboratories). Tissue was postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer, and slides were air dried and stored at -20°C until processed for *in situ* hybridization.

For immunohistochemistry analysis, rats at postnatal day 4 (day of birth=postnatal day 0) were overdosed with sodium pentobarbital (10 mg/100 g) and transcardially perfused with 2% paraformal dehyde in 0.1 M phosphate buffer. After decapitation, the heads were postfixed in 2% paraformaldehyde at 4°C for 1 h, and then transferred to 20% sucrose in 0.1 M phosphate buffer for 48 h at 4°C. Coronal sections through the head region were cut at 30 mm in a cryostat, collected into 0.1 M phosphate buffer, and immediately processed for immunostaining.

In Situ Hybridization:
The ^{35}S-labeled cRNA antisense probes for rat BDNF and rat NT3 were transcribed from cDNA templates in the presence of alpha ^{35}S-UTP. Antisense BDNF mRNA and antisense NT3 mRNA were generated from rat recombinant plasmids with T3 RNA polymerase following linearization with PvuII. The cRNA probe for BDNF contains 384 bases complementary to rat BDNF mRNA. The antisense probe for NT3 contains 550 bases with 392 bases complementary to rat NT3 mRNA. The antisense probe for trkB was made from a recombinant plasmid cut with EcoRI using T7 RNA polymerase. The trkB template corresponds to bases 1120 to 1458 of the

To determine if neurotrophins might play a role in bulb formation, we used *in situ* hybridization to localize the mRNAs for BDNF, NT3, trkB, and trkC in the developing rat olfactory system. Levels of mRNA expression

rat trkB sequence; the cRNA detects full length trkB mRNA. The trkC template was cut with HindIII and transcribed using T3 RNA polymerase. Tyrosine kinase C cDNA encodes a region corresponding to bases 349 to 490 of the rat trkC sequence.

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For *in situ* hybridization localization of mRNAs for BDNF, NT3, trkB and trkC, the tissue was sequentially rinsed in 0.1 M glycine in phosphate buffer, 0.1 M phosphate buffer, 0.25% acetic anhydride in 0.1 M triethanolamine, (pH 8.0 for 15 min at room temperature), and 2X saline sodium citrate buffer (SSC; 1X SSC = 150 mM NaCl, 15 mM sodium citrate; pH 7.0). Afterwards, the tissue was dehydrated through increasing concentrations of ethanol, defatted in chloroform, and rehydrated. Hybridization solution (10% dextran sulfate, 50% formamide, 35X Dendhardt's solution, 0.15 mg/ml yeast tRNA, 0.30 mg/ml salmon sperm DNA, 40 mM dithiothreitol, and 1 million cpm radiolabeled cRNA transcript/100 ml) was applied to each slide (250 ml). Slides were coverslipped and incubated at 60°C for 17 to 19 h. Following the incubation, the slides were rinsed in 4X saline sodium citrate buffer containing 0.16 g sodium thiosulfate/100 ml at 60°C for 60 min. Tissue was then treated with ribonuclease A (12 kunitz units/ml in 0.1 M Tris, 0.5 M NaCl, 0.01 M EDTA, pH 8.0) at 45°C for 45 min. The sections were rinsed in decreasing concentrations of saline sodium citrate buffer containing sodium thiosulfate for 17 to 20 h, rinsed in 0.05 M phosphate buffer and dried. Slides were exposed to Amersham bmax-Hyperfilm for 3 to 5 d, then dipped in Kodak NTB2 nuclear track emulsion (1:1 dilution with water) and exposed for 3 to 9 w. The emulsion was developed using Kodak D19 developer. Sections were stained with cresyl violet or neutral red, and coverslipped with Permount. Tissue sections were examined for localization of BDNF, NT3, trkB, and trkC mRNAs using dark field microscopy.

