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Null Mutations of NT-3 and *Bax* Affect Trigeminal Ganglion Cell Number but Not Brainstem Barrelette Pattern Formation

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Abstract

Trigeminal ganglion (TG) neurons innervate the grid-like array of whisker follicles on the face of the mouse. Central TG axons project to the trigeminal (V) brainstem nuclear complex, including the nucleus principalis (PrV), and the spinal subnucleus interpolaris (SpVi), where they innervate barrelettes that are organized in a pattern that recapitulates the whisker pattern on the face. Neurotrophin-3 (NT-3) supports a population of TG cells that supply slowly adapting mechanoreceptors in the whisker pad. We examined mice at embryonic day 17 (E17) and on the day of birth (P0) with null mutations of NT-3, *Bax*, a proapoptotic gene associated with naturally occurring cell death, and *Bax*/NT-3 double knockout mutants to determine if: 1) the number of TG cells would be reduced; 2) eliminating the *Bax* gene would rescue the NT-3 dependent neurons; and 3) the central projections of the rescued axons in the *Bax*/NT-3 double knockout mice would fail to develop the barrelette patterns in the PrV and SpVi subnuclei. In mice at E17, NT-3^{-/-} mutants had 65% fewer TG neurons than found in age matched wild-type (WT) mice, and at P0, the number was reduced by 55% ($p < 0.001$ for both). *Bax* null mutant mice at E17 had 132% of the WT number of TG cells ($p < 0.001$), although the numbers returned to WT levels by P0. *Bax*/NT-3 double knockout mice at E17 had TG cell numbers equal to those seen in WT, but the double knockout failed to retain WT TG neuron numbers in P0 mice (39% fewer cells; $p < 0.001$). In all cases of reduced experimental neuron numbers, and in the E17 *Bax*^{-/-} mice with supernumerary cells, the barrelette patterns in the PrV and SpVi were normal. Only a slight qualitative reduction in overall barrelette field area and clarity of barrelettes were seen. These results suggest that NT-3 is not necessary for barrelette pattern formation in the brainstem.

Keywords

trigeminal; neurotrophins; barrels; cell death; principalis; interpolaris

INTRODUCTION

In the trigeminal (V) brainstem nuclear complex, nucleus principalis (PrV) is the initial site of integration between peripheral sensory input and central somatosensory processing and is

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the essential nucleus directing pattern formation in the contralateral ventroposteromedial (VPM) thalamus and the subsequent primary somatosensory (S1) cortex (Jacquin et al., 1988, 2008; Killackey and Fleming, 1985). Central projections from neurons in the trigeminal ganglion (TG) influence the development of whisker representations in the brainstem (Woolsey, 1990). Primary mechanoreceptive afferents reliably project to the PrV which emits the ascending lemniscal pathway (Shortland et al., 1996) carrying detailed sensory information rostrally. The spinal subnucleus interpolaris (SpVi), which largely comprises the initial locus of the parallel paralemniscal system (Williams et al., 1994), receives collateral innervation from the same primary afferent inputs (Jacquin et al., 1986), yet lesions of the PrV interrupt whisker-related pattern formation in the contralateral VPM, but lesions of the SpVi do not (Killackey and Fleming, 1985).

During development, neuron survival depends on successful competition for a limited supply of neurotrophic factor(s) acquired from target tissues (Barde, 1994; Snider, 1994). Since the discovery of nerve growth factor (NGF) (Levi-Montalcini, 1987), three additional neurotrophic molecules in the NGF family have been identified: brain derived neurotrophic factor (BDNF) (Barde et al., 1982); neurotrophin-3 (NT-3) (Ernfors et al., 1990; Jones and Reichardt, 1990); and neurotrophin-4/5 (NT-4/5) (Berkemier et al., 1991; Ip et al., 1992). Each neurotrophin binds with highest affinity to a specific tyrosine kinase receptor (trk) protein: trkA binds NGF; trkB binds BDNF and NT-4/5; and trkC binds NT-3. Transgenic gene deletion experiments have demonstrated that each neurotrophin and its trk receptor influence survival in different populations of sensory neurons (Fundin et al., 1997; Rice et al., 1998; Wright and Snider, 1995; see Snider, 1994 for review).

