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Neurotrophin-3 synthesized by olfactory sensory neurons is anterogradely transported to axon terminals in the olfactory bulb

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Immunolocalization of neurotrophins in the rodent olfactory epithelium (OE) demonstrates that olfactory sensory neurons (OSNs) contain the neurotrophin peptides nerve growth factor, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). Expression of mRNAs for these factors, however, is not seen with in situ cRNA hybridization, so it is unclear whether OSNs can express the peptides themselves. Using quantitative-RT-PCR of RNA laser-captured from OSNs in situ, we verified that NT3 transcript is the most abundant of three neurotrophins. To test if OSNs synthesizing NT3 target the protein to appropriate secretory pathways and transport the peptide in their axons, we constructed adenovirus vectors encoding fusion peptides consisting of pro-NT3 and green fluorescent protein (GFP) or hemagglutinin (3xHA) epitope tag. Infection of cultured COS and neuroblastoma cells demonstrated synthesis and packaging of the fusion peptides occurred in secretogranin II-immunoreactive secretory granules, and peptide secretion was detected by ELISA. Nasal irrigation with adenovirus was used to infect mouse OSNs in vivo, and the expression and distribution of the fusion peptide was monitored by confocal and electron microscopy from 5 to 14 days after treatment. Within the epithelium, OSNs expressing the tagged peptide displayed punctate GFP labeling in the Golgi of the cell soma, secretory vesicles along the dendrites, and the axon terminals in the olfactory bulb glomeruli. Moreover, immunolocolization showed 3xHA-NT3 can transport across the synapse, accumulating in the TrkC-positive mitral/tufted neurons. Our data demonstrate for the first time that OSNs provide a specific anterograde trophic signal to the target bulb neurons they innervating.

Neurotrophin-3 trafficking in the olfactory system: Expression, anterograde transport and postsynaptic transfer to target neurons

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Abstract

The adult olfactory system is unique in its ability to generate new neurons throughout life, both peripherally in the nasal cavity, and centrally in the olfactory bulb. Peripheral olfactory sensory neurons (OSNs) are gradually eliminated and replaced by new neurons generated from resident stem cells in the olfactory epithelium (OE), while progenitors in the forebrain subventricular zone (SVZ) give rise to new olfactory bulb interneurons, some of which survive to replace previously-born neurons eliminated by apoptosis. Sensory axons synapse on the dendrites of olfactory bulb mitral, tufted and periglomerular cells, and turnover of the OSN population is accompanied by the constant remodeling of this input. As a result of this ongoing structural plasticity, the adult olfactory pathway maintains features characteristic of developing neural systems.

Neurotrophic factors are well known as regulators of neuronal survival, differentiation, and connectivity during development, and trophic interactions between the olfactory bulb and its innervating sensory neurons have long been hypothesized. A wide variety of identified factors and their cognate receptors have been localized to the olfactory system. These include the nerve growth factor family of neurotrophins and their Trk receptors. Neurotrophins function as target-derived, retrograde survival factors for developing peripheral neurons, but are also important mediators of dendritic maturation and maintenance, as well as synaptic plasticity, in the CNS, actions that are not limited to development. In some instances, these signals are provided via anterograde transport, with innervating axons supplying endogenous neurotrophins to central postsynaptic targets. An example is the anterograde transport of neurotrophin-3 (NT3) by developing optic nerve projections (Wang et al., 2003).

Although not detectable with in situ cRNA hybridization, NT3 appears to be expressed in a subset of OSNs in adult transgenic mice expressing beta-galactosidase (b-gal) under the NT3 promoter (Vigers et al., 2003). Bulb neurons receiving input from OSNs express TrkC (Deckner et al., 1995), the preferred NT3 receptor, suggesting that like the developing visual system, the adult olfactory pathway may utilize NT3 as an anterograde trophic messenger. Immunolocalization of the peptide has produced conflicting results from different laboratories using different antibodies, and as the peptide can be transported, site of synthesis is unclear. Here we use a combination of immunocytochemistry, Q-RT-PCR of laser-captured RNA, and viral-mediated gene transfer of epitope-tagged NT3 fusion peptides, to show that OSNs express and anterogradely transport NT3 in their axons in vivo. Additionally we show that sensory neuron-supplied NT3 can be acquired by postsynaptic neurons in the adult olfactory bulb, providing for the first time direct evidence that innervating OSNs provide specific trophic signals to their target bulbar neurons. Such support may contribute to the ongoing anatomical plasticity of the pathway, including the integration of adult-born periglomerular neurons.