Immunohistochemistry:
Tissue sections were rinsed in 0.1 M phosphate buffer, then pre-incubated in 2% bovine serum albumen and 0.25% Triton X-100 for 1 h. Sections were washed in phosphate buffer and incubated in chicken antibody to NT3 (Promega; 22 mg/ml; 19 h at 4°C) or in rabbit antibody to trkB (Transduction Labs; 1:100; 19 h at 4°C). Tissue was then rinsed in phosphate buffer and incubated in biotinylated anti-chicken immunoglobulin G (1:200) for 2 h at room temperature. After rinsing in phosphate buffer, tissue was incubated in horseradish peroxidase avidin-biotin complex (Vector Laboratories, Burlingame, CA) for 90 min at room temperature. Sections were rinsed in phosphate buffer, then reacted with diaminobenzidine (0.1 mg/ml) in phosphate buffer containing 0.03% hydrogen peroxide

Results

Figures 2 and 3 illustrate the distribution of BDNF, NT3, trkB, and trkC cRNA hybridization in sagittal sections through the embryonic rat head. In the dark field photomicrographs, autoradiographic silver grains, which indicate labeling, appear white.

Brain-derived neurotrophic factor mRNA:
As seen in Figures 2B and 2C, low levels of BDNF mRNA were expressed in the developing rat olfactory system. Hybridization of BDNF cRNA occurred in the olfactory epithelium at embryonic day 14 (Figure 2B). At this age, high levels of labeling were seen in the heart and the choroid plexus. By embryonic day 20, BDNF mRNA was detected in the tongue, in the facial mesoderm, and in the mesoderm underlying the olfactory epithelium. Very little labeling was seen in the olfactory epithelium at this age (Figure 2C). Hybridization was not seen in the olfactory bulb at any age examined.

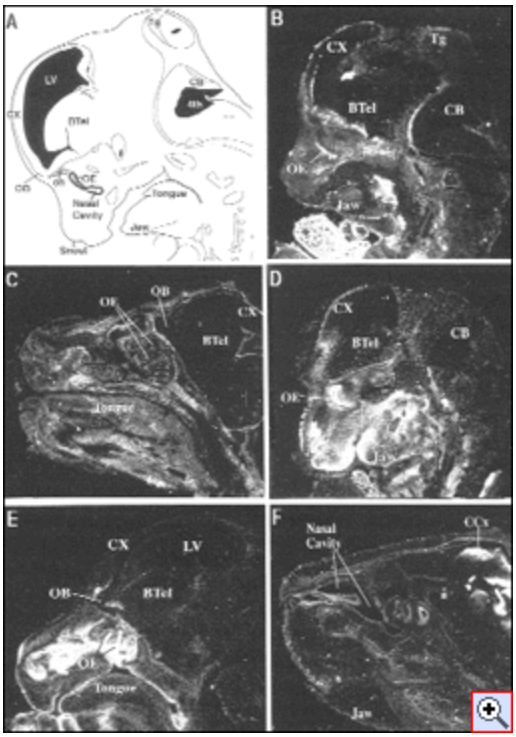

Figure 2
A. Schematic diagram of a sagittal section through a rat head at embryonic day (E) 14. Sagittal tissue sections hybridized with cRNA for brain-derived neurotrophic factor (BDNF; B, C), and neurotrophin-3 (NT3; D-F). BDNF mRNA is low in olfactory epithelium (OE) from E14 (B) to E20 (C). High levels of NT3 mRNA are seen at E14 (D) and E16 (E), but decrease by E20 (F). Moderate levels

and 0.06% nickel chloride for 10 to 20 min, and rinsed. Sections were mounted onto gelatin-coated slides, and air-dried. Tissue was dehydrated through alcohols, cleared in Americlear, and coverslipped with Permount.

Tissue sections were examined for immunoreactivity using light field microscopy.

occur in cortex (CX), and high levels are seen in olfactory bulb (OB) by E16. LV, lateral ventricle; BTel, basal telencephalon; Tg, tegmentum; CB, cerebellum; on, olfactory nerve; CCx, cingulate cortex.

