Developmental Biology 340 (2010) 419–429

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

BDNF is required for the survival of differentiated geniculate ganglion neurons

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ARTICLE INFO

Article history:
Received for publication 18 May 2009
Revised 5 January 2010
Accepted 22 January 2010
Available online 1 February 2010

Keywords:
BDNF
Gustatory/geniculate neurons
Taste development
Bax
$BDNF^{LacZ}$
Activated caspase-3

ABSTRACT

In mice lacking functional brain-derived neurotrophic factor (BDNF), the number of geniculate ganglion neurons, which innervate taste buds, is reduced by one-half. Here, we determined how and when BDNF regulates the number of neurons in the developing geniculate ganglion. The loss of geniculate neurons begins at embryonic day 13.5 (E13.5) and continues until E18.5 in BDNF-null mice. Neuronal loss in BDNF-null mice was prevented by the removal of the pro-apoptotic gene Bax. Thus, BDNF regulates embryonic geniculate neuronal number by preventing cell death rather than promoting cell proliferation. The number of neurofilament positive neurons expressing activated caspase-3 increased on E13.5 in *bdnf*$^{-/-}$ mice, compared to wild-type mice, demonstrating that differentiated neurons were dying. The axons of geniculate neurons approach their target cells, the fungiform papillae, beginning on E13.5, at which time we found robust $BDNF^{LacZ}$ expression in these targets. Altogether, our findings establish that BDNF produced in peripheral target cells regulates the survival of early geniculate neurons by inhibiting cell death of differentiated neurons on E13.5 of development. Thus, BDNF acts as a classic target-derived growth factor in the developing taste system.

Introduction

A small cranial sensory ganglion called the geniculate ganglion innervates chemosensory receptors responsible for the sense of taste that are on the front two-thirds of the tongue, the soft palate and the nasoincisor ducts of the hard palate. These neurons carry information from peripheral chemosensory cells to the central nervous system. The development of peripheral circuitry in the taste system is highly regulated, in that each taste bud is innervated by a specific number of neurons (Krimm and Hill, 1998; Zaidi and Whitehead, 2006). The mechanisms underlying this numerical precision are unclear, but the final number of taste neurons available to innervate each taste bud is determined by the relative contributions of cell proliferation, differentiation, and cell death within the geniculate ganglion during development. Early in geniculate ganglion development, cells are added rapidly (Altman and Bayer, 1982). By embryonic day 14.5 (E14.5) in rat (around E12.5–E13 in mouse), cell proliferation has slowed and thereafter the total number of neurons remains constant throughout the embryonic period (Carr et al., 2005). Like most regions of the nervous system, the geniculate ganglion overproduces neurons and undergoes a period of cell death, the peak of which is approximately E16.5 in rat (approximately E14–E14.5 in mouse) (Carr et al., 2005). However, total neuron number doesn't change after E13.5 in rat indicating that neuronal proliferation and differentiation progress at equivalent rates throughout much of embryonic development (Carr et al., 2005). A host of factors including neurotrophins likely regulate neuronal proliferation, differentiation, and death in the geniculate ganglion.

In mammals, the neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT-4) (Huang and Reichardt, 2001). These neurotrophins are sometimes thought to be produced by peripheral target tissues, where they influence neuron number by preventing the death of ganglion neurons that innervate them (Huang and Reichardt, 2001; Vogel and Davies, 1991). Alternatively, they are sometimes expressed within sensory ganglia and along projection pathways where they influence neuron development before target innervation (Farinas et al., 1996). Neurotrophins not only regulate neuronal numbers by influencing survival of differentiated neurons, but can also influence survival, differentiation, and proliferation of neuronal precursors during early development (Ernfors, 2001; Farinas et al., 2002; Liebl et al., 2000; Lopez-Sanchez and Frade, 2002).

