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Use of laser microdissection in the investigation of facial motoneuron and neuropil molecular phenotypes after peripheral axotomy

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Abstract

The mechanism underlying axotomy-induced motoneuron loss is not fully understood, but appears to involve molecular changes within the injured motoneuron and the surrounding local microenvironment (neuropil). The mouse facial nucleus consists of six subnuclei which respond differentially to facial nerve transection at the stylomastoid foramen. The ventromedial (VM) subnucleus maintains virtually full facial motoneuron (FMN) survival following axotomy, whereas the ventrolateral (VL) subnucleus results in significant FMN loss with the same nerve injury. We hypothesized that distinct molecular phenotypes of FMN existed within the two subregions, one responsible for maintaining cell survival and the other promoting cell death. In this study, we used laser microdissection to isolate VM and VL facial subnuclear regions for molecular characterization. We discovered that, regardless of neuronal fate after injury, FMN in either subnuclear region respond vigorously to injury with a characteristic “regenerative” profile and additionally, the surviving VL FMN appear to compensate for the significant FMN loss. In contrast, significant differences in the expression of pro-inflammatory cytokine mRNA in the surrounding neuropil response were found between the two subnuclear regions of the facial nucleus that support a causative role for glial and/or immune-derived molecules in directing the contrasting responses of the FMN to axonal transection.

Keywords

motoneuron; neuropil; facial nerve axotomy; facial motor nucleus; subnuclei

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INTRODUCTION

Functional recovery from traumatic nerve injury requires survival of the injured neuron, and subsequently, target reconnection following axonal regrowth (Lieberman, 1971; Engh and Schofield, 1972; Liuzzi and Tedeschi, 1991). Facial nerve transection, at the stylomastoid foramen, is a well established injury model that has been extensively utilized to understand the basic mechanisms of neural injury and repair (Moran and Graeber, 2004; Jones et al., 2005). Both peripheral and central reactions occur after facial nerve damage and consist of dynamic cellular interactions involving motoneurons, microglia, astrocytes, Schwann cells, and components of the innate and adaptive immune systems (Hall, 1989; Fu and Gordon, 1997; Stoll and Muller, 1999; Raff et al., 2002). Within the facial motor nucleus in the brainstem, facial motoneurons (FMN) typically respond to facial nerve injury by altering gene expression indicative of cell survival, axonal regrowth, and target reconnection (Table 1). In the microenvironment, or neuropil, surrounding injured FMN cell bodies, microglia, astrocytes, and infiltrating immune cells become extensively activated following peripheral nerve injury (Aldskogius and Kozlova, 1998; Raivich et al., 1998; Streit et al., 1998; Kalla et al., 2001), and their activation permits necessary interactions with FMN important for neuronal survival prior to the onset of axonal regrowth (Jones et al., 2005; Sanders and Jones, 2006).

Resting microglia continuously monitor the CNS microenvironment and are capable of rapidly responding to pathological disturbances (Kreutzberg, 1996a; Hanisch, 2002). The processes of resting microglia *in vivo* dynamically extend and retract to sample the extracellular space, providing complete surveillance of the neuronal parenchyma every few hours (Davalos et al., 2005; Nimmerjahn et al., 2005; Raivich, 2005). Microglia are activated and proliferate within days following facial nerve axotomy (Graeber et al., 1988; Schoen et al., 1992; Harrison et al., 1998; Mader et al., 2004). Microglial CX3CR1 activation by CX3CL1 (fractalkine), which is secreted and/or cleaved from FMN, activates microglia and causes migration towards the injured FMN cell bodies (Harrison et al., 1998), which they ensheath soon after axotomy (Kreutzberg, 1996a; Streit, 2002). Activated microglia support injured FMN by assisting with the displacement of excitatory input through the removal of afferent synapses to the FMN (Jones et al., 1999a), a process known as synaptic stripping (Blinzinger and Kreutzberg, 1968; Bruce-Keller, 1999; Schiefer et al., 1999; Streit, 2002), and allows injured FMN to functionally switch out of transmission mode to a developmental/regenerative mode (Jones and Lavelle, 1986; Fawcett and Keynes, 1990; Schwaiger et al., 1998; Oliveira et al., 2004). After axotomy, hypertrophic astrocytes extend their cytoplasmic processes into perineuronal positions, thereby also ensheathing the injured FMN and replacing the microglia (Graeber and Kreutzberg, 1988; Kreutzberg, 1996b). Reactive astrocytes continue to shield injured FMN in order to provide prolonged synaptic insulation (Graeber and Kreutzberg, 1988; Kreutzberg, 1996b; Laskawi and Wolff, 1996). The initial perineuronal contact by microglia, followed by astrocytic ensheathment of the axotomized FMN, permits the continuous exchange of growth factors and other molecules, and provides evidence for pro-survival and pro-regenerative neural-glial interactions following peripheral nerve injury.

Evidence from numerous laboratories suggests that neuronal injury provokes a robust immune response within the CNS (Streit, 1993; Raivich et al., 1998; Galiano et al., 2001), as immune cells are capable of cyclically reentering the CNS upon recognition of their specific antigen (Hickey et al., 1991). Our laboratory has also discovered an essential role for the adaptive immune system in maintaining wild-type (WT) levels of FMN survival following axotomy (Serpe et al., 1999; Serpe et al., 2000; Jones et al., 2005). CD4⁺ T cells are the primary adaptive immune cells responsible for maintaining WT levels of FMN survival in immunodeficient mice (Byram et al., 2003; Serpe et al., 2003; DeBoy et al., 2006), and are

initially activated via an injury-specific antigen presented by a major histocompatibility complex class II (MHC II) positive antigen-presenting cell (Byram et al., 2004). In the facial motor nucleus, microglial cells also upregulate MHC II which is required to re-activate CD4⁺ T cells following axotomy (Byram et al., 2004). Other laboratories have localized CD3⁺, CD4⁺, and CD8⁺ T cells within the axotomized facial motor nucleus (Raivich et al., 1998; Ha et al., 2006). Moreover, T cell infiltration into the axotomized facial motor nucleus is severely reduced in the absence of chemoattractant ligands and their receptors (Huang et al., 2007). Recently, we have shown that astrocytes induce the expression of CCL11, a CD4⁺ Th2 cell-specific chemokine, at a time-point in accordance with the peak hypertrophy of astrocytes (Wainwright et al., 2009b). CCR3, the cognate receptor for CCL11, is required for WT FMN survival levels following facial nerve axotomy and, furthermore, CD4⁺ T cells must express CCR3 to mediate their neuroprotective effects (Wainwright et al., 2009a).