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Neurotrophin-3 mRNA:
As shown in Figure 2, NT3 mRNA was expressed at much higher levels than BDNF mRNA in the developing olfactory system. At embryonic day 14, dense hybridization of NT3 cRNA occurred in the olfactory epithelium, in the respiratory epithelium, and in the epidermis of the snout and jaw. There was also diffuse labeling in the superficial neocortex at this age (Figure 2D). By embryonic day 16, the olfactory bulbs were clearly visible and exhibited distinct labeling in the outer layers (Figure 2E). However, only sparse labeling was seen where the olfactory nerve fibers contacted the ventral bulb; very little hybridization was seen in the accessory olfactory bulb. At embryonic day 16, expression of NT3 mRNA was still very high in the olfactory epithelium, with slightly less labeling seen in the underlying mesoderm (Figure 2E). The dense distribution of silver grains throughout the olfactory epithelium made individually labeled cells unidentifiable. By embryonic day 20, the density of NT3 cRNA hybridization had declined in the olfactory epithelium, although the surrounding mesoderm was still moderately labeled. At this age, dense labeling was seen in the developing cingulate cortex (Figure 2F).

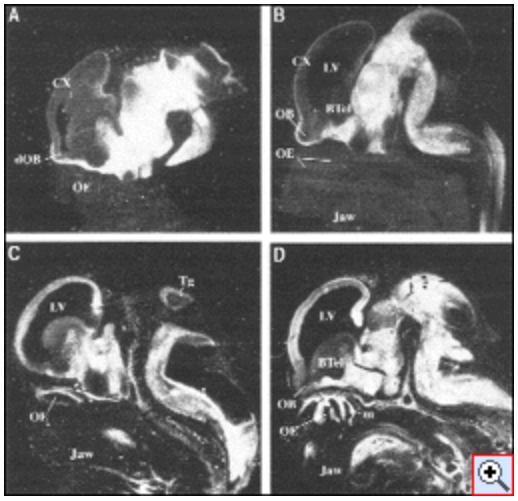

Figure 3
Sections hybridized with cRNA for trkB (A-B) and trkC (C-D). High levels of trkB mRNA are seen in the developing olfactory bulb (dOB) by E14 (A), and by E16 (B) expression is higher in the outer layer (B). TrkC cRNA labels most of the brain at E14 (C) and E16 (D), including the bulbs. At E16 (D), the mesoderm (m) under the olfactory epithelium is densely labeled. LV, lateral ventricle; BTel, basal telencephalon; Tg, tegmentum.

Tyrosine receptor kinase B mRNA: Hybridization of trkB cRNA occurred throughout most of the neuraxis at embryonic day 14 to 16 (Figure 3A). At embryonic day ers. Hybridization density was also higher in the superficial portions of the neocortex compared to deeper portions. By embryonic day 16, many regions of the brain and spinal cord were heavily labeled (Figure 3B). At this age, a distinct laminar pattern of labeling was seen in the olfactory bulb, with the outer region more densely labeled than the inner region; by embryonic day 18, this labeling appeared denser. Hybridization of trkB cRNA in the olfactory epithelium was not detected at any of the time points examined.