Experiments in vitro

Adenovirus constructs

The coding sequences of mouse prepro-NT3 (Genbank Access# NM-008742; IMAGE #1177923) and prepro-BDNF (Genbank access#AI098498; IMAGE #139728) were PCR amplified from ATCC clones using primers that introduced flanking restriction sites, modified the Kozak sequences, and removed the stop codons. Sequences were ligated into pEGFP vector (Clontech) to produce fusion sequences with GFP (~238 AAs) at the C-terminal ends. Sequences were cloned into pShuttle-CMV vector (AdEasy XL, Stratagene). For the NT3-3XHA-IRES-GFP construct, PCR amplification of the NT3 coding sequence was carried out and the product was cloned into pShuttle-IRES-hrGFP-2 (Stratagene) with an in-frame 3X hemagglutinin (HA) epitope (27 AAs) encoded at the C-terminus of the NT3 sequence. BJ5183 bacteria pre-transformed with pAdEasy-1 were transformed with shuttle vectors containing the constructs to produce recombinant adenovirus. Recombinants were amplified in XL-10-Gold bacteria and AD-293 cells were infected to produce viral stocks.

Western blot analyses of peptide expression in vitro

Neuroblastoma (N2a) or COS7 cells (ATCC) were seeded in DMEM with 20% FBS and maintained overnight. Media was removed, cells were washed with serum-free DMEM, then infected with recombinant adenovirus ON. Media was removed, serum-free DMEM was added, and cells were grown for 3 days. Cell lysates were subjected to SDS-PAGE and Western blotting. Membrane incubation was carried out ON at 4ºC using rabbit anti-NT3 (ours or Santa Cruz) combined with mouse anti-HA (Covance) or mouse anti-GFP (Santa Cruz). Licor infared dye-labeled secondary antibodies were used to localize bands using the Licor Oddysey imaging system.

Immunolabeling of cell cultures

Localization of tagged peptides was examined in COS7 and N2a cells at 3 days post-infection. Fixed cells (4% PFA) were incubated in rabbit anti-secretogranin II (Thermo), followed by AlexaFluor594 anti-rabbit IgG (Invitrogen). Labeled cells were imaged by confocal microscopy to evaluate co-localization with GFP. NT3-3XHA was localized in fixed cells at 3 days post-infection with Covance mouse antibody to HA (1:1200, clone HA.11).

Experiments in vivo

Antibody production and NT3 immunolocalization

Three synthetic peptides corresponding to regions of mouse NT3 having the least homology to BDNF and NGF were used as antigens to generate affinity-purified rabbit antibody (ProteinTech Group). Sequences were: CLGEIKTGNSPVKQY, TSENNKLVGWRWIC, CVTDKSSAIDIRGHQ. Specificity was verified by Western blot. Adult mice were perfused with buffered 2% PFA containing 0.2% parabenzoquinone. Antibody incubation (1:800) was done at 4ºC for 48 hrs. For comparison of NT3 protein localization and gene expression, sections from heterozygous mice NT3-LacZ mice (Vigers et al., 2003) were processed for beta-galactosidase histochemistry.

