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Anterograde trafficking of neurotrophin-3 in the adult olfactory system in vivo

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ABSTRACT

The olfactory system continuously incorporates new neurons into functional circuits throughout life. Axons from olfactory sensory neurons (OSNs) in the nasal cavity synapse on mitral, tufted and periglomerular (PG) cells in the main olfactory bulb, and low levels of turnover within the OSN population results in ingrowth of new axons under normal physiological conditions. Subpopulations of bulb interneurons are continually eliminated by apoptosis, and are replaced by new neurons derived from progenitors in the adult forebrain subventricular zone. Integration of new neurons, including PG cells that are contacted by sensory axons, leads to ongoing reorganization of adult olfactory bulb circuits. The mechanisms regulating this adaptive structural plasticity are not all known, but the process is reminiscent of early nervous system development. Neurotrophic factors have well-established roles in controlling neuronal survival and connectivity during development, leading to speculation that trophic interactions between OSNs and their target bulb neurons may mediate some of these same processes in adults. A number of different trophic factors and their cognate receptors are expressed in the adult olfactory pathway. Neurotrophin-3 (NT3) is among these, as reflected by beta-galactosidase expression in transgenic reporter mice expressing lacZ under the NT3 promoter. Using a combination of approaches, including immunocytochemistry, real-time PCR of laser-captured RNA, and adenovirus-mediated gene transfer of NT3 fusion peptides in vivo, we demonstrate that OSNs express and anterogradely transport NT3 to the olfactory bulb. We additionally observe that in mice treated with adenovirus encoding NT3 tagged with hemagglutinin (HA), a subset of bulb neurons expressing the TrkC neurotrophin receptor are immunoreactive for HA, suggesting their acquisition of the fusion peptide from infected sensory neurons. Our results therefore provide evidence that OSNs may serve as an afferent source of trophic signals for the adult mouse olfactory bulb.

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Introduction

The adult olfactory system is unique in its ability to incorporate new neurons throughout life, both peripherally in the nasal cavity, and centrally in the olfactory bulb. New olfactory sensory neurons (OSNs) are generated from resident basal stem cells in the olfactory epithelium (OE), a process that allows for slow replacement of mature sensory neurons as those subjected to normal environmental damage over time (due to their vulnerable location in the nasal cavity) are lost via apoptosis (Carter and Roskams, 2002; Deckner et al., 1997; Kondo et al., 2010; Magrassi and Graziadei, 1995; Mahalik, 1996). New olfactory bulb granule and periglomerular (PG) neurons are continuously generated from progenitors in the adult forebrain subventricular zone (SVZ; Nissant and Pallotto, 2011). A portion of these new interneuron populations survive and functionally incorporate into bulbar circuits, as subsets of previously established bulb neurons gradually undergo apoptosis, a process that is controlled in part by an animal's olfactory environment (Imayoshi et al., 2009; Lledo and Saghatelyan, 2005; Mandairon et al., 2006; Mouret et al., 2009; Petreanu and Alvarez-Buylla, 2002). OSN axons synapse on the dendrites of bulb mitral, tufted and type 1 PG cells, and adult-born PG cells establish functional connections with these axons as they integrate into the olfactory bulb's glomerular layer (Grubb et al., 2008; Kosaka and Kosaka, 2005). As a result of this considerable anatomical plasticity, the adult olfactory pathway maintains many features characteristic of developing neural systems (Nacher et al., 2001; Schwob, 2002; Verhaagen et al., 1989).

Neurotrophic factors are well known as regulators of neuronal survival, differentiation, and connectivity during development (Huang and Reichardt, 2001), and trophic interactions between the olfactory bulb and its innervating sensory neurons have long been postulated (Ardiles et al., 2007; Cowan et al., 2001; Ferrari et al., 2003; Hayward et al., 2004; Leo et al., 2000; Schwob et al., 1992). A wide variety of identified factors and their cognate receptors have been localized to the olfactory system (Langenhan et al., 2005; Mackay-Sim and Chuah, 2000; Schwob, 2002). These include the nerve growth factor (NGF) family of neurotrophins and their tropomyosin receptor kinase (Trk) receptors (Carter and Roskams, 2002; Deckner et al., 1993; Guthrie and Gall, 1991). Neurotrophins function as target-derived, retrograde