Thus, contributing factors that influence the ability of an injured neuron to survive include signals from the neuron accompanied by a tightly regulated environmental reaction involving CNS glial and peripheral immune cells. In a recent topographical mapping study (Canh et al., 2006), we analyzed the anatomical distribution pattern of FMN survival levels in the six facial motor subnuclei 28 days following axotomy and unexpectedly discovered distinct differences. While there is essentially 100% FMN survival in the ventromedial (VM) subnucleus after injury, the ventrolateral (VL) subnucleus exhibits significant cell death relative to the uninjured control side.

The objective of the current study was to exploit the differential effects of axotomy on FMN survival levels within distinct subregions of the same facial nucleus through the use of laser microdissection to delineate neuronal and/or neuropil contributions to neuronal survival after direct axonal injury. The initial working hypothesis for this study was that there would be two phenotypically distinct neuronal responses to injury in the VM vs. the VL subnuclei that could be categorized as “regenerative” or “degenerative”. The proposed “regenerative” neuronal phenotype would consist of upregulating genes that promote survival and regeneration and downregulating genes responsible for degeneration and cell death. In contrast, the proposed “degenerative” neuronal phenotype would involve downregulating genes supporting survival and regeneration and upregulating genes implicated in degeneration and cell death. Instead, we discovered that, regardless of neuronal fate after injury, FMN in either subnuclear region respond vigorously to injury with a characteristic “regenerative” profile and, indeed, appear to compensate in the VL for FMN loss. In contrast, significant differences in the surrounding neuropil response were revealed between the two subnuclear regions of the facial nucleus that support a causative role for glial and/or immune-derived molecules in directing contrasting responses of a specific cell type, the FMN, to the same injury, axonal transection.

MATERIALS AND METHODS

Animals and surgical procedures

Female wild-type mice on a C57BL/6 background were obtained from Jackson labs (Bar Harbor, ME) at seven weeks of age, permitted 1 week to acclimate to their environment before manipulation, and used at 8 weeks of age in all experiments. Mice were provided autoclaved pellets and water ad libitum, and were housed under a 12 h light/dark cycle in microisolator cages contained within a laminar flow system to maintain a pathogen-free environment. All surgical procedures were completed in accordance with National Institutes of Health guidelines on the care and use of laboratory animals for research purposes. Mice were anesthetized with 3% isoflurane prior to all surgical procedures. Using aseptic techniques, the right facial nerve was exposed at its exit from the stylomastoid foramen and completely transected (Jones and LaVelle, 1985; Serpe et al., 1999). The proximal and distal

facial nerve stumps were manually pushed away from each other in order to prevent reconnection. The left facial nerve remained intact, allowing the left facial nucleus to serve as an internal control for comparison purposes.

Tissue preparation and laser microdissection

Mice were euthanized by CO₂ asphyxiation without injury ($n = 3$), and at 3, 7, 14, or 28 days post-axotomy (dpa; $n = 5$ –9 per time-point). Brains were rapidly removed and frozen in a -30°C biphasic solution containing *n*-butyl bromide (62.5%) and 2-methyl butane (37.5%) for 5 minutes. Twenty-five micrometer coronal cryosections were collected throughout the rostrocaudal extent of the facial motor nucleus. The tissue sections were thaw-mounted onto glass polyethylene (PEN) foil slides (Leica) and stored at -80°C . The frozen sections were allowed to acclimate to -20°C prior to laser microdissection, and each slide containing 8–10 tissue sections was individually fixed, stained, and laser microdissected to minimize RNA degradation. The tissue was fixed in 100% ethanol for 1 minute, washed twice in 0.1% DEPC-treated water for 15 seconds each, quickly stained for 30 seconds with thionin (pH 3.5; 2 $\times$ concentration for laser microdissection of facial subnuclei or 4 $\times$ concentration for laser microdissection of FMN and neuropil), washed twice in 0.1% DEPC-treated water for 15 seconds each, and dehydrated via a graded ethanol series for 30 seconds each (70%, 90%, 100%, respectively). The tissue sections were then allowed to air dry for 3 minutes in an unsealed, covered container.

A laser microdissection protocol was established to accurately collect specific tissue samples from the facial motor nucleus (Fig. 1). A quick thionin stain was initially used for the identification and laser microdissection of the entire facial motor nucleus (Fig. 1A–C). Two separate laser microdissection experiments were conducted in the present study using a Leica AS laser microdissector. First, the ventromedial (VM) and ventrolateral (VL) facial subnuclear regions were isolated (Fig. 1D–F). Secondly, the facial motoneurons (FMN) from the entire facial motor nucleus were isolated separate from the surround facial nucleus microenvironment (neuropil; Fig. 1G–I). All laser microdissected tissue samples were collected into 65 μL extraction buffer (PicoPure RNA Isolation Kit; Arcturus) contained within the collection tube caps RNA isolation and extraction.

Previously, we developed a template to separate the facial nucleus into its six subnuclear regions (Fig. 1D; Canh et al., 2006) based on the published functionally and topographically distinct FMN groups (Ashwell, 1982). This template was superimposed over individual thionin-stained sections containing the facial motor nucleus, in order to identify the VM and VL subnuclei (Fig. 1E–F) for laser microdissection. The uninjured or axotomized VM and VL subnuclei were laser microdissected into separate collection tubes thereby resulting four samples per mouse.