In sensory ganglia, NT-3 supports a population of proprioceptive primary afferent neurons (Ernfors et al., 1990, 1994; Fariñas et al., 1994, 1998; Fundin et al., 1997; Genç et al., 2004; Oakley et al., 1995; Patel et al., 2003; Stephens et al., 2005). In addition, slowly adapting mechanoreceptors are lost in NT-3 null mutant (NT-3^{-/-}) mice, particularly Merkel cell innervation from the V nerve in the whisker pad (Fundin et al., 1997; Rice et al., 1998) and palate (Ichikawa et al., 2001), and from dorsal root ganglia (DRG) cells innervating cutaneous touchdomes (Airaksinen et al., 1996; Patel et al., 2003). Albers et al. (1996) reported that Merkel cell innervation was increased in NT-3 overexpressing mutants.

In NT-3^{-/-} knockout (KO) mice, Patel et al. (2003) reported a nearly 30% loss of NT-3-dependent dorsal root ganglion (DRG) neurons. Null mutant mice lacking the preapoptotic gene, *Bax*, were produced in which sensory neurons are rescued independent of neurotrophic support (Lentz et al., 1999; Middleton et al., 1996). Lentz et al. noted that, in *Bax*/NT-3^{-/-} double KO mice, the number of neurons was 150% of wild-type DRG cell numbers, and central axons from those ganglia retrogradely filled with DiI failed to accurately innervate ventral horn α -motor neuron targets. Genç et al., (2003) similarly assessed the central targeting of neurons in *Bax*/NT-3^{-/-} double KO mice by retrograde labeling with DiI and also noted ectopic distribution of axons in the spinal cord. In vitro assays demonstrated that exogenous application of NT-3 attracted axons and induced precocious branching in the V tract (Ozdinler et al., 2004; Ulpinar et al., 2000).

For the present study, we hypothesized that, in NT-3^{-/-} mice, the number of neurons in the TG would be significantly reduced, and this loss of cells would impair development of whisker-related brainstem patterns in the PrV and SpVi nuclei. Further, in *Bax*/NT-3^{-/-} double KO mutants, neurons in the TG would survive, but since their central projections are abnormal, the whisker-related barrelette patterns also will be abolished.

MATERIALS AND METHODS

Generation, maintenance, and genotyping of mutant mice

NT-3^{-/-}, *Bax*^{-/-}, and *Bax*/NT-3 double KO mice were generated and genotyped in the Washington University Transgenic Core (Chen et al., 2001). The date when the vaginal plug was observed was designated embryonic (E) day 0.5. Transgenic mice were housed in the mouse facility according to protocols approved by the Division of Comparative Medicine at the Washington University School of Medicine.

Light microscopy

Age matched mutant and wild-type control groups were sacrificed on E17 or on the day of birth, postnatal day 0 (P0). Newborn mice were anesthetized by hypothermia and perfused through the heart with saline, followed by 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Heads were postfixed at 4°C overnight, and brains and TG were removed. Brainstems and cerebral cortices were frozen and sectioned transversely or tangentially at 40 µm on a sliding microtome. Sections were plated on chrome–alum subbed slides, and processed for cytochrome oxidase staining to reveal the presence or absence of whisker-related patterning in the PrV and SpVi nuclei and barrel cortex (Henderson et al., 1994). Sections were incubated in phosphate buffer containing 0.24 mg/ml cytochrome C (Sigma, St. Louis, MO), 0.5 mg/ml diaminobenzidine (Sigma, St. Louis, MO), and 44 mg/ml sucrose (Sigma, St. Louis, MO) for 2–4 h at 37°C under gentle agitation or until whisker-related patchiness was visible in the stained sections. Sections were mounted onto gelatin coated glass slides and examined on an Olympus BX51 microscope (Tokyo, Japan). TG were serially sectioned on a cryostat at 15 µm, mounted on chrome–alum subbed slides, and Nissl stained with cresyl violet (Sigma, St. Louis, MO), followed by dehydration in graded alcohols and clearing in xylenes. Coverslips were mounted with DPX (BDH, Poole, UK).

Data analysis

For all estimates of total cell number in the TG, the optical disector probe was used as implemented by the Stereo Investigator software package (MicroBrightField, Williston, VT). This procedure was performed at a final screen magnification of 3330×. Every other section was sampled throughout the entire structure. In the TG, numerous spindle shaped non-neuronal cell types are readily identified and excluded from the count; the size cutoff was 3 µm. Whisker-related patterning in cytochrome oxidase-stained sections through the brainstem was qualitatively evaluated by the use of previously described methods (Henderson et al., 1994). Cell numbers in TG were subjected to statistical analyses. A multifactor ANOVA was performed that considered genotype, neuronal structure, and age as independent variables. When justified based on significant ($p < 0.05$) main effects, *post hoc*

comparisons were performed between experimental groups (genotypes) using Bonferroni's correction for multiple comparisons. Sample sizes are listed in the illustrations and legends.

