The role of SHP -1 in deafferentation-induced neuronal death in the murine auditory brainstem

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THE ROLE OF SHP-1 IN DEAFFERENTATION-INDUCED NEURONAL DEATH IN THE MURINE AUDITORY BRAINSTEM

by

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for the degree of

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Deafferentation is the loss of sensory input from a portion of the body to the central nervous system (CNS), usually caused by interruption of the peripheral sensory fibers. Removal of afferent input, or changes in afferent activity, has been shown to have a dramatic impact on neuronal structure and function during a “critical period” of development in the auditory system. Inner ear cochlear ablation results in the death of anteroventral cochlear nucleus (AVCN) neurons from birth to approximately postnatal day 14 (P14) in the murine auditory brainstem. However, the underlying physiological mechanisms that define the critical period of deafferentation-induced neuronal death are still incompletely understood. Glial cells, especially microglia, have received much attention as being important CNS immunoregulatory cells and active participators in the pathogenesis of neuron damage during injury, infection, and neurodegenerative diseases. Increasing evidence has suggested that once activated, microglia are capable of inducing neuronal death by secreting neurotoxic substances. Whether microglial activation helps determine the critical period for deafferentation-induced neuronal death in the auditory brainstem remains to be elucidated. Activation of glia often involves phosphorylation events controlled by the antagonistic actions of protein tyrosine kinases and protein tyrosine phosphatases. SHP-1 is a cytoplasmic protein tyrosine phosphatase predominantly expressed in hematopoietic cells and has been implicated as a negative regulator in cytokine signaling as well as in the proliferation and differentiation of various cell types, including glia. These studies utilize the SHP-1 deficient motheaten (me/me) mouse model to investigate the role of SHP-1 and activated microglia in deafferentation-induced AVCN neuronal death. In vivo studies demonstrate that following cochlear ablation 1) there is an extended critical period (beyond P14) of neuron loss concomitant with exaggerated microglial activation in the me/me AVCN; and 2) a down-regulated expression of the anti-inflammatory cytokines IL-4 and IL-10 in combination with an up-regulated expression of the pro-inflammatory cytokine IL-1β in the injured me/me hindbrain post-ablation. In vitro studies corroborate the deleterious role of activated me/me microglia in contributing to neuronal death by demonstrating an increased release of neurocytotoxic substances by cultured me/me microglial cells.
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INTRODUCTION

Loss or impairment of peripheral sensory input from a portion of the body to the central nervous system (CNS) is referred to as deafferentation. Removal of presynaptic afferent input or changes in afferent activity have been shown to have a striking impact on the structure and function of postsynaptic neurons during a "critical period" of development in a number of different systems (Hubel and Wiesel, 1970; Lasiter and Kachele, 1990; Brunjes, 1994; Baldi et al., 2000; Mostafapour et al., 2000). In the auditory system, loss of hair cells, the peripheral auditory receptors, by either mechanical lesions of the cochlea or biochemical lesions of the hair cells by ototoxic drugs, can profoundly influence the morphology, functional performance and survival of developing cochlear nucleus (CN) neurons in the central auditory system. Although the effect of changes in neural input during these critical periods has been well characterized, the underlying biological and physiological mechanisms remain to be fully elucidated.

Changes in Auditory Brainstem Neurons following Deafferentation

The auditory brainstem of birds and mammals is an excellent model in which to study the effects of afferent deprivation and the biological basis of the critical period. Neurons within the chicken cochlear nucleus n. Magnocellularis (NM) receive a single excitatory input from the eighth cranial nerve. When this afferent activity is removed, 25-30% of NM neurons die within the first few days of activity loss (Born and Rubel, 1985; Born and Rubel, 1988; Born et al., 1991). Some of the early changes that occur in NM neurons in response to deafferentation include decreases in transcription and protein...
synthesis (Steward and Rubel, 1985; Garden et al., 1994; Kelley et al., 1997) and increases in intracellular calcium (Zirpel et al., 1995; Zirpel and Rubel, 1996). The latter is thought to activate the transcription factor calcium/cAMP response element-binding protein (CREB) (Zirpel et al., 2000), and CREB phosphorylation has been shown to be important for neuronal survival in a variety of different cells (Hu et al., 1999; Walton et al., 1999; Walton and Dragunow, 2000). In contrast, neurons that are destined to die following deafferentation show polyribosomal degradation among various other intracellular changes (Garden et al., 1994; Hartlage-Rubsamen and Rubel, 1996).

Unlike birds, the switch from afferent-dependent to afferent-independent neuronal survival in the CN of the mammalian auditory brainstem is temporally related to hearing onset. Recent work has indicated that in both gerbil and mouse, there is an extremely narrow window during which CN neurons are susceptible to deafferentation-induced cell death (Tierney et al., 1997; Mostafapour et al., 2000). Cochlear removal at or before P7 results in 50-80% neuron loss within the cochlear nucleus, while at P9-14 or later, no significant neuronal death occurs.

Synaptic maturation during development may play a key role in defining this critical period of neuronal susceptibility within the mammalian auditory system. However, other intrinsic biological properties of neurons also appear to be important. Bcl-2 has been shown to be an effector of cell survival as AVCN neurons switch from afferent-dependent to afferent-independent mechanisms because bcl-2 knockout mice remain susceptible to death following cochlear removal through P21 (Mostafapour et al., 2000) and bcl-2 overexpression eliminates neuronal death within AVCN in very young mice (Mostafapour et al., 2002). Another apoptotic mediator, caspase-3 has also been
implicated in the cell death pathways of CN neurons following cochlear ablation (Mostafapour et al., 2000). It has become increasingly clear that a complex series of events determines whether a neuron will live or die following deafferentation. Among these, the role of glial cells should not be overlooked since interactive communications between neurons and glia exist, particularly in response to injury.

**Glial Response to afferent injury of the auditory brainstem**

Besides neural injury, CNS lesion also leads to a cascade of events resulting in glial activation (Kreutzberg et al., 1989). Two major types of glia, astrocytes and microglia, play an important role in the repair of injured tissue and these cells have been shown to respond to injury in ways that are both supportive and inhibitory of neuronal survival and regeneration. In particular, in response to injury, quiescent astrocytes become hyperplastic and hypertrophied, proliferating to fill the space left by degenerating neurons (Reier and Houle, 1988). Microglia, considered to be the immune cells of the brain, are also extremely sensitive to CNS damage (Gehrmann et al., 1995; Hanisch, 2002) and act as phagocytes (Aldskogius and Kozlova, 1998). The microglial response to injury is often characterized as a “double-edged sword” depending on the magnitude of the reaction and the type of stimulus; microglia can either contribute to host defense and repair, or intensify the CNS damage.

Cochlear removal in the avian results in astrocyte proliferation and hypertrophy within the deafferented brainstem nucleus NM (Canady and Rubel, 1992; Rubel and MacDonald, 1992; Lurie and Rubel, 1994). Previous studies in our laboratory have

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found that immunoreactivity for the protein tyrosine phosphatase SHP-1 increases in a subset of astrocytes following cochlear removal in chicken and these SHP-1+ astrocytes do not appear to enter the cell cycle (Lurie et al., 2000). This suggests that SHP-1 negatively regulates astrocytic cell division. Later studies in vitro also support this hypothesis. Addition of the protein tyrosine phosphatase inhibitor sodium orthovanadate (Na$_3$VO$_4$) to mixed neural/glial cultures of the avian brainstem results in a dose-dependent increase in astrocyte proliferation (Sorbel et al., 2002).

To date, no studies have documented the microglial response in the auditory brainstem following cochlear removal in either avian or mammalian models. However, other types of afferent injury such as axotomy of peripheral facial nerves, leads to a massive upregulation of SHP-1 in activated microglia and astrocytes (Horvat et al., 2001). Moreover, deafferentation by axonal interruption of motorneurons results in microglial proliferation and expression of new proteins such as vimentin or major histocompatibility complex (MHC) antigens (Kreutzberg et al., 1989), which may subsequently elicit immune functions of microglia.

Microglia And CNS Immunoregulation after Injury

Although the main function of microglia is to serve as immune surveillance for the CNS, they have increasingly received more attention as their activation and/or dysregulation is involved in most neurological disorders (Aloisi, 2001; Nelson et al., 2002; Schwartz, 2003). One of the most remarkable properties of microglia is their ability to respond to endogenous or exogenous signals and become activated for the
purposes of tissue repair or the induction of immune responses (Aloisi, 2001).