Laser capture microdissection and Q-RT-PCR

OE cryosections (8μm) were collected on LCM membrane slides. Ethanol-fixed tissue samples were collected at 20X using the ArcturusXT Laser Microdissection instrument. UV laser cuts were used to separate a region of sensory neurons located in the middle of the epithelium. Cut samples were pulsed with the IR laser to capture them on LCM caps. Total RNA was isolated with the PicoPure kit and RNA quality was measured by Pico Chip assay using the Agilent 2100 Bioanalyzer. RNA was amplified with Stratagene's Brilliant II One-step RT-PCR kit and FAM-labeled Taqman probe/primer assays for mouse NT3, BDNF, NGF, OMP and beta-actin transcripts. Reactions were performed in triplicate using 45ng of RNA (RIN >6.5) and random primers. Each triplicate assay was performed twice.

Viral transduction of olfactory sensory neurons and detection of fusion peptides in vivo

Viruses were diluted in sterile PBS to 2-5 x 10^{10} pfu/ml. Adult mice were anesthetized and 30ul of solution was delivered to the right nasal cavity using multiple 6 ul doses. Irrigation was repeated over the next 2 days. Mice survived 4-10 days after the first treatment and were euthanized and perfused with 4% PFA. Horizontal sections were processed free-floating using AlexaFluor594- or 488-conjugated secondary antibodies to detect antigens labeled with the following primary antibodies: Anti-TrkC (goat, R and D Systems), anti-olfactory marker protein (OMP, goat, Wako), anti-doublecortin (DCX; goat, Santa Cruz), or anti-HA (mouse, Covance).

Ultrastructural localization of NT3-GFP in sensory axons: Pre-embedding immunogold labeling

Two adult female C57Bl6 mice received intranasal treatment with Ad-NT3-GFP over 3 days. On the sixth day after the first treatment, mice were euthanized and perfused with cold PB followed by 4% PFA.. Brains were dissected and postfixed for 3 days. Coronal vibratome sections through the olfactory bulbs were cut at 50 um. Sections were treated with 0.1 M glycine followed by 0.1% sodium borohydride. Tissue was permeabilized in 50% ethanol for 30 min and incubated in Aurion goat blocker solution (EM Sciences). Sections rinsed in 0.15% acetylated BSA-c (Aurion), followed by 20 hr incubation in rabbit anti-GFP at 4ºC (1:500, Millipore). Sections then incubated overnight at 4ºC in Ultra Small immunogold F(ab')2 fragment of goat anti-rabbit IgG (1:100, Aurion), were postfixed in 2.5% gluteraldehyde, and treated with silver enhancing solution (Ted Pella). Tissue was counterstained, fixed with 0.5% OsO_4, dehydrated and embedded in propylene oxide/Epon-12-based resin. Ultramicrotome sections were cut at 60-80nm, and stained with uranyl acetate and lead citrate on mesh grids for TEM examination of the olfactory nerve layer.

Endogenous NT3 expression by OSNs

Transcript abundance and localization of peptide and reporter gene expression

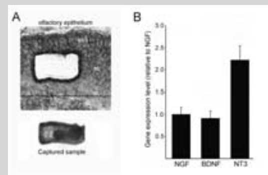

Figure 1. Laser capture microdissection of sensory neurons isolated from the middle of the epithelium (**A, top**). UV-cut samples were pulsed with an IR laser to capture them on LCM caps (**A, bottom**). Total RNA was isolated for Q-RT-PCR using FAM-labeled Taqman probe/primer assays for mouse NT3, BDNF, NGF, OMP and beta-actin transcripts. Relative levels of neurotrophin transcripts (normalized to b-actin) are shown in **B** (with NGF set to 1.0). **Mean Ct values: NT3=26.70, BDNF=27.60, NGF=27.55, b-actin=22.89, OMP=13.95, no template=39.92.** SUS, sustentacular cell bodies; NL, neuronal cell layer; BL, basal lamina).

Figure 2. (A) Western blot showing specificity of the rabbit NT3 antibody. Each lane contains 1ug of recombinant peptide. **(B-C)** Immunostaining of mouse olfactory epithelium showing labeled OSNs. **(D)** Staining is also seen in olfactory bulb glomeruli (gl), where sensory axons terminate. **(E)** Distribution of NT3 gene expression in the epithelium of a mouse heterozygous for NT3-LacZ. **(F-G)** Immunostaining controls include positive labeling of Purkinje cells (F), and lack of OE cell labeling with pre-immune serum.