We also investigated $trkA^{+/-}$ heterozygotes, $trkA^{-/-}$ homozygotes, $trkB^{-/-}$, and $trkC^{-/-}$ mutant KO mice for the number of TG cells and the presence of cortical barrel fields. TG cell counts were semi-quantitative and not stereologically calculated, thus the data is not included in the statistics described above for $NT-3^{-/-}$, $Bax^{-/-}$, and $Bax/NT-3$ double KOs. Cortical tissues were processed and assessed as described above.

RESULTS

Cell Numbers

In $Bax^{-/-}$, $NT-3^{-/-}$, $Bax/NT-3$ double KOs, and wild-type mice on E17 and on the day of birth, genotype and age predicted variance in estimates of neuron numbers in the TG. Table 1 contains the quantitative estimates of cell numbers showing diminished numbers in the $NT-3^{-/-}$ cases, compared to wild-type controls, as depicted in Graph 1. Although the main effect of age was not statistically different ($F=0.088$, $p=0.768$), the main effect of genotype and the cross-effect of age and genotype were significantly different ($F=101.925$, $p < 0.001$ and $F=12.014$, $p < 0.001$, respectively). *Post hoc* comparisons of E17 animals revealed significant differences between $Bax^{-/-}$ and wild-type ($p < 0.001$), and between $NT-3^{-/-}$ and wild-type ($p < 0.001$), however, the number of cells in $Bax^{-/-}$ was higher, and in $NT-3^{-/-}$ lower, than in wild-types. $Bax/NT-3$ double KO counts were statistically equal to those in wild-types ($p = 0.076$). At P0, $Bax^{-/-}$ cell numbers were not significantly different from wild-types ($p = 0.058$), but counts in both $NT-3^{-/-}$ and $Bax/NT-3$ double KOs were lower ($p < 0.0001$ in both).

Pattern Formation

Figure 1 illustrates the barrelette patterns in both the PrV (Fig. 1a–d) and SpVi (Fig. 1e–h) nuclei in P0 mice for all genotypes. In the PrV, individual rows and barrelettes are visible in wild-type sections (Fig. 1a) and in $Bax^{-/-}$ mutants (Fig. 1b). Although the barrelette patterns are recognizable in both $NT-3^{-/-}$ (Fig. 1c) and $Bax/NT-3$ double KO cases (Fig. 1d), qualitatively, the area of the PrV nuclei in both mutant strains seem slightly reduced and the individual barrelettes less distinct, corresponding to 55% and 39% reduction in TG cell number, respectively (Table 1).

Barrelette patterns in the SpVi are clear under all conditions (Fig. 1e–h). As in the PrV, individual rows and barrelettes in the SpVi are distinct in wild-type (Fig. 1e) and $Bax^{-/-}$ (Fig. 1f) mice. In $NT-3^{-/-}$ (Fig. 1g) and in $Bax/NT-3$ double KO cases (Fig. 1e), the barrelette patterns also appeared to be diminished in size, coincident with losses of TG cells (Table 1).

Graph 1: Graphic representation of the data presented in Table 1. * indicates significant difference to age-matched wild-type data ($p < 0.001$). † indicates significant difference between P0 and E17 TG cell number ($p < 0.001$).

trk Knockouts TG Cell Number and Cortical Barrels

Homozygous *trkA*^{-/-} null mutations reduced TG cell number by 63%, and in heterozygous *trkA*^{+/-}, TG cell number was reduced by 49%. In *trkB*^{-/-}, 28% of TG neurons were lost. TG cell counts could not be made in knockouts of *trkC*, the cognate receptor for NT-3, however, TG volume was qualitatively reduced. Figure 2 presents CO-labeled S1 cortical barrels in homozygous *trkA*^{-/-}, *trkB*^{-/-}, and *trkC*^{-/-} mutants. In all cases, the barrel fields were normal in comparison to age-matched wild-type cortices.

DISCUSSION

TG Neuron Number

We initially hypothesized that null mutations of the NT-3 gene would significantly reduce the number of neurons in the TG. Indeed, the number of TG neurons was reduced by 65% in E17 mice and by 55% in P0 mice. Others have reported similar losses in sensory ganglia in NT-3^{-/-} null mice (Ernfors et al., 1994; Huang et al., 1999; Patel et al., 2003).