Expression of a large array of surface receptors on microglia, including pattern recognition receptors, complement receptors, cytokine/chemokine receptors, etc., enables these cells to exert their immunoregulating functions in response to a variety of infectious and inflammatory stimuli (Aloisi, 2001). Significantly, microglia are thought to be both a source and target of immunoregulatory cytokines (Hanisch, 2002), and many of these immune mediators are involved in the initiation of different signaling pathways that could lead to glial activation and neural/glial damage. Microglial-induced synthesis of cytokines (such as the anti-inflammatory cytokines IL-10, IL-1 receptor antagonist and TGF-β, as well as the pro-inflammatory cytokines IL-1, IL-6, IL-12 and TNF-α) has been demonstrated in vivo and in vitro as a response to trauma and infection or as a hallmark of neuropathological changes (Hanisch, 2002). The complex cytokine network that controls the CNS immune system can be disturbed and augmented by CNS injury, which may in turn potentiate microglial activation resulting in a deleterious interaction between neurons and glia.

Microglial Induction of Neuronal Death

Accumulating evidence suggests that activated microglia can induce neurons to die both in vivo and in vitro through a variety of mechanisms. It has been suggested that the release of reactive oxygen species nitric oxide (NO) (Loihl and Murphy, 1998; Liberatore et al., 1999; Bal-Price and Brown, 2001), pro-inflammatory cytokines TNF-α and IL-1β (Chao et al., 1995; Viviani et al., 1998) and the excitatory amino acid
glutamate (Barger and Basile, 2001) by activated microglia all contribute to neuronal death following injury and disease. In particular, the release of iNOS (inducible nitric oxide synthase)-derived NO is thought to inhibit neuronal respiration, causing glutamate release, and the subsequent excitotoxic death of neurons (Bal-Price and Brown, 2001). In addition, activated microglia themselves secrete glutamate (Barger and Basile, 2001) and may also induce glutamate release from astrocytes via NO induction (Bal-Price and Brown, 2001). Activated microglia show increased induction of iNOS and TNF-α through activation of the extracellular signal-regulated kinase (ERK) and p38 MAP kinase subgroups of MAP kinase (MAPK) (Bhat et al., 1998). Inhibition of microglial activation and proliferation by the tetracycline derivative, minocycline, results in increased neuronal survival in neural/glial cultures of rat brain via inhibition of NO and IL-1β release (Tikka et al., 2001). In vivo studies also suggest that microglial activation in the parkinsonian substantia nigra can be inhibited by minocycline treatment and a large number of nigral neurons rescued from degeneration (He et al., 2001).

It is not known whether there is microglial activation in response to cochlear removal in the auditory brainstem, nor whether such activation could contribute to deafferentation-induced cell death or the timing of the critical period. Since SHP-1 immunoreactivity is also upregulated in microglia after deafferentation (Kreutzberg et al., 1989; Horvat et al., 2001; Wishcamper et al., 2003), SHP-1 could be a component of the signaling pathways involved in the modulation of microglial activation following injury. Significantly, SHP-1 has been shown to limit ERK activity (Shibasaki et al., 2001) and may be indirectly involved in the down-regulation of p38 MAP kinase (Kumar et al.,
1999). This raises the possibility that SHP-1 could negatively regulate microglial activation and influence microglia-induced neuronal death.

The Protein Tyrosine Phosphatase SHP-1

SHP-1 (also known as PTP1C, SHPTP-1, and SHP) is a cytoplasmic protein tyrosine phosphatase containing two src homology 2 (SH2) domains. It was first isolated from human breast cancer cells (Shen et al., 1991) and has been most thoroughly characterized in hematopoietic cells, where it is expressed at high levels and plays important physiological roles (Plutzky et al., 1992; Yi et al., 1993; David et al., 1995; Krautwald et al., 1996). In the CNS, SHP-1 is involved in neuronal differentiation (Mizuno et al., 1997) and somatostatin receptor sst2 growth inhibitory signaling via direct action on neuronal nitric oxide synthase (nNOS) (Lopez et al., 2001). Moreover, SHP-1 is thought to modulate cytokine activity by limiting activation through the erythropoietin (Epo), interleukin 3 (IL-3) and type I interferon (IFN) receptor pathways by binding to and dephosphorylating JAK (Janus kinase) and STAT (signal transducers and activators of transcription) proteins (Massa and Wu, 1996) and by controlling expression of IFN-inducible genes of the transcription factor NF-κB in astrocytes (Massa and Wu, 1998).

Motheaten (me/me) Mice

The animal model used in the present studies to examine neural/glial interactions and glial factors contributing to neuronal death in the auditory brainstem following afferent injury is a mouse mutant that lacks functional SHP-1 protein, called motheaten.
(me/me) mice. This mutation disrupts the SHP-1 gene on mouse chromosome 6 and is caused by a deletion of a cytidine residue which generates a frameshift and premature truncation of SHP-1 mRNA such that me/me mice are effectively null for SHP-1 protein (Kozlowski et al., 1993; Shultz et al., 1993; Tsui et al., 1993). Loss of SHP-1 leads to many hematopoietic abnormalities including an overproduction of macrophages and neutrophils (Shultz et al., 1984), a severe reduction in the number of early B cell progenitors (Schweitzer and Shultz, 1988; Shultz, 1988) and abnormal mast cell development (Shultz et al., 1997). The mutation confers an easily recognizable phenotype characterized by patchy dermatitis, reduced body size and weight, progressive arthritis, limb necrosis, and hemorrhagic pneumonitis, the latter of which is the primary cause of death (Shultz et al., 1993). The me/me mice die at a mean age of 3 weeks due to intra-alveolar hemorrhage as a consequence of infiltration of macrophages and neutrophils (Shultz et al., 1984). However, these animals remain healthy and viable up until a few days before death.
SPECIFIC AIMS

Specific Aim I. Precisely define the temporal relationship between neuronal susceptibility and glial activation following afferent injury

- Quantify the time course and magnitude of the neuronal, astrocyte and microglial response within AVCN following cochlear ablation in wild-type and me/me mice

Specific Aim II. Determine the role of SHP-1 in regulating inflammatory mediators involved in deafferentation-induced brain injury

- Establish the pro- and anti-inflammatory cytokine profiles of wild-type and me/me brains before and after deafferentation

Specific Aim III. Ascertain whether loss of SHP-1 alters the function of activated microglia implicated in inducing neurotoxicity

- Identify whether activated me/me microglia release increased amounts of the neurotoxic substances nitric oxide (NO), tumor necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β) compared to wild-type microglia in vitro
References


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CHAPTER ONE

Cochlear ablation in mice lacking SHP-1 results in an extended period of cell death of anteroventral cochlear nucleus neurons

Abstract

Cochlear ablation results in the death of anteroventral cochlear nucleus (AVCN) neurons from birth to approximately postnatal day 14 (P14) in the murine brainstem. It is not known whether microglial activation contributes to AVCN neuronal death following deafferentation. In order to determine whether microglial activation helps to define the period of neuronal susceptibility within AVCN, we performed unilateral cochlear ablation on mice lacking the protein tyrosine phosphatase SHP-1' (me/me). These mice have been shown to have an exaggerated microglial response following ischemic injury. In the present study, the glial and neuronal response to deafferentation within AVCN was examined in wild-type and me/me mice at P5, P14, and P21. Lack of SHP-1 results in robust microglial but not astrocyte activation within the ablated P14 me/me AVCN. These mice also exhibit approximately 28% neuronal death at P14, a time when normal wild-type littermate controls show little cell death. Glial activation and neuronal loss at P5 and P21 were similar between the two phenotypes, suggesting a role of activated microglia in inducing neuronal death beyond P14 but not P21. These results indicate that activated microglia may participate in determining whether neurons in AVCN live or die following deafferentation.
1. Introduction

Removal of afferent input or changes in afferent activity have been shown to have a dramatic impact on the development of mature neuronal structure and function during a "critical period" of development in a number of different systems (Hubel and Wiesel, 1970; Brunjes, 1994; Mostafapour et al., 2000). Within the auditory system, the sensitivity of cochlear nucleus neurons to removal of afferent input decreases over time. Born and Rubel (1985) first described this change from afferent-dependent to afferent-independent survival in the avian cochlear nucleus and studies in mammals have suggested that this transition to afferent-independent survival is temporally related to hearing onset within the auditory brainstem (Trane, 1982; Nordeen et al., 1983; Webster, 1983; Webster, 1988; Hashisaki and Rubel, 1989; Kitzes et al., 1995). Recent work has indicated that in both the gerbil and the mouse, there is an extremely narrow window during which the anteroventral cochlear nucleus (AVCN) neurons are susceptible to deafferentation-induced cell death (Tierney et al., 1997; Mostafapour et al., 2000). Cochlear removal at or before postnatal day 7 (P7) results in 50-88% neuron loss within the mammalian cochlear nucleus, while the same procedure performed at P14 or later results in no significant cell death.