Mammalian cells express and secrete tagged NT3 in vitro

Adenovirus-mediated gene transfer

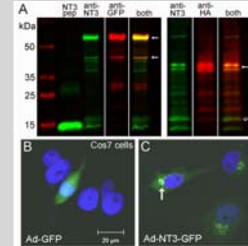

Table 1. Protein determination by ELISA.

Dilution factor	Ad-NT3-GFP	AdCMV-GFP control
undiluted	942 pg/ml	4 pg/ml of NT3
1:2	668 pg/ml	0 pg/ml of NT3
1:4	582 pg/ml	0 pg/ml of NT3

Figure 3. **(A)** Western blots of lysates from AdNT3-GFP or AdNT3-3XHA-IRES-GFP infected Cos7 cells. Blots were probed with antibodies to NT3 and GFP, or NT3 and HA. The NT3 antibody recognizes the control peptide monomer (~13.5 kD), and both proNT3 and mature NT3 tagged with either GFP or 3XHA (arrows). The propeptide is abundant in infected COS cells. **(B)** COS7 cells infected with Ad-GFP show diffuse GFP fluorescence. **(C)** Infection with AdNT3-GFP produces labeling in the Golgi/ER (arrow), indicating that fusion peptide is processed in the secretory pathway. **Table 1** above shows ELISA measurements of secreted NT3 (total) in media collected from Cos7 cells 3 days post-infection with AdNT3-GFP or Ad-GFP.

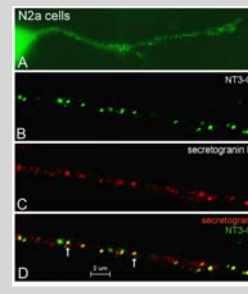

Figure 4. (A). Cultured N2a cells contain NT3-GFP in their processes. **(B-D)** Colocalization with secretogranin II shows that the tagged neurotrophin is contained in dense core secretory granules (arrows in D) within these processes.

Figure 5. **(A-C)** N2a cells treated with AdNT3-3XHA-IRES-GFP express NT3-3XHA that localizes to the secretory apparatus (red) and GFP (green) throughout the entire cell. **(D-F)** Within processes, GFP is diffusely distributed, whereas NT3-3XHA is distributed in a punctate pattern indicative of localization in secretory granules.

Olfactory sensory neurons express and transport epitope-tagged NT3 in vivo

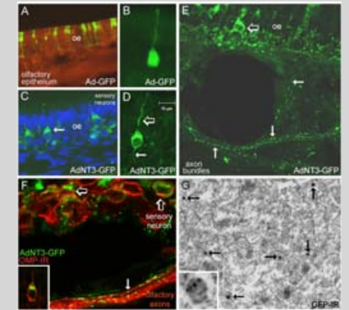

Figure 6. Olfactory epithelium (oe) at 6-10 days post-infection. **(A-D)** Comparison of GFP distribution in Ad-GFP-infected (A-B) and AdNT3-GFP infected olfactory sensory neurons (OSNs; C-D). OSNs expressing NT3-GFP show bright labeling in the Golgi apparatus (arrow in C), and in dendritic and axonal processes (arrows in D). **(E)** NT3-GFP+ puncta are contained within bundles of sensory axons (arrows) exiting the epithelium. **(F)** GFP+ puncta colocalize with OMP in axon bundles (solid arrow), and in OSNs (open arrows and inset). **(G)** Electron micrograph of a section through the bulb's olfactory nerve layer showing immunogold-GFP labeling in sensory axons. Densely-labeled structures (arrows) are consistent with secretory granules (inset).