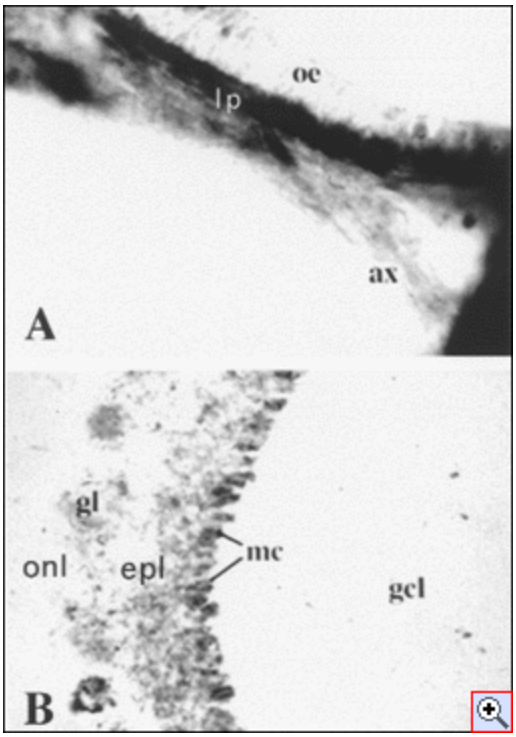

Figure 4
High magnification, light field photomicrographs of tissue sections through the olfactory epithelium (oe in A) and olfactory bulb (B) of a postnatal day 4 rat. (A) NT3 immunoreactivity is detected in olfactory nerve axons (ax) located below and within the lamina propria (lp). (B) TrkB antibody stains the mitral cells (m) of the olfactory bulb, with cell bodies more densely labeled than their dendrites which are distributed in the external plexiform layer (epl) and within individual glomeruli (g). No trkB immunostaining is detected in the olfactory nerve layer (onl). [gcl: granule cell layer]

Tyrosine receptor kinase C mRNA:
At embryonic day 14, trkC cRNA hybridized throughout most of the brain and spinal cord (Figure 3C). Dense labeling was also seen in areas of mesoderm. At embryonic day 16, high levels of trkC cRNA hybridization occurred in the mesoderm underlying the olfactory epithelium and developing

14, labeling was denser in the superficial region of the developing bulb than in deeper lay

neocortex, while moderate levels of hybridization were seen in the olfactory bulb

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(Figure 3D). In comparison to trkB cRNA hybridization, trkC labeling was more uniformly distributed in the cortex and olfactory bulbs; it did not appear laminated. Tyrosine kinase C labeling was high in the mesoderm, whereas trkB labeling was not detected here. At later ages, expression of trkC mRNA in the mesoderm declined, while labeling distinctly increased in the olfactory bulbs. Tyrosine kinase C mRNA was not detected in the olfactory epithelium at any of the observed ages.

Neurotrophin-3 immunoreactivity:
Neurotrophin-3 protein expression was not seen in the olfactory bulbs of rats at postnatal day 4. However, immunoreactivity was detected in olfactory axons distributed between the olfactory epithelium and the olfactory bulb (Figure 4A). No immunoreactivity was detected in the sensory neuron cell bodies in the olfactory epithelium.

Tyrosine receptor kinase B immunoreactivity:
At postnatal day 4, trkB antibody stained the mitral cells of the olfactory bulbs. The cell bodies were densely stained; lower levels of staining were seen in their dendrites within the external plexiform layer and the glomeruli (Figure 4B). Developing granule cells and their dendrites were faintly labeled at this age. Tyrosine kinase B immunoreactivity was also seen in a few cells in the glomerular layer of the olfactory bulb.

Discussion

The results of this study demonstrate that in the developing rat olfactory system, high levels of NT3 mRNA and lower levels of BDNF mRNA are expressed in the olfactory epithelium at a time when the olfactory axons make contact with the rostral telencephalon. At this time, cells in the developing forebrain express the mRNAs for the neurotrophin receptors, trkB, and trkC. Furthermore, in neonatal rats, NT3 immunoreactivity is detected in olfactory nerve axons, although not in sensory neuron cell bodies; trkB immunoreactivity is seen in the mitral cells of the olfactory bulbs. The spatial and temporal distribution of neurotrophin and trk mRNA expression, and the distribution of NT3 and trkB immunoreactivity, suggest that neurotrophins may act in an anterograde manner on trk-expressing cells in the developing forebrain and olfactory bulbs. Neurotrophins in the olfactory epithelium could also act locally, or on innervating fibers from the trigeminal nerve. Neurotrophin-3 mRNA is seen in the bulb during early development, but is not detected in adult rats; therefore, local actions of NT3 may occur in studies have provided evidence that neurotrophins can be anterogradely transported in the central nervous system (Figure 1).[3,4,7,6] Studies of the developing chick visual system have shown that radiolabeled NT3 and BDNF are anterogradely transported from the retina to the tectum when injected into the eye.[6] In the retinotectal pathway, neurotrophin mRNA is expressed in the retina while trk mRNA is expressed in the postsynaptic target, the tectum. Such patterns of distribution suggest anterograde transport of endogenous neurotrophins in this pathway. Additional evidence for anterograde transport of trophic factors has come from studies of the motor-cortical region of the zebra finch brain.[3] If presynaptic input from the lateral magnocellular nucleus of the anterior neostriatum is removed, there is massive neuronal death in the target, the robust nucleus of the archistriatum (RA). However, if lesions are followed by infusions of neurotrophins into the RA, cell death in the RA is suppressed.[3] Further evidence of anterograde transport in the brain has also been obtained in the rat. Brain-derived neurotrophic factor protein is present in processes in the striatum, not in cell bodies, but BDNF mRNA is not expressed here. Inhibition of axonal transport decreases BDNF immunoreactivity in the striatum and increases it in regions that project to the striatum.[4] Taken together, these observations suggest that anterograde transport of neurotrophins may be a mechanism common to many brain systems.