Although the effect of changes in neural input during this critical period has been well characterized, the underlying physiological mechanisms that define critical periods are not well understood. Within AVCN, the transition from afferent-dependent to afferent-independent neuronal survival is thought to be largely regulated by the intrinsic properties of neurons including the apoptotic mediators Bcl-2 and caspase-3.
(Mostafapour et al., 2000; Mostafapour et al., 2002). However, little attention has focused on the role that glial cells may play in defining critical periods. Accumulating evidence suggests that activated microglia can kill neurons by secreting neurotoxic substances such as nitric oxide, TNF-α and/or glutamate in several CNS disorders including inflammatory, infectious, traumatic, and neurodegenerative diseases (Bhat et al., 1998; Bal-Price and Brown, 2001). Collectively, these results suggest that activated microglia could induce death in a population of neurons that have been weakened by deafferentation, but might otherwise survive the deafferentation injury.

Previous studies in our laboratory have demonstrated that the protein tyrosine phosphatase SHP-1 is upregulated in glial cells following injury (Wishcamper et al., 2003). Importantly, mice lacking SHP-1 (motheaten) have an exaggerated microglial response following ischemic injury to the brain (Wishcamper, Brooks, and Lurie, Personal Observation). SHP-1 has been implicated as a negative regulator in a number of different signaling pathways including the direct modulation of the JAK/STAT pathway (Finbloom and Larner, 1995; Bousquet et al., 1999; Hilton, 1999; Starr and Hilton, 1999) and indirect modulation of ERK and p38 MAP kinases (Kumar et al., 1999; Shibasaki et al., 2001). Both the ERK and p38 MAP kinase signaling cascades are involved in microglial activation (Bhat et al., 1998) and activated microglia may play a role in determining whether neurons live or die following deafferentation.

In the present study, glial activation and neuronal cell loss within the auditory brainstem in response to cochlear ablation is compared between motheaten mice and wild-type controls in order to evaluate whether the microglial response plays a role in determining neuronal survival within AVCN following deafferentation. P5, P14, and P21
me/me and wild-type mice received a unilateral cochlear ablation and were allowed to survive for 2, 4 or 6 days. Microglial and astrocyte activation, and neuronal cell loss, were examined within AVCN at 4 days after injury. Confirming the results of previous studies (Mostafapour et al., 2000), there is approximately 50% neuronal cell loss within the P5 AVCN 4 days following cochlear ablation in wild-type as well as me/me mice. However, in contrast to their normal littermates, motheaten mice continue to demonstrate significant amounts of deafferentation-induced neuronal death at P14, although by P21, there is no difference in neuronal loss between wild-type and me/me mice. Importantly, there is increased microglial activation following deafferentation in P14 me/me mice compared to wild-type, and this microglial activation may induce death in AVCN neurons that would otherwise survive following deafferentation at this time point. These findings suggest that activated microglia play a role in determining the critical period of neuronal susceptibility following cochlear ablation in the murine auditory brainstem.

2. Materials and methods

2.1. Animals

The founding mice (C3FeLe.B6-a/a-Hcph<sup>me</sup>/a) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice used in this study were propagated by +/me matings, producing me/me mutants, and +/+ and +/me wild-type control littermates. Mice were maintained in microisolator units and kept in the University of Montana specific pathogen free (SPF) animal facility. Mice were given free access to food and water and
were placed in a 12-hour light/dark cycle, constant temperature and humid environment. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Montana Institutional Animal Care and Use Committee.

2.2. Mouse Genotyping

Polymerase chain reaction (PCR) amplifying a 426-bp fragment, which encompasses the site of the me/me mutation, was utilized to genotype the mice. The PCR reaction was carried out using forward (5'-CGCTGCTGCTCATGTATCTC-3') and reverse (5'-GCCTACAGAGGAAGAGGCT-3') primers (Operon Technologies, Alameda, CA) and the PCR Master Mix (Promega Corporation, Madison, WI). At weaning, 2-3 mm tail samples were digested in 100 mM NaOH at 65°C for 3 hours. Digestion was terminated by adding 0.75 M Tris-HCl (pH 7.5) prior to centrifugation. DNA was subjected to 35 cycles of the following PCR conditions: 30 seconds at 95°C, 1 minute at 56°C, and 1 minute at 72°C. The amplified PCR products were digested for 1 hour with 1.0 µl Taq I enzyme (New England BioLabs, Beverly, MA) at 65°C and separated by 1.2% agarose gel electrophoresis in 1 x TAE at 120 V for 100 minutes. PCR products were stained with ethidium bromide and visualized by UV light.

2.3. Unilateral Cochlear Ablation
P5, P14, and P21 (n = 4-5/age group) mice were anesthetized with 2,2,2-
tribromoethanol (375 mg/kg, i.p.) and received a unilateral cochlear ablation. A separate
group of both wild-type and moheteen P21 control mice (n = 3) were anesthetized and
allowed to recover. Surgeries were performed as previously described with some
modifications (Mostafapour et al., 2000). Briefly, for P5 animals whose ear canals have
not developed, a horizontal incision was made 2-3 mm inferior to the pinna of the right
ear using fine surgical scissors. The middle ear mesenchyme was cut and the ossicles
were removed, exposing the basal turn of the cochlea. Using a 27-gauge needle with a
blunt tip connected to a vacuum pump, the bony wall of the cochlea was penetrated and
the contents aspirated. The skin incision was closed with cyanoacrylic glue. For animals
P14 and older, the right ear pinna inferior region was shaved and an incision was made
on the inferior side of the pinna. The ear canal was enlarged by making further incision at
its entry, and the tympanic membrane was punctured and the ossicles were removed
using a pair of fine forceps. The basal turn of the cochlea was visualized and destroyed
with a 27-gauge needle followed by aspiration of its contents. The incision was closed
with cyanoacrylic glue. All surgical procedures were performed on a warming pad and
animals were given oxygen inhalation after surgery on a heating element until they were
fully awake. Following the unilateral cochlea ablation, mice were allowed to survive for
2, 4, or 6 days. Non-surgical controls were sacrificed 4 days following anesthesia.

2.4. Immunohistochemistry
Immunohistochemistry was performed as previously described (Lurie and Durham, 2000; Lurie et al., 2000). At 2, 4, or 6 days after cochlear ablation, mice were deeply anesthetized with tribromoethanol and transcardially perfused with phosphate-buffered saline (PBS) for 10 minutes followed by 10 minutes with 4% Na-periodate-lysine-paraformaldehyde (PLP) fixative. Brains were removed and post-fixed in PLP fixative at 4°C for an additional 24 hours. The tissue were then rinsed several times in PBS over a 4-6 hour period and placed in a Shandon Citadel™ tissue processor (Shandon Scientific Limited, Runcorn, Cheshire, England) to be dehydrated in increasing ethanol (70%, 95%, and then 100%) and embedded in paraffin. Ten-micron thick coronal sections of the brainstem containing the AVCN were cut and collected, and a 1 in 6 series mounted onto Superfrost plus micro slides (VWR Scientific, West Chester, PA). The first series of tissue sections were stained with either thionin or hematoxylin and eosin (Shandon, Pittsburgh, PA) to identify neurons. Subsequent series were processed for the appropriate antibodies using the avidin-biotin-peroxidase complex (ABC) method with reagents from Vectastain kits (Vector Laboratories, Burlingame, CA) as previously described (Lurie et al., 2000). Briefly, sections were deparaffinized, rinsed in 0.1 M Tris buffer (pH 7.4) and then blocked with 4% normal goat serum (for polyclonal antibodies) or 4% normal horse serum (for monoclonal antibodies) for 20 minutes. This and all other immunohistochemistry reagents (except for ABC reagent) were prepared in 0.5% bovine serum albumin (BSA)/0.1% sodium azide in Tris buffer. Sections were incubated overnight in anti-glial fibrillary acidic protein (GFAP; polyclonal, 1: 2000; Dako, Carpinteria, CA) or for 2 days in anti-F4/80 (monoclonal, 1:75; Serotec, Raleigh, NC) at 4°C in a humidified chamber. Sections were then washed in Tris, incubated in
biotinylated goat anti-rabbit or horse anti-mouse serum (Vector Laboratories) at 1:400 in
the humidified chamber for 1 hour, washed in Tris, then incubated in the avidin-biotin
complex (Vectastain ABC Elite kit, Vector Laboratories) for 1 hour. The chromogen used
was diaminobenzidine (0.25 mg/ml; Sigma, St. Louis, MO) prepared with 0.1 M
imidazole and 30% hydrogen peroxide in Tris buffer. Sections were then rinsed in water,
dehydrated, and coverslipped with DPX (BDH Laboratory Supplies, Poole, England).