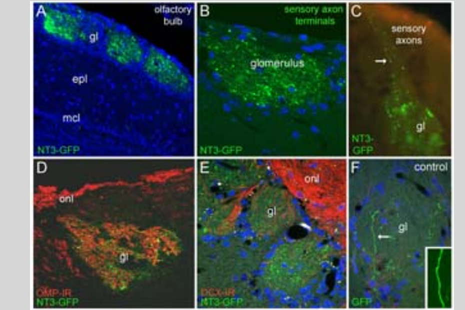

Figure 7. Confocal microscope images of the olfactory bulb (OB) glomerular layer at 7-10 days post-infection. **(A)** Low magnification image showing GFP distribution in the lateral glomerular layer of a mouse treated with AdNT3-GFP. **(B)** Higher magnification of NT3-GFP associated with sensory axon terminals in a glomerulus. **(C)** Punctate GFP is distributed in incoming sensory axons (arrow). **(D)** NT3-GFP co-distributes with OMP-IR in glomeruli. **(E)** Bulb section immunostained for DCX (red) showing sensory axons entering glomeruli containing NT3-GFP. **(F)** Infection with control Ad-GFP also labels sensory axons in glomeruli (arrow), but the uniform GFP distribution contrasts with the vesicular-like pattern seen with expression of NT3-GFP. gl, glomeruli; epl, external plexiform layer; mcl mitral cell layer.

Anterogradely transported NT3-3XHA is acquired by postsynaptic target neurons

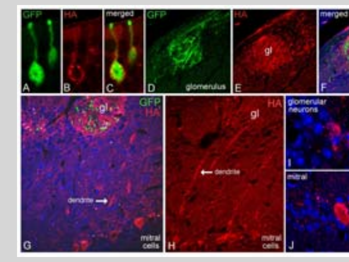

Figure 8. **(A-C)** Olfactory sensory neurons transduced with AdNT3-3XHA-IRES-GFP express HA-tagged NT3 (red) in the secretory apparatus, and GFP throughout the entire cell. **(D-F)** 5 days after infection, HA is detected in glomeruli that receive GFP+ axons, and in nearby neurons. **(G-J)** Confocal images of HA-immunoreactive bulb neurons at **5 days** after initial OSN infection. **(G)** HA+ mitral cells are seen below a GFP+/HA+ glomerulus (gl). The arrow indicates a labeled dendritic segment. **(H)** Mitral cells and their apical dendrites (arrow) are labeled by the HA antibody. **(I-J)** Higher magnification confocal images of HA+ cells bordering a glomerulus (I, gl) and HA+ mitral cell bodies (J).

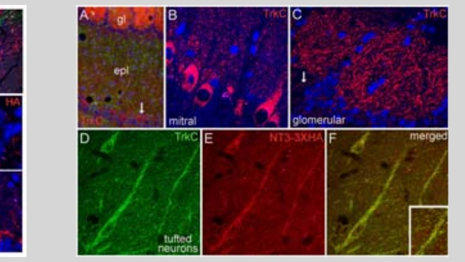

Figure 9. Olfactory bulb neurons express TrkC. **(A-C)** Mitral cell bodies and dendrites, including dendritic terminals within glomeruli (gl), are labeled by TrkC antibody. The arrow in C indicates an incoming TrkC+ dendritic process. **(D-F)** Confocal images showing co-localization of HA and TrkC immunoreactivity in tufted cells in the external plexiform layer (epl), 5 days after olfactory sensory neurons were transduced in vivo with Ad-NT3-3XHA-IRES-GFP.

SUMMARY

Adapted from BW Strowbridge

Olfactory sensory neurons express endogenous NT3.

Mammalian cells infected with our viral vectors express, package and secrete GFP- and HA-tagged NT3 fusion peptides in vitro.

OSNs transduced with NT3-GFP and NT3-3XHA vectors in vivo synthesize and anterogradely transport the tagged peptides in their axons.

Postsynaptic bulb neurons accumulate sensory neuron-supplied NT3-3XHA.

Our data demonstrate that NT3 is anterograde trophic signal in the adult olfactory pathway: OSNs provide NT3-mediated trophic support to TrkC-expressing target neurons in the olfactory bulb.

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References: Deckner et al. (1993) NeuroReport, 5 :301-4; Vigers et al. (2003) J Comp Neurol. 463:221-35; Wang et al. (2003) J Comp Neurol. 458:62-77.