To analyze mRNA expression within FMN or the neuropil, we identified and laser microdissected FMN based on their morphology and displaying a clear nucleus and nucleolus (Fig. 1G, indicated by *). Once all clearly identified FMN were collected via laser microdissection (Fig. 1H), any visible cell body fragments were laser microdissected separately for disposal (Fig. 1G, indicated by arrows). The remaining facial nucleus tissue was laser microdissected for analysis as the neuropil sample (outlined in Fig. 1I). Laser microdissection of the FMN cell body fragments was completed in order to minimize the contamination of neuropil mRNA in the FMN samples and of FMN mRNA in the neuropil samples. A total of four separate samples were collected for each mouse, consisting of the control FMN, axotomized FMN, control neuropil, and axotomized neuropil.

Semi-quantitative real-time RT-PCR

From laser microdissected facial motor nucleus or facial nucleus subnuclei collected samples, total cellular RNA was isolated with the PicoPure RNA Isolation Kit (Arcturus), including a DNase treatment step (Qiagen). Complementary DNA was obtained using Superscript First Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer's instructions (Sharma et al., 2009). Semi-quantitative real-time RT-PCR was performed using the iCycler iQ detection system (BioRad) for 25 μ L PCR reactions (Jones et al., 2000; Fargo et al., 2008; Sharma et al., 2009). RT-PCR cycle parameters included a 95°C initial denaturing step for 10 minutes, followed by 45 cycles of three repeated steps, first step denaturing at 95°C for 30 seconds, second step annealing for 30 seconds at the optimal annealing temperature (T_A ; Table 2) for that primer set, and third step extension at 65°C for 30 seconds. PCR primers were designed from published mouse sequences, consisting of 15–24 nucleotides each and produced ~100 base-pair amplicons (Table 2). The T_A for each primer set was determined experimentally using either mouse brain cDNA or mouse spleen cDNA as the template over a proper temperature gradient according to the designed primer's estimated annealing temperature. To determine the specificity of the amplified product, a melt curve analysis was performed immediately after the amplification protocol and compared to the experimentally determined melt temperatures (T_m ; Table 2) for each gene investigated. Relative mRNA expression levels acquired during the PCR amplification were analyzed using the $\Delta\Delta C_T$ method against a well-established axotomized facial nucleus internal standard, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Raivich et al., 1998; Ando et al., 2003; Sharma et al., 2009). As previously published by our laboratory (Fargo et al., 2008), the relative mRNA expression is given as the percent change, by $(2^{-\Delta\Delta C_T} - 1) * 100$, of the axotomized samples relative to the control samples. The percent changes of mRNA expression were statistically compared using a two-way ANOVA (group $\times$ time), followed by the Student-Newman-Keuls multiple comparison post-hoc test, with significance at $p \leq 0.05$ (GB-Stat School Pak).

Gel electrophoresis

Gel electrophoresis was accomplished using RT-PCR products for genes induced by axotomy, with below detectable levels of mRNA expression in the uninjured controls. Electrophoresis was performed using PCR amplified products on Criterion precast 10% TBE gels (BioRad) in a Criterion cell vertical electrophoresis system (BioRad) for 90 minutes at 100V, using 1 $\times$ TBE running buffer (10 $\times$ TBE nucleic acid electrophoresis buffer; BioRad) which contained 100 μ L SYBR Green I nucleic acid gel stain (Invitrogen). PCR amplified products were collected from the associated wells within 96 well PCR plates and diluted 4:1 with nucleic acid sample loading buffer (BioRad; 12 μ L sample: 3 μ L buffer). Each gel included a 100 base-pair (bp) molecular marker (BioRad), GAPDH (positive control), and non-reverse transcribed PCR products (negative control). The gels were scanned on a STORM 860 PhosphorImager (Molecular Dynamics) using ImageQuant software for visualization.

RESULTS

Axotomy-induced gene expression in the VM vs. VL facial motor subnuclear regions

Cytoskeletal genes, such as actin, α_I tubulin, β_{II} tubulin, and β_{III} tubulin, are upregulated by axotomy to initiate and enhance neuronal restructuring and regeneration (Tetzlaff et al., 1988a; Tetzlaff et al., 1991; Jones and Oblinger, 1994; Jones et al., 1999b; Sharma et al., 2009). An upregulation in β_{II} tubulin mRNA expression in response to axotomy occurred in both the VM and the VL facial motor subnuclear regions with a main effect of post-operative time ($F_{4,46}=13.75$, $p<0.0001$; Fig. 2A). In the axotomized VM, a significant upregulation in β_{II} tubulin mRNA expression was observed at 3, 7, 14, and 28 days post-

axotomy (dpa; $108 \pm 31\%$, $196 \pm 29\%$, $96 \pm 26\%$, $56 \pm 25\%$, respectively; $p < 0.05$), relative to the uninjured control VM (Fig. 2A). In the axotomized VL, a significant increase in β_{II} tubulin mRNA expression at 3, 7, 14, and 28 dpa ($123 \pm 16\%$, $206 \pm 32\%$, $248 \pm 41\%$, $83 \pm 28\%$, respectively; $p < 0.05$), relative to the uninjured control VL (Fig. 2A), also occurred. An overall main effect of subnuclear region following axotomy was observed ($F_{1,46}=4.37$, $p=0.042$), as β_{II} tubulin mRNA expression was found to be significantly increased in the axotomized VL at 14 dpa compared to the VM (Fig. 2A; $p < 0.05$).