2.5. Image Analysis and Statistics

All slides were coded and analysis of tissue sections was performed by an
individual blinded as to phenotype. Every stained tissue section from the 1 in 6 series was
examined using standard light microscopy with a Nikon Eclipse E800 microscope.
Thionin or hematoxylin and eosin staining was used to determine the borders of AVCN.
The boundary between the AVCN and the posteroventral cochlear nucleus (PVCN) was
defined by the appearance of the granular layer of the cochlear nucleus (GrC).

Image analysis for GFAP and F4/80 antibody immunostained sections was
performed with the NIH Image Analysis V1.61 software as previously described
(Wishcamper et al., 2001). A Powermac 8600/200 computer connected to a Nikon E800
microscope by a Cohu black & white video camera was used. Sections chosen from the
rostral, middle, and caudal portions of AVCN on both the ablated and uninjured sides
were analyzed for staining density. The boundary of AVCN was outlined and a fixed
threshold was set. The sum of the stained area (\(\mu m^2\)), which reached the threshold within
the outlined region, was divided by the sum of the entire AVCN area (\(\mu m^2\)) from the
three representative sections, and the ratio of staining within each side of the brainstem was obtained.

Neuron counts were performed using a 40 × objective and images were captured with a Nikon digital camera DXM1200 connected to a CG computer. All normal appearing neurons within AVCN in a given section were counted on both the ipsilateral and contralateral sides of the brainstem. The criteria for a neuron to be counted were a well-defined cytoplasm and nuclear membrane and clear nucleoli within the nucleus. The total AVCN neuron number in each side of the brainstem was defined as: Total number of neurons per side = sum of No. of neurons in all counted sections × 6. The contralateral AVCN was used as a within-animal control. Percentage of AVCN neuron loss on the injured side was calculated as: 100 × [(Total No. of neurons on control side – Total No. of neurons on ablated side)/Total No. of neurons on control side]. “Double counting” was not considered because the nucleolus is small compared to section thickness (Mostafapour et al., 2000).

Statistical analysis was performed using the non-parametric Mann-Whitney U-test. The data are presented as the mean ± standard error of the mean (SEM), p < 0.05 was considered significant.

2.6. Photography

Digital images were captured with a Nikon E800 microscope attached to a Cohu black & white video camera by NIH Image Analysis V1.61 software on a Powermac
8600/200 computer. Captured images were processed with Adobe Photoshop 5.5 to provide consistent size; and text, magnification bars and arrows were added.

3. Results

3.1. Microglial activation following deafferentation is upregulated in me/me mice

To elucidate the role of SHP-1 following deafferentation, the neuronal and glial response to cochlear ablation was examined in mice lacking functional SHP-1 (me/me). A unilateral cochlear ablation was performed at P5, P14, and P21 in me/me and wild-type littermates. Animals were allowed to recover for 4 days and the AVCN was examined for neuronal loss and glial activation. Microglial activation was quantified by immunolabeling with the F4/80 antibody which labels activated microglia, and astrocyte activation was quantified using an antibody against GFAP. Neurons were counted within the deafferented AVCN and compared to the uninjured side to determine neuronal loss following cochlear ablation.

At P5, microglial activation increases two-fold in both the wild-type and me/me deafferented AVCN compared to the unablated side (Fig. 1) and is not significantly different between the two phenotypes (Fig. 1 and Table 1). However, me/me mice at P14 display a very prominent, significant increase in microglial activation within the deafferented AVCN compared to age-matched wild-type mice (Fig. 2 A-D). Although, some microglial activation following deafferentation does occur in P14 wild-type mice,
Figure 1. F4/80 immunoreactivity in AVCN following deafferentation
Figure 1: Percent of F4/80 immunoreactivity within the control and ablated sides of AVCN in wild-type and me/me mice after deafferentation. At P5, there is a similar but not significant increase in density of F4/80 immunostaining in the ablated side compared to the control side of AVCN in both me/me (3.8 ± 0.9% vs. 2.1 ± 0.5%; n = 4) and wild-type littermates (3.7 ± 1.1% vs. 1.6 ± 0.3%; n = 4). When cochlear ablation is performed at P14, me/me mice demonstrate a significant upregulation of F4/80 immunoreactivity in the ablated AVCN compared to the ablated wild-type AVCN (5.0 ± 1.2% vs. 2.2 ± 0.7%; n = 4; p < 0.05). In older animals at P21, there is a higher density of F4/80 staining in both the control and ablated AVCN in me/me mice compared to wild-type. Note that the basal level of F4/80 immunoreactivity in me/me mice on the control side (2.6 ± 1.4%) is significantly higher than that in wild-type mice (0.2 ± 0.06%). Wt-ctr, wild-type control side; wt-abl, wild-type ablated side; me-ctr, me/me control side; me-abl, me/me ablated side. Error bars indicate standard error of the mean. Asterisk, Mann-Whitney U-test, p < 0.05.
Table 1. Quantification of F4/80 immunoreactivity

<table>
<thead>
<tr>
<th>Age at surgery</th>
<th>Phenotype</th>
<th>n</th>
<th>Percent of F4/80 staining (mean ± SEM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>Ablated</td>
</tr>
<tr>
<td>P5</td>
<td>Wild-type</td>
<td>4</td>
<td>1.56 ± 0.29</td>
<td>3.66 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>4</td>
<td>2.08 ± 0.51</td>
<td>3.81 ± 0.89</td>
</tr>
<tr>
<td>P14</td>
<td>Wild-type</td>
<td>4</td>
<td>1.09 ± 0.18</td>
<td>2.20 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>4</td>
<td>1.81 ± 0.51</td>
<td>5.01 ± 1.19 *</td>
</tr>
<tr>
<td>P21</td>
<td>Wild-type</td>
<td>4</td>
<td>0.19 ± 0.06</td>
<td>2.09 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>5</td>
<td>2.57 ± 1.39 *</td>
<td>3.65 ± 1.14</td>
</tr>
</tbody>
</table>

Table 1. Percent of AVCN immunopositive for F4/80 staining in wild-type vs. motheaten mice 4 days following cochlear ablation. The percent AVCN immunopositive for F4/80 staining was obtained by dividing the sum of the stained area (μm²) that reached threshold within AVCN by the sum of the area of AVCN (μm²) from three representative sections. Comparisons are made between age-matched wild-type and me/me mice. *Mann-Whitney U-test, p < 0.05.
Figure 2. F4/80+ microglia in P14 wild-type vs. me/me AVCN
Figure 2: F4/80 immunostaining in AVCN of wild-type vs. motheaten mice at P14, 4 days following cochlear ablation. Wild-type AVCN shows small numbers of F4/80+ microglia within the control side (A) and a small increase in the ablated side (B), (arrows). However, there is a higher basal level of F4/80+ microglia in the control side of the me/me AVCN (C), and a significantly upregulated expression on the ablated side (D), (arrows). Notice that me/me microglia are transformed from the ramified, inactivated state (C), (arrow heads), to the amoeboid, activated state (D), (arrow heads), after cochlear ablation. Scale bar = 20 μm.
quantification of the F4/80 immunoreactivity reveals that the deafferented me/me AVCN contains twice as much F4/80 immunolabeling compared to the deafferented wild-type AVCN at this age (Fig. 1 and Table 1). In addition to increased F4/80 expression, me/me microglial cells at P14 appear to transform from the ramified, resting state observed in the uninjured AVCN (Fig. 2C) to the amoeboid, activated state found in the deafferented AVCN (Fig. 2D). In contrast to the me/me mice, far fewer amoeboid-like microglia are seen within the deafferented wild-type AVCN (Fig. 2B).

At P21, the microglial response to deafferentation in wild-type mice is similar to that observed at P14 in wild-type mice (Fig. 1 and Table 1). In contrast, P21 me/me mice demonstrate less F4/80 immunostaining within the deafferented AVCN compared to me/me P14 animals although there is still an increased microglial response in the injured P21 me/me AVCN compared to the control side.