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survival factors for populations of developing peripheral neurons, but are also important mediators of dendritic maturation and maintenance, as well as synaptic plasticity, in the central nervous system (CNS), actions that are not limited to development (Conner et al., 2009; Gorski et al., 2003; Lu, 2004; McAllister et al., 1996, 1999; Rauskolb et al., 2010). In some instances, these signals are provided via anterograde transport, with innervating axons supplying endogenous neurotrophins to postsynaptic CNS targets (Conner et al., 1997). Examples include the anterograde transport of brain-derived neurotrophic factor (BDNF) by corticostriatal afferents and of neurotrophin-3 (NT3) and BDNF by developing optic nerve projections (Baquet et al., 2004; Caleo et al., 2000; von Bartheld and Butowt, 2000).

NT3 expression has been indirectly localized to OSNs in heterozygous reporter mice in which most of the NT3 coding sequence is replaced by the *Escherichia coli* lacZ sequence under control of the NT3 promoter (Fariñas et al., 1994; Vigers et al., 2003). Bulb neurons that receive input from OSNs express TrkC, the preferred NT3 receptor (Deckner et al., 1993), suggesting that like the developing visual system, the olfactory pathway may utilize NT3 as an anterograde trophic signal. Here we use a combination of immunocytochemistry, semi-quantitative, real-time (Q-RT) PCR of laser-captured RNA, and viral-mediated gene transfer of NT3 fusion peptides, to show that OSNs express and anterogradely transport epitope-tagged NT3 to their target, the adult olfactory bulb, in vivo.

Material and methods

Laser capture microdissection and RNA isolation

All animal procedures were approved by the Florida Atlantic University Institutional Animal Care and Use Committee and adhered to National Institutes of Health guidelines. Six adult female C57Bl6 mice (19–23 g, 8–12 weeks, Charles River Laboratories, Raleigh, NC) were euthanized with sodium pentobarbital (150 mg/kg, i.p.) and decapitated. Nasal cavities were dissected, embedded in OCT medium and frozen in isopentane (−50 °C). The hippocampus was dissected and frozen on dry ice. Coronal cryosections (8 μm) through the nasal cavities/olfactory epithelium were collected on LCM membrane slides (Arcturus, Applied Biosystems, Carlsbad, CA) and stored at −80 °C. Additional sections were cut and dropped intact into extraction buffer for RNA purification (Arcturus PicoPure RNA isolation kit). Immediately prior to laser capture, slides were removed from storage, fixed in 75% ethanol (−20 °C), then stained and dehydrated through increasing concentrations of ethanol using the Arcturus Histogene staining kit according to instructions. Final dehydration was carried out in 100% ethanol for 2 min followed by 6 min in xylene. Olfactory epithelium was visualized and samples captured at 20-40X objective magnification using the ArcturusXT Laser Microdissection instrument. In NT3-lacZ mice, OSN beta-galactosidase expression is detected throughout the OE, with particularly high levels seen in posterior endoturbinates (Vigers et al., 2003). In order to obtain sufficient amounts of material for RNA isolation, our samples were captured throughout the OE, and were not limited to particular locations. UV laser cuts were used to separate and lift a region containing olfactory sensory neurons (OSNs) located in the middle depth of the epithelium, to avoid collecting progenitor cells situated more deeply, and sustentacular cells located superficially. When necessary, UV-cut samples were pulsed with the IR laser (16–20 ms, 20 μm spot size, 65–72 mW) to facilitate attachment to Arcturus HS LCM collection caps (Fig. 1A). Samples lifted from 2 to 3 coronal sections were collected on each cap. Collection membranes were removed from caps, placed in 50 μl of RNA extraction buffer (Arcturus), and incubated at 42 °C for 30 min. Membranes were removed and samples were stored in extraction buffer at −80 °C prior to isolation of total RNA using the Arcturus PicoPure procedure with DNase treatment according to kit instructions. RNA from dissected hippocampus was isolated using Qiagen's RNeasy Plus kit (Qiagen, Valencia, CA). RNA concentration and quality was assessed by Qubit assay (Invitrogen, Carlsbad, CA), and by Pico Chip assay (Agilent Technologies, Santa Clara, CA) using the Agilent 2100 Bioanalyzer. The resulting electropherograms were examined and samples with RNA integrity numbers (RIN) lower than 6.0 were excluded from further analysis.