Growth- associated protein of 43 kilodaltons (GAP-43) and hemopoietic- and neurologic-expressed sequence-1 (Hn1) protein, which are important in MN development, have been shown to be part of the regenerative peripheral MN response to axotomy (Tetzlaff et al., 1991; Jones et al., 1997a; Akazawa et al., 2004; Zujovic et al., 2005; Sharma et al., 2009). An upregulation in GAP-43 mRNA expression in response to axotomy occurred in both the VM and the VL facial motor subnuclear regions with a main effect of post-operative time ($F_{4,45}=12.52$, $p < 0.0001$; Fig. 2B). In the axotomized VM, a significant upregulation in GAP-43 mRNA expression was observed at 3, 7, 14, and 28 dpa ($1611 \pm 198\%$, $2741 \pm 361\%$, $1985 \pm 447\%$, and $195 \pm 87\%$, respectively; $p < 0.05$), relative to the uninjured control VM (Fig. 2B). In the axotomized VL, a significant increase in GAP-43 mRNA expression at 3, 7, 14, and 28 dpa ($4831 \pm 1103\%$, $4456 \pm 859\%$, $4082 \pm 786\%$, and $662 \pm 182\%$, respectively; $p < 0.05$), relative to the uninjured control VL (Fig. 2B), also occurred. As observed with β_{II} tubulin an overall main effect of subnuclear region following axotomy was observed ($F_{1,45}=12.17$, $p=0.0011$), as GAP-43 mRNA expression was found to be significantly increased in the axotomized VL at 3 and 14 dpa compared to the VM (Fig. 2B; $p < 0.05$).

An upregulation in Hn1 mRNA expression in response to axotomy occurred in both the VM and the VL facial motor subnuclear regions with a main effect of post-operative time ($F_{4,45}=32.33$, $p < 0.0001$; Fig. 2C). In the axotomized VM, a significant upregulation in Hn1 mRNA expression was observed at 3, 7, 14, and 28 dpa ($528 \pm 71\%$, $647 \pm 97\%$, $279 \pm 72\%$, and $56 \pm 12\%$, respectively; $p < 0.05$), relative to the uninjured control VM (Fig. 2C). In the axotomized VL, a significant increase in Hn1 mRNA expression at 3, 7, 14, and 28 dpa ($752 \pm 54\%$, $636 \pm 64\%$, $355 \pm 68\%$, and $76 \pm 31\%$, respectively; $p < 0.05$), relative to the uninjured control VL (Fig. 2C), also occurred. As found with both β_{II} tubulin and GAP-43 mRNA expression, Hn1 mRNA expression was also found to be significantly increased in the axotomized VL at 3 dpa compared to the VM (Fig. 2C; $p < 0.05$).

BDNF has been localized to peripheral nerves, Schwann cells, MNs, and immune cells, and influences MN survival via paracrine- and autocrine-mediated neurotrophic actions during development and after injury (Serpe et al., 2005; Meyer et al., 1992; Kobayashi et al., 1996; Besser and Wank, 1999; Widenfalk et al., 2001; Azoulay et al., 2008; Gordon, 2009). An upregulation in BDNF mRNA expression in response to axotomy occurred in both the VM and the VL facial motor subnuclear regions with a main effect of post-operative time ($F_{4,42}=6.14$, $p=0.0006$; Fig. 2D). In the axotomized VM, a significant upregulation in BDNF mRNA expression was observed at 3 dpa ($310 \pm 87\%$; $p < 0.05$), relative to the uninjured control VM, whereas BDNF mRNA expression was not affected by axotomy at 7, 14, and 28 dpa ($60 \pm 24\%$, $15 \pm 27\%$, and $116 \pm 156\%$, respectively; Fig. 3A). In the axotomized VL, a significant increase in BDNF mRNA at 3 and 7 dpa ($536 \pm 170\%$ and $319 \pm 70\%$, respectively; $p < 0.05$) relative to the uninjured control VL (Fig. 2D) also occurred. As in the VM, there were no statistically significant changes in BDNF mRNA expression at 14 or 28 dpa (201 ± 115 and 207 ± 166 , respectively), relative to the uninjured control VL (Fig. 2D). An overall main effect of subnuclear region following axotomy was observed ($F_{1,42}=7.37$, $p=0.0096$), as BDNF mRNA expression was found to be significantly increased in the axotomized VL at 7 dpa compared to the VM (Fig. 2D; $p < 0.05$).

Reactive astrocytes elevate glial fibrillary acidic protein (GFAP) expression in the facial nucleus following facial nerve axotomy (Guntinas-Lichius et al., 1996; Jones et al., 1997b). An upregulation in GFAP mRNA expression in response to axotomy occurred in both the VM and the VL facial motor subnuclear regions with a main effect of post-operative time ($F_{4,44}=29.51$, $p<0.0001$; Fig 3A). In the axotomized VM, a significant upregulation in GFAP mRNA expression was observed at 3, 7, 14, and 28 dpa ($1305 \pm 144\%$, $867 \pm 185\%$, $2399 \pm 812\%$, and $440 \pm 254\%$, respectively; $p < 0.05$), relative to the uninjured control VM (Fig. 3A). In the axotomized VL, a significant increase in GFAP mRNA expression at 3, 7, 14, and 28 dpa ($1148 \pm 152\%$, $506 \pm 133\%$, $3480 \pm 666\%$, and $507 \pm 89\%$, respectively; $p < 0.05$), relative to the uninjured control VL, also occurred (Fig. 3A). No significant differences were observed in GFAP mRNA expression between the axotomized VM and VL subnuclei.

The pro-inflammatory cytokines, tumor necrosis factor alpha (TNF α) and interferon gamma (IFN γ), are expressed in the facial motor nucleus in response to axotomy (Olsson et al., 1989; Kiefer et al., 1993; Raivich et al., 1998; Hanisch, 2002; Raivich et al., 2002; Raivich et al., 2003). In the present study, TNF α and IFN γ pro-inflammatory cytokine mRNA expression was undetectable in either the VM or VL control facial motor subnuclear regions (Figs. 3B and 3C). In the axotomized VM, TNF α mRNA expression was induced as early as 3 dpa, maintained until 14 dpa, and below detectable levels at 28 dpa (Fig. 3B). In contrast, in the axotomized VL, while the initial induction time at 3 dpa was similar to the VM, there was sustained expression of TNF α at 28 dpa (Fig. 3B). In both the axotomized VM and VL, IFN γ mRNA expression was induced at 14 dpa (Fig. 3C). While this expression was transient in the VM, IFN γ mRNA expression was sustained at 28 dpa in the VL (Fig. 3C).