To determine whether loss of SHP-1 results in an increase in the magnitude of microglial activation versus an earlier peak response, P5 and P14 wild-type and me/me mice received a unilateral cochlear ablation and were allowed to survive for 2, 4 or 6 days. The brains were then processed for F4/80 immunohistochemistry. At P5, microglial activation is similar in wild-type and me/me mice within the ablated AVCN at all time points following cochlear ablation. As shown in Figure 3A, both the time course and the magnitude of the microglial response is similar in both phenotypes with maximal activation occurring at 6 days following deafferentation. However, at P14, motheaten mice have significantly higher levels of microglial activation 4 days after cochlear ablation compared to wild-type (Fig. 3B). Day 4 after cochlear ablation appears to be an
Figure 3. Time course of microglial activation in P5 & P14 AVCN
Figure 3: Time course of F4/80 immunoreactivity at 2, 4, and 6 days following cochlear ablation in the deafferented AVCN of P5 and P14 wild-type and me/me mice. A. Both the time course and magnitude of the F4/80 immunolabeling are similar in P5 wild-type and me/me mice. B. P14 me/me display a significantly higher level of F4/80 immunoreactivity compared to the wild-type at 4 days following cochlear ablation (5 ± 1.2% vs. 2.2 ± 0.7%, n =4; p < 0.05), but similar F4/80 staining is observed at 2 and 6 days in animals of both phenotypes. Error bars indicate standard error of the mean. Asterisk, Mann-Whitney U-test, p < 0.05.
important time point at P14 because the microglial response is similar between me/me and wild-type mice at days 2 and day 6 following deafferentation (Fig. 3B).

3.2. Me/me mice lacking SHP-1 are more vulnerable to deafferentation-induced neuronal death

Many studies now indicate that activated microglia are able to kill neurons (Klegeris and McGeer, 2000; Bal-Price and Brown, 2001; He et al., 2002; Park et al., 2002) and the large microglial response observed in P14 me/me mice following cochlear ablation may affect neuronal survival in AVCN following deafferentation. To further elucidate the relationship between activated microglia and neuronal survival, P5, P14, and P21 me/me and wild-type littermates received a unilateral cochlear ablation. Animals were allowed to recover for 4 days and the AVCN was then stained with either thionin or hematoxylin and eosin (H&E). Neurons displaying a normal morphology (including a well-defined cytoplasm and nuclear outlines, and a clearly visible nucleus with one or more nucleoli) were considered to be healthy cells and neuron counts were obtained for both the ablated and unablated AVCN.

Figure 4A-D demonstrate that in P5 mice, cochlear ablation results in a large amount of neuronal cell loss within AVCN in both me/me and wild-type mice. Neuron counts reveal that 54% of neurons within AVCN die following deafferentation in wild-type mice while 52% are lost in the deafferented me/me AVCN compared to the uninjured contralateral AVCN (Fig. 5 and Table 2). These results are consistent with
Figure 4. H&E staining of neurons in P5 & P14 AVCN

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Figure 4: H&E staining of AVCN neurons in P5 and P14 wild-type and me/me mice after unilateral cochlear ablation and 4 day survival. Arrows point to some of the surviving neurons. At P5, approximately half numbers of neurons die within the ablated AVCN in both wild-type (B) and me/me (D) mice compared to their respective control sides (A and C), (arrows). Cochlear ablation at P14 results in a small amount of neuronal loss in the ablated wild-type AVCN (F) compared to the control side (E). In contrast, deafferentation in P14 me/me mice results in a significant amount of neuronal death within the ablated AVCN (H) compared to the control side (G), (arrows). This neuronal loss is less than that observed in both phenotypes at P5, but significantly higher than that seen in the P14 wild-type. Scale bar = 20 μm.
Figure 5. Percent AVCN neuron loss after cochlear ablation.
Figure 5: Percent neuron loss in AVCN in wild-type vs. motheaten mice at P5, P14 and P21, 4 days following unilateral cochlear ablation. At P5, approximately 50% of the neurons are lost in both wild-type and me/me mice following deafferentation (53.8 ± 11.5%, vs. 51.5 ± 7.2%, n = 4). At P14, me/me mice continue to demonstrate significant neuron loss (27.9 ± 3.7%, n = 4) compared to wild-type mice (12.7 ± 2.1%, n = 4). At P21, cochlear ablation does not induce significant neuron loss in animals of either phenotype. Wt, wild-type; me, me/me. Error bars indicate standard error of the mean. Asterisk, Mann-Whitney U-test, p < 0.05.
Table 2. Neuron number in AVCN 4 days post-ablation

<table>
<thead>
<tr>
<th>Age at surgery</th>
<th>Phenotype</th>
<th>n</th>
<th>Total neuron number per side (mean ± SEM)</th>
<th>Percentage neuron loss¹ (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>Ablated</td>
</tr>
<tr>
<td>P5</td>
<td>Wild-type</td>
<td>4</td>
<td>7241 ± 749</td>
<td>3453 ± 1157</td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>4</td>
<td>6489 ± 501</td>
<td>3204 ± 564</td>
</tr>
<tr>
<td>P14</td>
<td>Wild-type</td>
<td>4</td>
<td>7415 ± 815</td>
<td>6507 ± 806</td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>4</td>
<td>6198 ± 171</td>
<td>4485 ± 174</td>
</tr>
<tr>
<td>P21</td>
<td>Wild-type</td>
<td>4</td>
<td>5702 ± 89</td>
<td>4976 ± 133</td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>5</td>
<td>5781 ± 379</td>
<td>5058 ± 341</td>
</tr>
<tr>
<td>P21 (Anesthesia Only)</td>
<td>Wild-type</td>
<td>3</td>
<td>6328 ± 265</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>3</td>
<td>6768 ± 350</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Neuron number within AVCN 4 days following unilateral cochlear ablation in wild-type vs. motheaten mice. Comparisons are made between age-matched wild-type and me/me mice. *Mann-Whitney U-test, p < 0.05.

¹The percentage of neuron loss is obtained as follows: 100 × (Total neuron number within control AVCN – Total neuron number within ablated AVCN)/Total neuron number within control AVCN.
those previously reported in B6/129 mice where cochlear ablation at P5 results in 50-60% neuronal cell loss within AVCN (Mostafapour et al., 2000).

In contrast, P14 wild-type mice exhibit a very small amount of deafferentation-induced neuronal cell death within AVCN (Fig. 4E and F). Neuron counts reveal that only 13% of neurons are lost within the deafferented AVCN of P14 wild-type mice (Fig. 5, Table 2). However, a significant amount of neuronal cell death continues to occur in P14 me/me mice within the ablated AVCN (Fig. 4G and H). In these mice, about twice as much neuronal cell death (28%, p < 0.05) occurs in AVCN following deafferentation compared to wild-type mice (Fig. 5, Table 2) and this increased neuronal death is correlated with the increased microglial response observed in the P14 deafferented me/me AVCN. By P21, cochlear ablation results in very little neuronal loss within AVCN in both me/me and wild-type mice. Approximately 12% of P21 me/me AVCN neurons die following deafferentation while 13% of wild-type neurons are lost (Fig. 5 and Table 2). Taken together, these results demonstrate that loss of SHP-1 extends the window of neuronal susceptibility to cochlear ablation within AVCN beyond P14 but not beyond P21, and this neuronal death is correlated with the robust activation of microglial cells in these mice.

In order to confirm that neither loss of SHP-1 nor anesthesia affects the normal number of neurons within AVCN, a group of P21 wild-type and me/me mice received anesthesia alone, and neuron counts within AVCN were obtained. Table 2 demonstrates that there is no significant difference in the neuron number between non-surgical controls and the unablated sides of AVCN of all the three ages of injured animals, suggesting neither anesthesia nor lack of SHP-1 affects neuronal survival within AVCN.
3.3. *Astrocyte activation does not appear to be correlated with neuronal death*

In contrast to the microglial response to deafferentation, the astrocyte response to cochlear ablation does not appear to be correlated in any way with neuronal survival and is similar in both the deafferented wild-type and *me/me* AVCN. Figure 6 and Table 3 illustrates a similar trend towards increased GFAP immunoreactivity in AVCN at P5 and P21 in both wild-type and *me/me* mice 4 days following deafferentation, although this increase is not significant. Neither wild-type nor *me/me* mice at P14 display changes in GFAP immunolabeling within AVCN after unilateral cochlear ablation. It should be noted that immunoreactivity for GFAP appears to increase in the unablated AVCN at P14 (Fig. 6, 7A &C, and Table 3). The reasons for this are unclear.