Bax^{-/-} null mutation rescued TG cells to 132% of wild-type numbers at E17. By P0, this number had diminished to approximately the same number as seen in wild-type TG. In *Bax*/NT-3 double KO mice at E17, sufficient numbers of neurons survived to yield TG cell counts equal to those observed in wild-types. Similar neuronal rescue was observed in *Bax*/NT-3 double KOs (Patel et al., 2003), in *Bax*/*Drg11* double KOs (Jacquin et al., 2008), and in *Bax*/*Lmx1b* double KO mice (Xiang et al., 2010). The number of TG cells in *Bax*/NT-3 double KOs at P0 was reduced to around 60% of wild-type cell number. Our observations suggest that a substantial population of NT-3-dependant neurons is lost between E17 and P0 under a mechanism that does not employ the *Bax* apoptotic cascade (ElShamy and Ernfors, 1996; Jacquin et al., 2008).

In *trk* KO mice similar TG cell losses were semi-quantitatively examined. *TrkA*^{-/-} mutants had the most severe cell losses at 68% reduction from WT mice, comparable to several previous reports: Patel et al. (2000) saw a 90% reduction; and both Klein (1994) and Piñon et al. (1996) reported nearly 70% losses. *TrkA*^{+/-} heterozygotes lost fewer cells than *trkA*^{-/-} homozygotes, suggesting a “dose dependent” survival effect for *trkA*. Our observed 28% loss of TG cells in *trkB* KOs approximates the 30% reduction in DRG cells reported by Hofer and Barde (1998), but falls short of the 60% cell loss reported by Klein et al. (1993). Klein et al. (1994) and Piñon et al. (1996) both described approximately 20% fewer DRG neurons in *trkC*^{-/-} mutants. It is interesting to note that the loss of sensory neurons in NT-3 KOs greatly exceeds the loss seen with *trkC* KOs, suggesting that NT-3 can support neurons by acting through *trk* receptors other than the preferred *trkC* (Fariñas et al., 1998; Klein et al., 1994).

Barrelette Pattern in the PrV and SpVi

Often gene deletions in neurotrophin null mutants exert significant deleterious effects that lead to early death, hindering analyses of complex central developmental mechanisms taking place in postnatal life. Newborn NT-3^{-/-} mutant mice experienced difficulty feeding and survived only a short postnatal time (Tessarollo et al., 1994). It has been demonstrated that

malnutrition delays barrel formation in the somatosensory cortex (Medina-Aguirre et al., 2008; Vondokmai, 1980). Combined, these conditions limited our analyses of pattern formation to P0 and to brainstem nuclei, since the VPM barreloid pattern appears at P2 (Ma, 1991), and the barrelfield at P4 (Woolsey and Van der Loos, 1974; see reviews by Jones and Diamond, 1995; Woolsey, 1990).

NT-3, and to a lesser degree BDNF, have been implicated as chemotropic factors for peripheral and central projections of TG neurons in the developing maxillary process (Lumsden and Davies, 1986; O'Connor and Tessier-Levigne, 1999; and see Erzurumlu et al., 2006 for a review). In vitro examinations of the chemoattractant role of NT-3, in which neurotrophin-soaked beads were ectopically implanted, demonstrated that NT-3 induced and directed elongation of sensory axons, and affected precocious axon arborization and erroneous central projection (Erzurumlu et al., 2006; Genç et al., 2004; Ozdinler et al., 2004; Ulpinar et al., 2004). These observations led to our hypothesis that loss of NT-3 in null mutations would alter the central morphology of trigeminal primary afferents, eliminating pattern formation in the trigeminal brainstem nuclei. We now report that, in NT-3 KO mice, loss of 65% of the NT-3-dependant TG cells at E17 or 55% at P0, or loss of nearly 40% of TG cells in newborn *Bax*/NT-3 double KO mice does not disrupt the whisker-related barrelette pattern in either the PrV or SpVi. A similar lack of impairment of pattern formation was seen in guinea pig pups whose dams were treated with antibodies against NGF (Sikich et al., 1986). Even with up to 80% TG cell loss in NGF-deprived mice, the barrelette pattern appeared normal in the PrV. Conversely, Henderson et al. (1994) injected fetal mice with NGF at E15 and E18 and induced a 36% increase in the number of TG neurons and disrupted the development of barrelettes in the PrV, suggesting that naturally occurring cell death of a NGF-dependent TG cell population is necessary for the sculpting of whisker-related patterns in the V brainstem. Excess numbers of NT-3 dependent TG cells in embryonic *Bax* KO mice in this study had no effect on the morphology of the PrV or SpVi barrelette pattern. These results suggest that there is a substantial difference in the effects exerted on V brainstem pattern formation between NT-3-dependent and NGF-dependent cells. Similarly, the loss of substantial TG cell populations in all four of our *trk* KO mutant mouse strains failed to prevent cortical barrel formation.