A FMN-specific chemokine, fractalkine (CX3CL1), and its microglial-specific cognate receptor CX3CR1 may provide a mechanism by which FMN and microglia interact following facial nerve axotomy (Harrison et al., 1998; Verge et al., 2004; Zhuang et al., 2007). A downregulation in CX3CL1 mRNA expression in response to axotomy occurred in both the VM and the VL facial motor subnuclear regions with a main effect of post-operative time ($F_{4,46}=54.85$, $p<0.0001$; Fig 4A). In the axotomized VM, a significant downregulation in CX3CL1 mRNA expression was observed at 3, 7, and 14 dpa ($-71 \pm 9\%$, $-93 \pm 2\%$, $-69 \pm 13\%$, respectively; $p < 0.05$), relative to the uninjured control VM, whereas CX3CL1 mRNA expression was not affected by axotomy at 28 dpa ($13 \pm 30\%$; Fig. 4A). In the axotomized VL, a significant decrease in CX3CL1 mRNA at 3, 7, and 14 dpa ($-96 \pm 0.5\%$, $-91 \pm 3\%$, and $-81 \pm 6\%$, respectively; $p < 0.05$) relative to the uninjured control VL (Fig. 4A) also occurred. As in the VM, there were no statistically significant changes in CX3CL1 mRNA expression at 28 dpa ($0 \pm 19\%$), relative to the uninjured control VL (Fig. 4A). CX3CL1 mRNA expression was found to be significantly decreased in the axotomized VL at 3 dpa compared to the VM (Fig. 4A; $p < 0.05$).

Neuroprotective capabilities of pituitary adenylate cyclase-activating polypeptide (PACAP) have been revealed in a variety of *in vitro* and *in vivo* models, with PACAP acting as a neurotransmitter, neurotrophic factor, and neuroimmune modulator (Gomariz et al., 2006; Shioda et al., 2006). PACAP was not found to be expressed in either the VM or VL control facial motor subnuclear regions (Fig 4B). In the axotomized VM, PACAP mRNA expression was induced as early as 3 dpa and maintained throughout the time-course investigated (Fig. 4B). In the axotomized VL subnucleus, there was a delay in the induction of PACAP mRNA expression after injury until 14 dpa, with maintenance of induced expression at 28 dpa (Fig. 4B).

Caspase 8 is an upstream initiator caspase in apoptotic signaling cascades that is activated following death receptor activation after nerve injury (Martin et al., 2005). An upregulation

in Caspase 8 mRNA expression in response to axotomy occurred in both the VM and the VL facial motor subnuclear regions with a main effect of post-operative time ($F_{4,45}=6.88$, $p=0.0002$; Fig 4C). In the axotomized VM, a significant upregulation in Caspase 8 mRNA expression was observed at 3, 7, 14, and 28 dpa ($67 \pm 14\%$, $633 \pm 140\%$, $218 \pm 104\%$, and $75 \pm 14\%$, respectively; $p < 0.05$) relative to the uninjured control VM (Fig. 4C). In the axotomized VL, a significant upregulation in Caspase 8 mRNA expression was also observed at 3, 7, 14, and 28 dpa ($256 \pm 40\%$, $321 \pm 90\%$, $867 \pm 238\%$, and $203 \pm 115\%$, respectively; $p < 0.05$) relative to the uninjured control VL (Fig. 4C). Caspase 8 mRNA expression was found to be significantly increased in the axotomized VL at 3 and 14 dpa compared to the VM (Fig. 4C; $p < 0.05$). Caspase 8 mRNA changes following axotomy revealed an interaction between subnuclear region and post-operative time ($F_{4,45}=3.81$, $p=0.0094$), as Caspase 8 mRNA expression in the axotomized VM peaked at 7 dpa relative to the other post-operative time-points ($p < 0.05$), and Caspase 8 mRNA expression in the axotomized VL peaked at 14 dpa relative to the other postoperative time-points ($p < 0.05$).

FMN and neuropil mRNA localization following axotomy

To determine the localization for the mRNA gene expression that was induced following axotomy, the FMN and surrounding neuropil were laser microdissected and collected separately from the control and axotomized facial motor nuclei (Fig. 1G–I). Induction of TNF α and IFN γ mRNA was restricted to the axotomized neuropil surrounding the axotomized FMN within the facial nucleus, and no expression was detected in the control FMN, axotomized FMN, or control neuropil samples for either gene (Fig. 5 panels 1 and 2). PACAP mRNA induction following axotomy was localized primarily to the axotomized FMN, and no expression was detected in the control FMN or control neuropil samples (Fig. 5 panel 3). Although PACAP mRNA expression was in the detectable range in the axotomized neuropil samples, this expression is most likely the result of small axotomized FMN cell body fragments that were unable to be laser microdissected for disposal prior to collection of the neuropil sample.

DISCUSSION

The cellular and molecular determinants in the fate of injured neurons are not well delineated. Are such determinants inherent in the phenotypic responses of individual neurons, or are they embedded with the neuronal microenvironment? The current study was undertaken to address these questions, through the utilization of laser microdissection to examine contrasting responses of individual subpopulations of FMN within the mouse facial nucleus as a whole (Canh et al., 2006). Based on our previously published studies of immune cell-mediated neuroprotection in the mouse facial nerve injury model (Jones et al., 2005), we originally hypothesized that the FMN would display distinct phenotypes in two subnuclear regions with differing neuronal survival levels after axotomy. Unexpectedly, we discovered that 1) FMN in the VM subnucleus, where there is no cell loss, and VL, where there is substantial cell loss, respond with similar molecular changes supporting survival and regeneration, and that 2) there are substantial differences in axotomy-induced gene expression within the surrounding neuropil of the two subnuclei that could be causative in the axotomy-induced fate of FMN in the VL.