To determine whether the timing of astrocyte activation is similar between the two phenotypes, P5 and P14 wild-type and *me/me* mice received a unilateral cochlear ablation and were allowed to survive for 2, 4 or 6 days. The brains were then processed for GFAP immunohistochemistry. At both P5 and P14, astrocyte activation is similar in both the time course and the magnitude in wild-type and *me/me* mice within the ablated AVCN at all time points following cochlear ablation as showed in Figure 8A and B.

In summary, GFAP immunoreactivity tends to increase in the ablated AVCN following deafferentation but this increase is not significant. Thus, astrocyte activation does not appear to be correlated with the increased neuronal cell death observed at P14 in *me/me* mice.
Figure 6. GFAP immunoreactivity in AVCN following deafferentation

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Figure 6: Percent of GFAP immunoreactivity within the control and ablated sides of AVCN in wild-type and me/me mice following deafferentation. At P5, there is a similar but non-significant increase in GFAP immunostaining in the ablated side compared to the control side in both wild-type (11.7 ± 3.3% vs. 5.9± 1.1%; n = 4) and me/me mice (16.4 ± 2.7% vs. 10.0 ± 1.97%; n = 4). At P14, changes in GFAP immunoreactivity continue to be similar between the two phenotypes. There is a small increase in GFAP immunostaining in the ablated AVCN compared to the control AVCN in wild-type mice (19.3 ± 1.8% vs. 17.5 ± 1.8%; n = 4); and a larger but not significant elevation of GFAP immunoreactivity in the ablated AVCN compared to the control AVCN in me/me mice (22.7± 0.8% vs. 15 ± 2.4%; n = 4). At P21, both phenotypes exhibit a similar increase in GFAP immunoreactivity after cochlear ablation. Note that the basal level of GFAP immunoreactivity in me/me mice on the control side (11.9 ± 2%) is significantly higher than that in wild-type mice (4.4 ± 1.3%). Wt-ctr, wild-type control side; wt-abl, wild-type ablated side; me-ctr, me/me control side; me-abl, me/me ablated side. Error bars indicate standard error of the mean. Asterisk, Mann-Whitney U-test, p < 0.05.
Figure 7. GFAP+ astrocytes in P14 wild-type vs. me/me AVCN
Figure 7: GFAP immunostaining in AVCN of wild-type vs. motheaten mice at P14, 4 days following cochlear ablation. Wild-type AVCN shows slightly increased GFAP labeling on the ablated side (B) compared to the control side (A) after the cochlear ablation (arrows). There is a larger, but non-significant increase in GFAP+ astrocytes on the ablated side (D) compared to the control side (C) of me/me AVCN (arrows). Scale bar = 20 μm.
Table 3. Quantification of GFAP immunoreactivity

<table>
<thead>
<tr>
<th>Age at surgery</th>
<th>Phenotype</th>
<th>n</th>
<th>Percent of GFAP staining (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>P5</td>
<td>Wild-type</td>
<td>4</td>
<td>5.87 ± 1.14</td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>4</td>
<td>10.03 ± 1.97</td>
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<tr>
<td>P14</td>
<td>Wild-type</td>
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<td>17.50 ± 1.78</td>
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<tr>
<td></td>
<td>Me/me</td>
<td>4</td>
<td>14.99 ± 2.36</td>
</tr>
<tr>
<td>P21</td>
<td>Wild-type</td>
<td>4</td>
<td>4.43 ± 1.34</td>
</tr>
<tr>
<td></td>
<td>Me/me</td>
<td>5</td>
<td>11.94 ± 2.01 *</td>
</tr>
</tbody>
</table>

Table 3. Percent of AVCN immunopositive for GFAP staining in wild-type vs. motheaten mice 4 days following cochlear ablation. The percent AVCN immunopositive for GFAP staining was obtained by dividing the sum of the stained area (μm²) that reached threshold within AVCN by the sum of the area of AVCN (μm²) from three representative sections. Comparisons are made between age-matched wild-type and me/me mice. *Mann-Whitney U-test, p < 0.05.
Figure 8. Time course of astrocyte activation in P5 & P14 AVCN
Figure 8: Time course of GFAP immunoreactivity at 2, 4, and 6 days following cochlear ablation in the deafferented AVCN of P5 and P14 wild-type and me/me mice. Both the time course and magnitude of the GFAP immunolabeling are similar in both P5 (A) and P14 (B) wild-type and me/me mice at all three time points following cochlear ablation. Error bars indicate standard error of the mean. Asterisk, Mann-Whitney U-test, p < 0.05.
4. Discussion

The current study demonstrates that lack of the tyrosine phosphatase SHP-1 results in a significant increase in microglial activation following unilateral cochlear ablation in P14 mice, that is correlated with an extension of the critical period for deafferentation-induced neuronal cell death within AVCN beyond P14. While previous studies have suggested that the transition of AVCN neurons from afferent-dependent to afferent-independent mechanisms relies on the establishment of mature synaptic connections from the eighth nerve (Rubel and Fritzsch, 2002), we hypothesize that the large microglial response in me/me mice may significantly contribute to neuronal cell loss following deafferentation. It is interesting to note that astrocyte activation does not appear to be correlated with the survival of AVCN neurons.

4.1. Synaptic maturation in AVCN

The onset of hearing in the mouse occurs at P11-14 (Limb and Ryugo, 2000) suggesting that the critical period for deafferentation-induced cell death is defined by the activation of eighth nerve synapses onto AVCN neurons (Rubel and Fritzsch, 2002). Our data indicates that microglial activation as well as synaptic maturation are coexisting factors that determine the extension of the critical period for neuronal susceptibility in me/me mice. In addition, our results indicate that at P5, activated microglia are able to induce neuronal loss in both phenotypes at a time when synaptic connections onto AVCN neurons are very immature. At P21, activated microglia are no longer able to induce
neuronal death in either phenotype because functional synaptic connections are mature and confer some protection onto AVCN neurons. However, at P14, synaptic connections are just maturing, and AVCN neurons are still vulnerable to microglia-induced neuronal death if the microglial response is large enough, as is the case in the me/me mice.

An important point is that the motheaten auditory system appears to mature at the same rate as the wild-type auditory system. Examination of wholemounted me/me cochlea reveals no obvious differences in hair cell number or distribution compared to wild-type (Dabdoub and Kelley, personal communication). In addition, both wild-type and me/me mice show a startle response at P14, and both phenotypes display a similar pattern of synaptophysin immunoreactivity within AVCN at P14 (Zhao, Brooks and Lurie, personal observations). Synaptophysin immunoreacts with presynaptic vesicles of CNS neurons, indicating that presynaptic connections within AVCN are morphologically similar between wild-type and me/me mice at P14. Thus, the extension of the critical period in me/me mice is not due to delayed maturation of the auditory system, lending further support to our hypothesis that activated microglia play a role in defining the critical period of neuronal loss within AVCN following deafferentation.

4.2. Role of SHP-1 in microglial activation

The extensive activation of microglia observed in the me/me mutants following deafferentation suggests that lack of the protein tyrosine phosphatase SHP-1 may play a role in regulating the glial response to injury. Previous studies in our laboratory have demonstrated that SHP-1 increases in a subset of glial cells following deafferentation of
the avian auditory brainstem and both in vivo and in vitro studies show that these SHP-1+ glia do not appear to enter the cell cycle (Lurie et al., 2000; Sorbel et al., 2002). We have also found that ischemic damage to the mouse forebrain results in the upregulation of SHP-1 in non-proliferating mammalian glial cells, with the expression of SHP-1 in microglia occurs at early times (day 1) after the ischemic injury (Wishcamper et al., 2003). Notably, ischemic lesion of the SHP-1 deficient me/me mouse brain results in a significantly larger microglial response compared to the same damage induced in wild-type mice (Wishcamper and Lurie, personal observation), suggesting that SHP-1 negatively regulates microglial activation in the mammalian brain following injury.