The final number of neurons that comprise the geniculate ganglion is influenced by BDNF. Relative to wild-type animals, *bdnf*$^{-/-}$ mice show a significant reduction in neurons in the geniculate ganglion by birth (Conover et al., 1995; Liu et al., 1995). Approximately 45–65% of the taste buds on the anterior tongue, whose survival requires gustatory innervation, are also lost in *bdnf*$^{-/-}$ mice (Mistretta et al., 1999; Nosrat et al., 1997; Sun and Oakley, 2002), indicating that many of the missing neurons are gustatory. The mechanism employed by BDNF during embryogenesis to influence the neuronal numbers within the adult geniculate ganglion is unknown. Since BDNF is expressed in the geniculate ganglion, tongue muscle, and gustatory targets of

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doi:10.1016/j.ydbio.2010.01.024

geniculate neurons (Nosrat, 1998; Nosrat et al., 1996; Nosrat and Olson, 1995; Schecterson and Bothwell, 1992), the removal of BDNF from multiple locations, at any embryonic age and through a variety of mechanisms could result in a reduction of geniculate neurons.

In this study, we sought to determine how and when BDNF regulates the final number of sensory neurons that comprise the geniculate ganglion. We established that BDNF expressed in taste epithelium promotes survival of differentiated gustatory neurons as they first innervate their targets, before the peak of naturally occurring cell death.

Materials and methods

Animals

All BDNF mutant (*bdnf*$^{-/-}$) and wild-type mice were on a C57BL/6J background strain. Homozygous *bdnf*$^{-/-}$ embryos were obtained by breeding mice with heterozygous targeted mutations of the *bdnf* gene. Heterozygous *bdnf*$^{-/+}$ mice were acquired from Jackson Laboratories (Bar Harbor, Maine, USA; #002266). Animals were genotyped by polymerase chain reaction, as described in protocols provided by Jackson Laboratories, http://jaxmice.jax.org/). *Bdnf*-lacZ mice were a kind gift from Kevin Jones (Dept. of Molecular, Cellular and Developmental Biology, University of Colorado at Boulder, Boulder, CO, USA) (Baquet et al., 2005; Gorski et al., 2003a,b). *Bdnf*-lacZ mice have been used successfully to examine BDNF-producing locations in the adult taste system (Yee et al., 2003). Animals were genotyped by polymerase chain reaction using the *lacZ* forward primer 5′-TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA-3′, and the *lacZ* reverse primer 5′-ATG TGA GCG AGT AAC AAC CCG TCG GAT TCT-3′. Embryonic mice were obtained from time-bred females that were placed with males just before the 8-h dark period and examined for plugs the following morning. The day a plug was observed was designated embryonic day 0.5 (E0.5). Heads from wild-type, *bdnf*$^{-/-}$, *bax*$^{-/-}$, and *bdnf*$^{-/-}$/*bax*$^{-/-}$ double knockout mice at birth were a kind gift from David M. Katz (Dept. of Neurosciences, Case Western Reserve University, Cleveland, OH, USA).

Single-label immunohistochemistry

Embryos aged 14.5 (E14.5) and older were immediately perfused using chilled 4% paraformaldehyde (PFA), and animals E13.5 and younger were fixed by immersion in PFA. All embryos were fixed overnight in 4% PFA. Following fixation, embryo heads were moved to 70% ethanol and processed for paraffin embedding. Immuno-detection of the cytoskeletal element class III β-tubulin (β-III tubulin) was used to visualize geniculate ganglion neurons. Serial transverse sections (5 μm) of paraffin-embedded embryo heads were collected on Fischer SuperFrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Paraffin was removed by immersion in CitriSolv (Fisher). Sectioned tissues were rehydrated in a graded series of alcohols, followed by 0.1 M phosphate-buffer saline (PBS), pH 7.4. Endogenous peroxidase activity was quenched by treatment for 15 min in a solution of 10% methanol/3% H_2O_2 in PBS. Slides were washed in dH_2O (3×5 min each). In the antigen retrieval step that followed, slides were boiled in the microwave for 5×2 min in citrate buffer (0.1 M citric acid/0.1 M Na citrate/dH_2O; pH 6) with a 5 min rest between the two boiling periods, and then cooled down to room temperature. The sections were washed in PBS and blocked for 30 min in blocking buffer (PBS/5% goat serum/0.25% Triton X-100) and were then incubated overnight at 4 °C in blocking buffer containing a 1:1000 dilution of monoclonal mouse anti-β-III tubulin antibody (TUJ1; Covance, Princeton, NJ, USA; catalog # MMS-435P). On the following day, the sections were washed in PBS (3×5 min) and incubated for 2 h at room temperature in blocking buffer containing a 1:200 dilution of biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA; #BA-2000), and visualized with an ABC diaminobenzidine reaction. The following numbers of mice embryos were used for quantification: wild-type ($n=3$ for E12.5, E14.5, E16.5, E18.5; $n=5$ for E13.5); *bdnf*$^{-/-}$ ($n=3$ for E12.5, E13.5, E16.5, E18.5; $n=4$ for E14.5).