In the developing rat olfactory system, patterns of neurotrophin mRNA expression are correlated with olfactory nerve innervation of the developing forebrain. At embryonic day 12 to 13, olfactory axons first contact the telencephalon; by embryonic day 14, the olfactory bulb primordium is morphologically distinguishable from the adjacent neocortex.[8,9] The coincident arrival of olfactory nerve afferents in the telencephalon with bulb formation suggests that the olfactory nerve contributes to olfactory bulb development. This hypothesis is supported by studies of arhinencephalic mutant mouse embryos. In these mice, the olfactory nerve axons fail to make contact with the developing forebrain, resulting in apparent death of developing mitral cells and the complete

the developing olfactory bulb.[10]

In contrast to the classical retrograde hypothesis proposed for the peripheral nervous system, several recent

absence of olfactory bulbs.[11] Furthermore, surgical removal of the olfactory placode in amphibian species results in a shrunken telencephalon and/or the absence of olfactory bulbs.[8] Data suggested that olfactory nerve fibers may contribute to bulb formation by altering cell cycle kinetics within the contacted olfactory bulb primordium, causing more cells in this area to exit the cell cycle and begin differentiation as compared to nearby neocortex.[9] Neurotrophins or other substances provided by the olfactory nerve may be causing changes in cell proliferation patterns in the olfactory bulb primordium.

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Neurotrophic factors regulate neuronal differentiation, as well as cell survival and proliferation.[1] A lack of neurotrophic support can result in cell death and atrophy of surviving neurons and their processes.[12] Studies of rabbit and rat olfactory systems have shown that removal of the olfactory epithelium causes degeneration of some neurons in the olfactory bulbs.[13] Additional studies of young rodents indicate that depriving the olfactory bulbs of active nerve input by naris occlusion results in reduced bulb size.[8] Restricting olfactory nerve activity by naris occlusion also results in increased levels of cell death in the olfactory bulbs.[14] It has recently been shown that neurotrophins can be released from the neurons, which synthesize them in an activity-dependent manner.[15] Since neurotrophins can be released from neurons during stimulation, and some olfactory bulb neurons can die from a lack of olfactory nerve stimulation, it may be that neurotrophic factors are transported to the bulb and released by active olfactory nerve afferents. Cell death in the deprived olfactory bulb therefore may be due to a lack of trophic support from the olfactory nerve.

This study demonstrates that neurotrophins are expressed by the developing rat olfactory epithelium, and NT3 immunoreactivity can be detected in olfactory nerve axons. Neurotrophin receptors are expressed in the developing forebrain and olfactory bulb. Based on our results, we propose that, as has been seen in other parts of the brain, there is anterograde transport of neurotrophins in the rat olfactory system. Future studies will examine transport of radiolabeled neurotrophins injected into the olfactory epithelium.

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