It should be noted that SHP-1 is predominantly expressed in hematopoietic cells and lack of SHP-1 results in a variety of hematopoietic abnormalities in me/me mice, including overproduction of macrophages and/or immune dysfunction (Shultz and Green, 1976; Shultz et al., 1997). The robust response of me/me microglia to deafferentation and other CNS injuries is possibly due to both local activation of resident microglia and peripheral infiltration of circulating macrophages as an inflammatory reaction (Priller et al., 2001; Hanisch, 2002; Raivich et al., 2003). Studies in our laboratory have also found that, due to lack of SHP-1, the uninjured P14 me/me brainstem expresses a reduced level of the anti-inflammatory cytokine IL-10 and an elevated level of the pro-inflammatory cytokine IL-1β. In addition, following cochlear ablation in me/me mice there is a decreased induction of the anti-inflammatory cytokines IL-4 and IL-10 and an increased induction of IL-1β at 1 day compared to the wild-type brain, suggesting a role for SHP-1 in mediating the activation of microglia (Zhao and Lurie, personal observation).
4.3. Microglial activation-induced AVCN neuronal death

Accumulating evidence indicates that activated microglia can kill neurons both in vivo and in vitro through a variety of mechanisms. It has been suggested that the release of nitric oxide (NO) (Loihl and Murphy, 1998; Liberatore et al., 1999; Bal-Price and Brown, 2001), reactive oxygen species (Beckman et al., 1994; Chao et al., 1995), TNF-α and IL-1β (Chao et al., 1995; Viviani et al., 1998; He et al., 2002), and glutamate (Barger and Basile, 2001) by activated microglia all contribute to neuronal death following injury and disease. In particular, the release of inducible nitric oxide synthase (iNOS) derived NO is thought to inhibit neuronal respiration, causing glutamate release and the subsequent excitotoxic death of neurons (Bal-Price and Brown, 2001). In addition, activated microglia themselves secrete glutamate (Barger and Basile, 2001) and may also induce glutamate release from astrocytes via NO induction (Bal-Price and Brown, 2001). Increased induction of iNOS and TNF-α through activation of the extracellular signal regulated kinase (ERK) and p38 MAP kinase subgroups of MAP kinases (MAPK) occurs in both microglia and astrocytes (Bhat et al., 1998).

One possible link between SHP-1 and microglial activation may be the ERK and p38 MAPK pathways. Endotoxin LPS and adenovirus infection-activated microglia show increased induction of iNOS and TNF-α through activation of both the ERK and p38 subgroups of MAPKs (Bhat et al., 1998; Bhat and Fan, 2002) and hypoxia induces iNOS expression and NO release from activated microglia via p38 MAPK (Park et al., 2002). Significantly, SHP-1 has been shown to inhibit ERK activity (Shibasaki et al., 2001) and to indirectly inhibit p38 MAP kinase (Kumar et al., 1999). This raises the possibility that
SHP-1 is associated with negative regulation of ERK and/or p38 MAPK within microglia, with loss of SHP-1 leading to increased activation of ERK and/or p38 MAP kinase in me/me microglia and subsequent induction and release of NO and TNF-α. This in turn might result in death of AVCN neurons following deafferentation, particularly at P14 when these neurons are still vulnerable.

Finally, it will be of interest to determine whether excessive microglial activation can induce death of AVCN neurons that have been protected from deafferentation-induced cell loss. Mostafapour et al. (2002) have recently shown that bcl-2 overexpression eliminates neuronal death within AVCN in very young mice, suggesting that upregulation of bcl-2 can rescue neurons from deafferentation-induced death even during the critical period when they are normally susceptible. It is not known whether microglial activation can induce neuronal death in AVCN neurons that overexpress bcl-2. Future experiments will address this issue.

5. Conclusion

In summary, cochlear ablation in mice lacking functional SHP-1 protein extends the critical period for deafferentation-induced neuronal death beyond P14. This neuronal loss is correlated with extensive microglial activation within the me/me AVCN. Our data suggests that the critical period for neuronal survival within the murine auditory brainstem may depend upon the timing and extent of microglial activation as well as other intrinsic factors such as synaptic maturation and/or apoptotic mediators.
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Wishcamper CA, Coffin JD, Lurie DI. (2001). "Lack of the protein tyrosine phosphatase SHP-1 results in decreased numbers of glia within the motheaten (me/me) mouse brain." J Comp Neurol 441(2): 118-33.

CHAPTER TWO

Loss of SHP-1 phosphatase selectively alters cytokine expression following cochlear ablation

Abstract

Inflammatory cytokines in the central nervous system are largely modulated by glial cells and play a crucial role in regulating neuronal responses to CNS injury. The protein tyrosine phosphatase SHP-1, an intracellular regulator of many cytokine signaling pathways, has also been implicated in mediating the activation of glia. Previous studies have demonstrated a direct correlation between abnormally activated microglia and neuron loss within the SHP-1 deficient motheaten (me/me) mouse auditory brainstem after afferent injury. In order to determine whether loss of SHP-1 creates an aberrant cytokine environment that could drive the abnormal activation of me/me microglia, the expression of five different inflammatory cytokines, interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) was examined by enzyme-linked immunoabsorbent assay (ELISA) in the intact and deafferented me/me and wild-type hindbrain. Normal uninjured me/me mice showed decreased IL-10 but increased IL-1β levels. One day following unilateral cochlear ablation, expression of IL-4 and IL-10 in me/me brains is significantly less than in wild-type, whereas the level of IL-1β in these mutants is significantly increased compared to wild-type. The production of TNF-α and IFN-γ is reduced in the me/me brain at day 4 after deaferentation. Taken together, these results indicate that lower levels of the anti-inflammatory cytokines IL-4 and IL-10, in combination with elevated expression of the pro-inflammatory cytokine IL-1β shortly after injury, may initiate a severe inflammatory reaction within the me/me brain, leading to activation of microglia and contributing to the neuronal degeneration in the deaferented auditory brainstem.
1. **Introduction**

Afferent deprivation of the auditory system leads to neuronal loss and degeneration within the cochlear nucleus of the auditory brainstem in both avian and mammals during early stages of postnatal development (Trune, 1982; Webster, 1983; Born and Rubel, 1985; Hashisaki and Rubel, 1989; Tierney et al., 1997; Edmonds et al., 1999; Mostafapour et al., 2000). However, the underlying physiological mechanisms that determine whether neurons survive or die following deafferentation are not fully understood. Glial cells, especially microglia, have recently received much attention as mediators of neuronal survival and/or death following injury, infection or other neuropathological states (Streit, 1996; Aschner et al., 1999; Bruce-Keller, 1999; Streit et al., 1999). Previous studies in our laboratory have demonstrated that after unilateral cochlear ablation, there is a direct correlation between microglial activation and neuronal cell loss within the auditory brainstem nucleus, anteroventral cochlear nucleus (AVCN) of the SHP-1 deficient motheaten (me/me) mice at postnatal day 14 (P14), a time when these neurons are normally resistant to deafferentation-induced death (Zhao and Lurie, submitted).

During neural injury and repair, a unique immunoregulatory function of microglia is to help drive the cytokine network by producing and responding to a variety of cytokines and chemokines (Sawada et al., 1999; Hanisch, 2002). The activation of microglia following injury is thought to be partially regulated by the cytokine environment through the expression and activation of pro- and/or anti-inflammatory cytokine receptors on these cells (Sawada et al., 1999; Basu et al., 2002; John et al.,
leading to the initiation of intracellular cytokine signaling and a subsequent inflammatory response. Many of the inflammatory cytokines produced by activated microglia are deleterious to neurons. A variety of *in vitro* studies (Ma et al., 2002; Eskes et al., 2003; Li et al., 2003) provide support for the concept that overactivation of microglia within damaged neural tissue *in vivo* could produce excessive amounts of harmful cytokines such as the pro-inflammatory cytokines IL-1β and TNF-α, triggering the degeneration of neighboring neurons.

Microglial proliferation and activation is thought to involve, at least in part, phosphorylation events that are governed by the antagonistic activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Horvat et al., 2001). SHP-1 is a cytoplasmic protein tyrosine phosphatase containing two src-homology 2 domains (SH2). It has been implicated in the regulation of various cytokine signaling pathways (You and Zhao, 1997; Hilton, 1999; Xie et al., 2000; Kile et al., 2001), as well as in modulating astroglial and microglial proliferation and differentiation (Massa and Wu, 1998; Lurie et al., 2000; Wishcamper et al., 2003). Loss of functional SHP-1 in motheaten mice results in both immunodeficiency and autoimmune defects including lack of a T and B cell response to antigen (Shultz and Green, 1976; Davidson et al., 1979; Stanton et al., 1985). However, little is known about how SHP-1 regulates the immune functions elicited by activated glia in the central nervous system following brain injury.