Axonal elongation during development or regeneration following injury involves assembly of a microtubule cytoskeleton consisting primarily of tubulin, and specifically of the class II isotype (Hoffman and Cleveland, 1988; Moskowitz and Oblinger, 1995). β_{II} tubulin mRNA expression is localized to a variety of neuronal populations throughout the brain (Paden et al., 1995), while, in the facial nucleus, tubulin mRNA is predominantly expressed by FMN (Tetzlaff et al., 1991). While an increase in β_{II} tubulin was expected in the axotomized VM, based upon the literature in rat and hamster (Tetzlaff et al., 1991; Jones and Oblinger, 1994;

Jones et al., 1999b; Sharma et al., 2009), it was surprising to find even greater expression in the VL, where substantial cell death occurs. GAP-43, which is involved in growth cone development and maintenance of compensatory sprouting or regenerating neuronal axons (Skene and Willard, 1981; Skene, 1989; Strittmatter et al., 1991; Palacios et al., 1994; Namgung and Routtenberg, 2000), was also upregulated in response to axotomy (Saika et al., 1993; Jones et al., 1997a). GAP-43 over-expression in transgenic mice resulted in spontaneous synthesis of axonal sprouting prior to, and enhanced following, injury (Aigner et al., 1995). As we found with β_{II} tubulin mRNA expression, GAP-43 mRNA expression was actually greater in the VL.

Hn1 expression was first localized to hematopoietic and neural tissue, with high expression in neurons within the fetal brain (Tang et al., 1997) and regenerating newt retina (Goto et al., 2006), and has been suggested to play a role as a regeneration-associated gene following injury (Harrison et al., 1998). As previously observed in the rat (Zujovic et al., 2005), the present study confirmed that Hn1 mRNA expression is upregulated in the mouse facial motor nucleus following facial nerve axotomy. As with β_{II} tubulin and GAP-43, Hn1 mRNA expression was significantly upregulated in the axotomized VL compared to the VM. Thus, surprisingly, there is an augmentation in survival- and regeneration-associated gene expression in the VL, despite substantial cell loss, that may reflect compensatory mechanisms inherent with the remaining FMN. Collectively, these data suggest that there is a single phenotypic response to axotomy by FMN within the facial nucleus that is geared toward survival and regeneration.

Neurotrophic factors, such as BDNF, have been shown to promote motoneuron survival by rescuing motoneurons from axotomy-induced cell death via paracrine and autocrine mechanisms (Sendtner et al., 1990; Sendtner et al., 1992; Yan et al., 1994; Zhao et al., 2004; Serpe et al., 2005). Facial nerve axotomy results in increased BDNF mRNA expression in rat FMN (Kobayashi et al., 1996), and the current results confirm increased BDNF mRNA expression in the axotomized mouse facial motor nucleus as well. However, recent evidence suggests that motoneurons can be susceptible to cell death via a BDNF-directed excitotoxic mechanism through activation of TrkB receptors, and that inhibiting TrkB receptor complex signals from BDNF stimulation is neuroprotective (Mojsilovic-Petrovic et al., 2006). Therefore, in the present study, the increased BDNF mRNA expression in the axotomized VL compared to the VM could either be promoting a compensatory response of the remaining FMN in the VL to support survival or causing BDNF-directed excitotoxic cell death of the axotomized VL FMN.

The neuropil contains a complex network of neuronal-astrocyte (de Haas et al., 2007; Wainwright et al., 2009a; Wainwright et al., 2009b), neuronal-microglial (Harrison et al., 1998; de Haas et al., 2007), neuronal-immune (Serpe et al., 1999; Serpe et al., 2000; Serpe et al., 2003), astrocyte-microglial (Chen et al., 2006), astrocyte-immune (Wainwright et al., 2009a; Wainwright et al., 2009b), and microglial-immune (Byram et al., 2004; Chen et al., 2006) cellular interactions. Microglial cells are the first responders following facial nerve axotomy (Blinzinger and Kreutzberg, 1968; Graeber et al., 1988), presumably sensing a change in the homeostatic environment between the injured motoneuron and the microglia (Gehrmann et al., 1993; Kreutzberg, 1996a). The axotomy-induced astrocytic response occurs subsequent to the microglial response (Graeber and Kreutzberg, 1988; Kreutzberg et al., 1989; Laskawi and Wolff, 1996) and to the activation of the immune system (Raivich et al., 1998).

GFAP constructs the cytoarchitecture of astrocytes (Bignami et al., 1972), and activated astrocytes increase GFAP mRNA and protein expression (Graeber and Kreutzberg, 1988; Kreutzberg, 1996b). Depending on the nerve injury and the severity of the injury, GFAP

mRNA is increased as early as 1 day post-operative (Tetzlaff et al., 1988b; Jones et al., 1997b; Hurley and Coleman, 2003; Storer and Jones, 2003), and GFAP protein has been detected for up to 1 year post-operative (Laskawi and Wolff, 1996). The data in the present study indicate similar astrocytic responses between the VM and VL subnuclei, with a peak in increased expression at 2 weeks post-axotomy. Astrocytes reach their highest level of activation within 3 weeks following axotomy, and extend their processes into the perineuronal environment to ensheath the injured FMN while concurrently replacing the microglial cells (Graeber and Kreutzberg, 1988; Kreutzberg, 1996b).