Semi-quantitative RT-PCR

RNA samples were reversed transcribed and PCR-amplified using Stratagene's Brilliant II One-step RT-PCR kit (Agilent). FAM-labeled Taqman probe/primer assays from Applied Biosystems were used to amplify mouse NT3 (#Mm01182924_m1), BDNF (#Mm01334042_m1), NGF (#Mm00443039_m1), NT4 (#Mm01701591_m1), and olfactory marker protein (OMP, #Mm00448081_s1). VIC-labeled mouse beta-actin assays (#4352341E) were used for normalization. Amplification standard curves were first generated for all sequences using serial dilutions of RNA isolated from intact cryostat sections through the nasal cavities/OE (15–240 ng per reaction). Amplification efficiencies for all neurotrophins ranged from 95 to 108% and r^2 values ranged from 0.984 to 0.998. Reactions using laser-captured OSN samples were performed in triplicate using 45 ng of total RNA pooled from all OE samples, random primers, the specific Taqman probes, and Stratagene's MxPro3000P Q-PCR instrument. Parametric studies have shown that tissue treatment procedures for laser capture microdissection (fixation, staining, laser pulsing, collection duration) tend to reduce RNA quality when compared to RNA isolated from fresh, non-laser captured tissue samples (Kerman et al., 2006). Recommended parameters for RT-PCR using total RNA isolated by laser capture microdissection include amplified product lengths of 200 bases or less, and RNA integrity numbers (RIN) greater than 5 (Erickson et al., 2009; Fleige et al., 2006). We discarded all samples with RIN values below 6, and our amplicon sizes ranged from 69 to 110 bases. The final RIN of our pooled, laser-captured OSN RNA was 6.5. As expected, the RIN for RNA isolated from non-laser captured hippocampal samples was higher (8.5). Reverse transcription reactions were carried out at 42 °C for 1 h, followed by 10 min denaturing at 95 °C, and 40 cycles of amplification (95 °C–15 s, 60 °C–1 min). Reactions using Taqman OMP primers were included to verify collection of RNA from OSNs, and reactions using total RNA from hippocampus (20 ng) were included as positive controls for detection of neurotrophin transcripts. Each triplicate assay was performed twice, for a total of 6 measurements per transcript, and template RNA was omitted from control assays. MxPro3000P software was used to normalize neurotrophin values (delta Ct) relative to the mean Ct of beta-actin. BDNF and NT3 transcript abundance in the OE were both calibrated and graphed relative to NGF (mean set to 1.0), also using MxPro3000P software. NT4/5 expression was not detectable in either the OE or hippocampus.

Antibody production and NT3 immunolocalization

Three synthetic peptides corresponding to regions within mature mouse NT3 having the least homology to BDNF and NGF were used as antigens in combination to generate a rabbit polyclonal antibody, with the same three peptides used for final affinity purification of the IgG fraction isolated from serum (ProteinTech Group Inc, Chicago, IL). The sequences were as follows: CLGEIKTGNSPVKQY, TSENNKLV GWRWIC, CVTDKSSAIDIRGHQ. Each peptide was coupled to keyhole limpet hemocyanin prior to in vivo administration to a New Zealand white rabbit. The specificity of the final antibody was verified by Western blot detection of human recombinant (hr)NT3 peptide, with hrBDNF, hrNT4 and mouse NGF (all mature peptides, ~13–15 kDa) used as controls (Peprotech Inc., Rocky Hill, NJ). Samples of purified peptide (1 μg) were subjected to SDS-PAGE (18%) under reducing conditions and transferred to nitrocellulose membranes. Blots were probed using the rabbit anti-NT3 antibody (1:5000) and goat HRP-labeled anti-rabbit IgG (Millipore, Temecula, CA, 1:20,000) followed