While the overall patterns in the PrV and SpVi are present, the barrelette fields are smaller and the barrelettes less distinct. In *Drg11* null mutant mice, patterning in the PrV is disrupted, but present in the SpVi and SpV subnucleus caudalis, although reduced in size and completeness (Ding et al., 2003; Jacquin et al., 2008). In the current study, it is possible that the loss of central projections from depleted TG underlies this reduction in barrelette field area and distinctness.

The present report documents the loss of significant numbers of TG neurons in embryonic and neonatal NT-3 null mouse mutants and in P0 *Bax*/NT-3 double KO mice. Loss of these cells had little effect on the presence of barrelette patterns in either the PrV or SpVi nuclei beyond an apparent decrease in overall size and the prominence of the barrelettes. Additionally, excess TG cells seen in E17 *Bax* KO mice failed to exert a significant impact on barrelette formation. While NT-3 is an essential factor in development of maxillary

afferent innervation (O'Conner and Tessier-Levigne, 1999), its impact on trigeminal brainstem whisker-related patterning is limited.

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Figure 1.

NT3^{-/-}, Bax^{-/-}, and Bax/NT3^{-/-} double KO cytochrome oxidase labeling in the PrV and SpVi at P0. Barrelette pattern in the PrV in newborn mice (A–D). A. Discrete rows of barrelettes in the wild-type PrV are easily discernable. Designations of each row are labeled a–e. B. Rows are similarly identifiable in the Bax^{-/-} KO PrV. C. Although rows are present in NT3^{-/-} KOs, the clarity of individual barrelettes is diminished. Qualitatively, the overall area of the barrelette field appears smaller than seen in wild-type PrV. D. In Bax/NT3 double KOs, the definition of individual barrelettes is reduced and the area apparently smaller than in wild-type PrV. Barrelette pattern in the SpVi in newborn mice (E–H). E. The whisker-related pattern in wild-type SpVi is clear at P0. Rows are labeled a–e as in A. F. As in the PrV, the pattern in the SpVi in Bax^{-/-} KO mice is distinct. G. NT3^{-/-} SpVi nuclei also appear somewhat reduced in area, although individual barrelettes remain more distinct than in NT3^{-/-} PrV. H. The area of the SpVi in Bax/NT3 double KOs is slightly reduced compared to wild-type nuclei, but barrelettes remain distinct.

Figure 2.
trkA^{-/-}, trkB^{-/-}, and trkC^{-/-} KO S1 cytochrome oxidase labeling in the barrel cortex. Cortical barrel pattern, size and density appear normal in trkA^{-/-} (A), trkB^{-/-} (B), and trkC^{-/-} (C) knockout mutants. Designations of each row are labeled a-e.

Figure 3.

TrkA/, trkB/, and trkC/ KO S1 cytochrome oxidase labeling in the barrel cortex. Cortical barrel pattern, size, and density appear normal in (a) trkA/, (b) trkB/, and (c) trkC/ KO mutants. Designations of each row are labeled a–e.

Table 1
Trigeminal Ganglion Neuron Counts

NT-3^{-/-}, *Bax*^{-/-}, and *Bax*/NT-3 double KO trigeminal ganglion cell counts. Numbers represent the entire sample used for this study. Averages and standard deviations are also provided.

E17				
	Wild Type	<i>Bax</i> ^{-/-}	NT3 ^{-/-}	<i>Bax</i> ^{-/-} NT3 ^{-/-}
	38219	34607	14665	31168
	37633	58113	10620	27790
	44903	47708	11185	
	37354	53020	13004	
	31327	48897	13140	
	31270	49666	13921	
Average	36784*	48668*	12756	29479
StDev	4640.4	7162.7	1428.3	1689.3
P0				
	Wild Type	<i>Bax</i> ^{-/-}	NT3 ^{-/-}	<i>Bax</i> ^{-/-} NT3 ^{-/-}
	41865	33798	17875	26056
	41077	34512	17452	25808
	43644	41436	19529	27587
	38606	36131	18389	23997
	45560	34343		23159
	43997	31353		22779
	37444	42184		23733
	34440	38465		
Average	40829 [†]	36528*	18311*	24731
StDev	3755.0	3837.3	897.4	1774.3

* indicates significant difference to age-matched wild-type data ($p < 0.001$).

[†] indicates significant difference between P0 and E17 TG cell number ($p < 0.001$).