In contrast to neuronal and astrocytic responses to axotomy, pro-inflammatory cytokine gene expression, localized exclusively in the microenvironment, was substantially different in the VM and VL subnuclei after axotomy. Unlike the transient expression of TNF α and IFN γ in the VM, there was prolonged expression in the VL. These data expand on previous research identifying axotomy-induced TNF α and IFN γ mRNA expression within the whole axotomized facial nucleus (Raivich et al., 1998). It has been demonstrated that activated microglia upregulate TNF α , while injured motoneurons upregulate a receptor for TNF α , tumor necrosis factor receptor-1 (TNFR1; Hanisch, 2002; Bigini and Mennini, 2004; Veglianesi et al., 2006). Upon stimulation by TNF α , the TNFR1 mediates the activation of Caspase 8 by recruiting pro-caspase 8 to the membrane where it is cleaved into its active form (Baker and Reddy, 1998; Friedlander, 2003). In addition, pro-inflammatory immune cells are thought to be the primary source of IFN γ (Schoenborn and Wilson, 2007; Xin et al., 2008). Interaction of microglia with immune cells and astrocytes results in the increased production of microglial TNF α mRNA (Chen et al., 2006). Thus, one interpretation of the data is that, in contrast to the VM, axotomy results in dysregulated FMN-neuropil interaction in the VL, and potentially triggering an apoptotic cell death pathway. A more pronounced decline in fractalkine, a chemokine involved in microglial-neuronal interactions (Harrison et al., 1998) by the VL subnucleus early after injury supports this interpretation.

The neuroprotective peptide, PACAP, has been shown to inhibit pro-inflammatory cytokine production, including TNF α , in activated microglia *in vitro* (Kim et al., 2000; Delgado et al., 2003). Axotomy has been shown to induce PACAP expression in mouse FMN, but only in the presence of a normal immune system (Armstrong et al., 2003; Armstrong et al., 2004a; Armstrong et al., 2004b; Armstrong et al., 2006), and may play a direct role in promoting injured FMN axonal regeneration (Armstrong et al., 2008), as PAC-IR has been localized to the growth cones of newly developing neuronal axons (Guirland et al., 2003). In addition, direct application of PACAP to the transected proximal facial nerve stump promotes axonal sprouting and elongation (Suarez et al., 2006). In the present study, PACAP induction after axotomy was found to be significantly delayed, in the VL relative to the VM, which could potentially impact TNF α secretion by activated microglial cells (Delgado et al., 1999a; Delgado et al., 1999b; Kim et al., 2000; Delgado et al., 2002a; Delgado et al., 2002b; Gomariz et al., 2006; Shioda et al., 2006). Thus, early PACAP mRNA induction in the axotomized VM FMN could result in the transient TNF α induction observed in the VM neuropil, whereas, delayed PACAP mRNA induction in the axotomized VL FMN could lead to sustained TNF α induction in the VL neuropil and increased FMN cell death.

Caspase 8, a cysteine protease, is an initiator caspase activated downstream in apoptotic signaling cascades, subsequent to activation of the death receptors, TNFR1 or Fas, by TNF α or FasL, respectively (Friedlander, 2003; Thorburn, 2004). FMN apoptosis after axotomy has been shown to be prevented in transgenic mice lacking functional Fas (Martin et al., 2005) or containing the combined deletion of both TNF α receptors, TNFR1 and TNFR2 (Raivich et al., 2002). Caspase 8 mRNA expression has not yet been investigated in response to facial nerve axotomy. In the present study, we discovered an upregulation in Caspase 8 mRNA expression in the facial motor nucleus following facial nerve axotomy,

with differential expression between the VM and VL subregions. In the VL, there was substantial, sustained expression of Caspase 8 mRNA expression that is temporally correlated with the delayed induction of PACAP mRNA and sustained induction of TNF α mRNA observed in the axotomized VL. These data and observations again support the interpretation of a dysregulated neuropil, resulting in faulty neuropil-neuronal interactions, and leading directly to motoneuron cell death following injury.

In contrast to the other genes examined in this study, PACAP, TNF α , and IFN γ mRNA expression was induced following facial nerve axotomy. In agreement with reports of neuronal localization of PACAP mRNA in the rat and mouse following peripheral nerve injury (Zhang et al., 1996; Zhou et al., 1999; Armstrong et al., 2003), we found that FMN were the primary source of PACAP mRNA induction after axotomy. TNF α and IFN γ mRNA expression following facial nerve axotomy has been shown in the mouse whole facial nucleus (Raivich et al., 1998). In the present study, we detected the induction of both the pro-inflammatory cytokines within the axotomized facial motor nucleus as well, with selective induction in the neuropil but not the FMN, in contrast to data demonstrating IFN γ -like immunoreactivity in axotomized motoneurons (Olsson et al., 1989). Since T cell subsets activated by facial nerve axotomy show increased expression for IFN γ (Xin et al., 2008), and have been reported in the axotomized facial motor nucleus (Raivich et al., 1998; Raivich et al., 2003; Ha et al., 2006), it may be infiltrating immune cells that are the primary source of IFN γ mRNA expression in the axotomized neuropil of the facial nucleus.

In summary, the data lead us to hypothesize that peripheral motoneurons are programmed to direct a molecular response geared toward viability and reconnection to target, but the ability of these motoneurons to survive injury is directly dependent upon molecular cues in the surrounding neuropil. Dysregulation of those molecular cues, in a context-dependent manner, ultimately directs motoneuron fate, and may play a significant role in the initiation and/or disease progression in fatal motoneuron diseases, such as amyotrophic lateral sclerosis (ALS). Future directions will include the use of in situ hybridization and immunohistochemistry to localize the molecular changes within the facial motor nucleus subregions at the cellular level, and the extrapolation of these findings to animal models of motoneuron disease.

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Figure 1. Laser microdissection of the facial motor nucleus

Thionin-stained section of axotomized facial nucleus (A), with partial laser microdissection of the axotomized facial nucleus (B,C). A template of the subnuclei (D) was superimposed on the computer screen to identify the VM and VL facial subnuclei for laser microdissection (E, F). FMN were laser microdissected based on their morphology with a visible nucleus and nucleolus (*, G, H), while FMN cell body fragments, indicated by the arrows (H), were laser microdissected separately and disposed of to eliminate FMN mRNA in the neuropil samples. After all FMN and cell body fragments were collected, the remaining facial nucleus tissue was laser microdissected as the neuropil sample (I). Scale bars = 100 μ m.