Triple-label immunohistochemistry

Pregnant females were injected 2 h before sacrifice with 50 mg/kg (intraperitoneal) of 2′-bromo-5′-deoxyuridine (BrdU; 5 ml/kg of a 10 mg/ml stock solution in 0.1 M Tris–HCl buffer, pH 7.5). Embryos were immersion-fixed overnight in 4% PFA in PBS, pH 7.4. They were then placed in 30% sucrose/PBS overnight for cryoprotection, and frozen on dry ice in OCT embedding medium. Embedded embryo heads were sectioned transversely (16-μm), mounted on SuperFrost/Plus slides (Fisher), and allowed to air-dry. Fixed, mounted transverse sections were treated with 2 N HCl and heated to 37 °C for 30 min. Slides were washed in dH_2O (3×5 min each). In the antigen retrieval step that followed, slides were boiled for 15 min in citrate buffer (0.1 M citric acid/0.1 M Na citrate/dH_2O; pH 6), and allowed to cool down to room temperature. Next, the slides were washed in PBS (3×5 min) and blocked in blocking buffer (described above) for 30 min. Sections were incubated overnight in the following primary antibodies: anti-BrdU (1:200, Accurate Chemicals, Westbury, NY, USA; #OBT0030), mouse anti-neurofilament (1:200, Millipore, Billerica, MA, USA; #MAB5266), and rabbit anti-cleaved caspase-3 (Asp175) (1:200, Cell Signaling, Beverly, MA, USA; #9661). The slides were washed in PBS (3×5 min), and then incubated for 2 h at room temperature in the following secondary antibodies: alexa-488 anti-rat (A11006), alexa-546 anti-mouse (A11030), and alexa-647 anti-rabbit (A21244;Invitrogen, Carlsbad, CA, USA) at a dilution of 1:200 in blocking buffer. Slides were dehydrated in a graded series of alcohols and cover slipped in CitriSolv with DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Immuno-labeled tissue was visualized using a fluorescence confocal microscope. Antigen-positive cells were quantified from $n=3$ each wild-type and *bdnf*$^{-/-}$ embryos.

β-Galactosidase histochemistry

Detection of β-Gal was performed either in whole mount embryo tissue or in both sagittal and transverse sections. The embryos were immersion-fixed in ice cold 0.5% glutaraldehyde in PBS/$MgCl_2$ solution (2 mM $MgCl_2$ in PBS) for 1–2 h. Fixative was removed by multiple washes in ice cold PBS/$MgCl_2$ and embryos were frozen on Dry Ice in OCT embedding medium. Tissue sections (25 μm) were mounted on SuperFrost/Plus slides and allowed to air-dry for 1 h at 4 °C. Whole mount tissues and sections were washed in ice cold PBS/$MgCl_2$ to thoroughly remove OCT and then immersed in X-gal staining solution (InvivoGen, San Diego, CA; 0.02% Igepal, 0.01% sodium deoxycholate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-gal in PBS/$MgCl_2$) for 2–6 h in a 37 °C incubator. Tissues were then washed and photographed. For immunohistochemistry following X-gal staining, sections were fixed for 1 h in ice cold 4% PFA. Slides were washed in PBS and were allowed to dry on a 37 °C heater overnight. β-Gal-stained tissue was labeled with mouse anti-neurofilament antibody using the single-label immunohistochemistry protocol described above. Sections were mounted in Glycergel mounting medium (Dako North America, Carpinteria, CA, USA) and imaged using a bright field camera.

Quantification of β-III tubulin geniculate ganglion neurons

Neurons of the geniculate ganglion were quantified in transverse serial sections of the mouse embryo head. Monoclonal mouse anti-β-III tubulin antibody (TUJ-1) was used to identify and count neuron profiles, only in sections where the nucleus was visible ("*" Fig. 2J inset). Neuronal profiles were counted in six sections per geniculate ganglion. The area containing the geniculate ganglion was measured