Figure 2. Pro-regeneration and pro-survival mRNA expression in the VM and VL facial motor subnuclear regions following facial nerve axotomy

Average percent of mRNA expression $\pm$ SEM in the transected VM and VL facial subnuclei relative to the unoperated control subnuclei (A–D). Time-course of mRNA expression includes no injury (0), 3, 7, 14, and 28 dpa for β II tubulin (A), GAP-43 (B), Hn1 (C), and BDNF (D). * represents significant differences of VL compared to VM, at $p < 0.05$.

Figure 3. Neuropil-specific mRNA expression in the VM and VL facial motor subnuclear regions following facial nerve axotomy

Average percent of mRNA expression $\pm$ SEM in the transected VM and VL facial subnuclei relative to the unoperated control subnuclei (A). Time-course of mRNA expression includes no injury (0), 3, 7, 14, and 28 days post-axotomy (dpa) for GFAP (A). Electrophoresis of RT-PCR products from control and axotomized VM and VL facial subnuclei at 3, 7, 14, and 28 days post-operative for TNF α (B; 102 bp amplicon) and IFN γ (C; 111 bp amplicon), with the 100 bp molecular weight band (MW), the internal standard (GAPDH), and a no reverse-transcriptase control (rtc). * represents significant differences of VL compared to VM, at $p < 0.05$.

Figure 4. Motoneuron-neuropil interaction mRNA expression in the VM and VL facial motor subnuclear regions following facial nerve axotomy

Time-course of mRNA expression includes no injury (0), 3, 7, 14, and 28 days post-axotomy (dpa) for CX3CL1 (A) and Caspase 8 (C). Average percent of mRNA expression $\pm$ SEM in the transected VM and VL facial subnuclei relative to the unoperated control subnuclei in wild-type mice (A, C). Electrophoresis of RT-PCR products from control and axotomized VM and VL facial subnuclei at 3, 7, 14, and 28 dpa for PACAP (B; 109 bp amplicon), with the 100bp molecular weight band (MW), the internal standard (GAPDH), and a no reverse-transcriptase control (rtc). * represents significant differences of VL compared to VM, at $p < 0.05$.

Figure 5. Localization of induced mRNA expression in facial motoneurons (FMN) or neuropil following facial nerve axotomy

Electrophoresis of RT-PCR products from control or axotomized FMN and neuropil at 7 days post-operative for TNF α (102 bp amplicon; first panel), IFN γ (111 bp amplicon; second panel), PACAP (109bp amplicon; third panel), and the internal standard (GAPDH; 78 bp amplicon; fifth panel), along with the 100 bp molecular weight band (MW) and a no reverse-transcriptase control (rtc).

Table 1

The previously identified molecular function of regenerative- and degenerative-associated genes used to phenotype FMN populations.

Gene	Localization	Function	References
β_{II} tubulin	MN	Regeneration	Sharma et al., 2009; Jones et al., 1999; Jones & Oblinger, 1994
GAP-43	MN	Regeneration	Akazawa et al., 2004; Jones et al., 1997; Palcios et al., 1994; Tetzlaff et al., 1991
Hn1	MN	Survival/Regeneration	Zujovic et al., 2005
CX3CL1	MN	Survival/Regeneration	Zhuang et al., 2007; Verge et al., 2004; Harrison et al., 1998
PACAP	MN	Regeneration	Armstrong et al., 2008; Suarez et al., 2006
BDNF	MN/Neuropil	Survival/Regeneration	Serpe et al., 2005; Kobayashi et al., 1996
Caspase 8	MN/Neuropil	Pro-apoptotic/Degeneration	Friedlander, 2003
GFAP	Neuropil	Survival/Regeneration	Hurley et al., 2003; Jones et al., 1997; Laskawi & Wolff, 1996; Graeber & Kreutzberg, 1988
IFN γ	Neuropil	Pro-inflammatory/Degeneration	Raivich et al., 1998
TNF α	Neuropil	Pro-inflammatory/Degeneration	Raivich et al., 1998

Table 2

Published murine complementary DNA sequences used to design RT-PCR primers.

Gene	Primer Sequence	Accession number	T _A (°C)	T _m (°C)	Amplicon Length (bp)
βII Tubulin	F 5'-TGCAACAAATATGTACCTC-3' R 5'-GAATGGTCCCGACCTC-3'	<u>NM009450</u>	54	84	79
GAP-43	F 5'-CCTAAGGAAAGTGCCCGACAG-3' R 5'-CAGGTGGGGCAACGTGG-3'	<u>BC028288</u>	54	84	102
Hn1	F 5'-TTGGGAACCTGCACAGCGTGAGC-3' R 5'-GCAGGAAAGACAGCGGTACAT-3'	<u>U90123</u>	55	80	90
CX3CL1	F 5'-GCCATGTTTGTGTTACAGAG-3' R 5'-TGGCACACAGGACGTATGAGTT-3'	<u>BC006650</u>	55	87	117
BDNF	F 5'-CCATAAGGACGCGGACTTG-3' R 5'-GACATGTTTGGCGCATCCA-3'	<u>X55573</u>	54	82	120
GFAP	F 5'-AGAACAACTGGCTGCGTAT-3' R 5'-TCTCTCTCCAGCGATTTC-3'	<u>AF332062</u>	55	84	93
Caspase 8	F 5'-CGGTGAAGAACTGCGTTTC-3' R 5'-CGCCAGTCAGGATGCTAAG-3'	<u>AF067834</u>	53	81	123
PACAP	F 5'-GGGGAATTCTGAGGTAATGAC-3' R 5'-GACAAATGCCCACTTGAGTTAG-3'	<u>D14716</u>	56	84	109
TNFα	F 5'-ATTCACTGGAGCCTCGAATG-3' R 5'-AGGAAGGCTGAGATCTTATC-3'	<u>X02611</u>	53	82	102
IFNγ	F 5'-GCCATCAGCAACACATAAGC-3' R 5'-GTTGACCTCAAACTTGGCAATA-3'	<u>K00083</u>	53	80	111
GAPDH	F 5'-GAACATCATCCCTGCATCCA-3' R 5'-CCAGTGAGCTTCCCGTTCA-3'	<u>M32599</u>	50-57	81	78