The current study aims to elucidate the role of SHP-1 in modulating the production of anti- and pro-inflammatory cytokines during brain injury. Previous studies have shown deafferentation results in abnormally activated microglia with a corresponding increase in neuronal loss in *me/me* mutants at age P14 but not at P5 or
P21. Here we examined the P14 wild-type and me/me brainstem for expression of the cytokines IL-4, IL-10, IL-1β, TNF-α, and IFN-γ before and after a unilateral cochlear ablation in order to determine whether the cytokine environment in me/me mice favors abnormal microglial activation following deafferentation. Normal, uninjured me/me mice show a reduced expression of the anti-inflammatory cytokine IL-10 and an enhanced expression of the pro-inflammatory cytokine IL-1β. Following deafferentation, down-regulation of both IL-4 and IL-10 in conjunction with up-regulation of IL-1β is observed in me/me brains when compared to the wild-type mice at day 1 post-injury. In contrast, levels of TNF-α and IFN-γ in these mutants decrease at later times (day 4 post-injury), well after the onset of microglial activation (Zhao and Lurie, submitted). These results suggest that a decrease in the anti-inflammatory cytokines combined with an elevation of the pro-inflammatory cytokine IL-1β early after the injury may drive the abnormal activation of me/me microglia following cochlear ablation. This in turn, may potentiate the death of AVCN neurons in the face of afferent deprivation.

2. Materials and methods

2.2. Animals

The founding mice (C3FeLe.B6-a/a-Hcphme+/+) were obtained from The Jackson Laboratory (Bar Harbor, ME). Homozygous me/me mutants and +/- wild-type progeny used in this study were propagated by +/-me matings. Mice were given food and water ad
libitum, and were placed in a 12-hour light/dark cycle, constant temperature and humid environment. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Montana Institutional Animal Care and Use Committee.

2.2. Mouse Genotyping

Polymerase chain reaction (PCR) amplification of a 426-bp fragment, which encompasses the site of the me/me mutation, was utilized to genotype the mice. The PCR reaction was carried out using forward (5'-CGCTGCTGCTCATGTATCTC-3') and reverse (5'-GCCTACAGAGGAAGAGGCT-3') primers (Operon Technologies, Alameda, CA) and the PCR Master Mix (Promega Corporation, Madison, WI). Briefly, 2-3 mm tail samples were digested in 100 mM NaOH at 65°C for 3 hours. Digestion was terminated by adding 0.75 M Tris-HCl (pH 7.5) prior to centrifugation. DNA was subjected to 35 cycles consisting of: 30 seconds at 95°C, 1 minute at 56°C, and 1 minute at 72°C. The amplified PCR products were digested for 1 hour with Taq I enzyme (New England BioLabs, Beverly, MA) at 65°C and separated by 1.2% agarose gel electrophoresis at 120 V for 100 minutes. PCR products were stained with ethidium bromide and visualized by UV light.

2.3. Unilateral Cochlear Ablation
Wild-type mice and \textit{me/me} mutants at P14 (\(n = 5\)) were anesthetized with 2,2,2-
tribromoethanol (375 mg/kg, i.p.) and received a unilateral cochlear ablation. Surgeries
were performed as previously described with some modifications (Mostafapour et al.,
2000). Briefly, the right ear pinna inferior region was shaved and an incision was made
on the inferior side of the pinna. The ear canal was enlarged by making further incision
at its entry, and the tympanic membrane was punctured and the ossicles were removed
using a pair of fine forceps. The basal turn of the cochlea was visualized and destroyed
with RS-5610 ultra fine scissors (Roboz Surgical Instrument Company, Rockville, MD)
followed by aspiration of its contents using a 27-gauge needle with a blunt tip connected
to a vacuum pump. The incision was closed with cyanoacrylic glue. All surgical
procedures were performed on a warming pad and animals were given oxygen inhalation
after surgery on a heating element until they were fully awake. Following the operation,
mice were returned to their parents and allowed to survive for 1, 2, or 4 days.

2.4. \textit{Brain Tissue preparation}

Unoperated P14 wild-type and \textit{me/me} mice were used for normal uninjured
control groups. Mice receiving a unilateral cochlear ablation were allowed to survive for
1, 2, or 4 days after the surgery. All mice used in this study were sacrificed by
decapitation after deep anesthetization with 2,2,2-tribromoethanol (> 375 mg/kg, i.p.).
The brains were rapidly removed and cut to separate brainstem-cerebellum from
forebrain and control side from ablated side. The brain pieces were then immediately
frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until use. A modified RIPA buffer
composed of 150 mM NaCl, 50 mM Tris-HCl, 1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, with addition of 1 mM PMSF immediately before application, was used to homogenize of the brain tissue pool of each group (n = 3-5) at 1 ml buffer/brainstem-cerebellum. Brain homogenates were centrifuged at 3220 x g for 10 minutes at 4°C, and supernatants containing extracted proteins were aliquoted and frozen at -80°C until needed for subsequent enzyme-linked immunosorbent assays (ELISA).

2.5. Measurement of cytokines by ELISA

The total protein concentrations in the brain tissue lysates were analyzed at 1:50 dilution by commercially available BCA Protein Assay Reagent (Pierce, Rockford, IL) to normalize cytokine contents within the lysates. Cytokine levels for IL-4, IL-10, IL-1β, TNF-α, and IFN-γ were measured using the respective mouse cytokine ELISA kit according to the manufacturer’s instructions (BD Pharmingen, San Diego, CA). Undiluted samples were used for all cytokine measurement with the exception of IL-1β which was measured at 1:50 dilution. Brain tissue protein extracts were added to Nunc-Immuno™ Maxisorp™ 96-well plates (VWR International) coated with anti-mouse IL-4, IL-10, IL-1β, TNF-α, or IFN-γ monoclonal antibodies. Specific cytokine contents were detected by treating the plates with respective biotinylated mouse monoclonal antibodies with avidin-horseradish peroxidase conjugate. Enzyme substrate solution (BD Pharmingen) containing tetramethylbenzidine (TMB) was added to generate a color product and the reactions were terminated by adding stop solution (1 M H₃PO₄)
immediately before the absorbance was read at 450 nm. A recombinant mouse antibody specific to each cytokine contained within the kit was utilized to generate a standard curve and included in each assay.

2.6. Statistical analyses

Data were produced from three independent preparations containing a pool of brains (n = 3-5). ANOVA unpaired t-test was used for statistical analyses on the cytokine expression of the uninjured wild-type and me/me brain tissue. The cytokine responses within the injured mouse brains were expressed as percentage of control (uninjured wild-type brains). The non-parametric Mann-Whitney U-test was employed. The data are presented as the mean ± standard error of the mean (SEM), p < 0.05 was considered significant.

3. Results

3.1. Loss of SHP-1 is associated with down-regulation of IL-10 and up-regulation of IL-1β in the mouse hindbrain

Previous studies have demonstrated that loss of SHP-1 is associated with abnormal activation of microglia and neuronal loss within AVCN of the mouse auditory brainstem following cochlear ablation at P14 (Zhao and Lurie, submitted). Because immune dysfunction is a trait of SHP-1 deficient me/me mice, this study is designed to
investigate whether inflammatory cytokines participate in the mediation of microglial activation and CNS neuronal survival after afferent injury to the murine auditory brainstem.

To determine whether loss of SHP-1 alters the production of cytokines in the intact mouse brainstem, normal uninjured P14 wild-type and me/me brainstem-cerebellum tissues were processed for multiple cytokine ELISAs. The expression of anti-inflammatory (IL-4 and IL-10) and pro-inflammatory (IL-1β, TNF-α and IFN-γ) cytokines were evaluated and compared between the two genotypes (Fig. 9).

The anti-inflammatory cytokines IL-4 and IL-10 are expressed at a lower level in the uninjured me/me hindbrain compared to wild-type, although only levels of IL-10 are significantly decreased (Fig. 9A1 & A2; p < 0.05). The expression of IL-10 is reduced from 32.6 pg/mg protein in the wild-type mice to 27.3 pg/mg protein in the me/me mice (Fig. 9A2). In contrast, there is a 33% increase in the levels of the pro-inflammatory cytokine IL-1β in the me/me brainstem compared to wild-type mice (Fig. 9B1, p < 0.01). The additional two cytokines usually involved in the triggering of inflammatory reactions, TNF-α and IFN-γ, are not significantly increased in the me/me mouse brainstem compared to wild-type (Fig. 9B2 & B3).

3.2. Me/me mice show reduced levels of anti-inflammatory cytokines 1 day after deafferentation

P14 wild-type and me/me mice were subjected to unilateral cochlear ablation and were allowed to survive for 1, 2, or 4 days. Both the deafferented (ipsilateral) and
Figure 9. Cytokine profile in the intact hindbrain

A1  IL-4  A2  IL-10

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>me/me</th>
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<tr>
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B1  IL-1β

<table>
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<tr>
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B2  TNF-α

<table>
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</thead>
<tbody>
<tr>
<td>TNF-α</td>
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</table>

B3  IFN-γ

<table>
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<tr>
<th></th>
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<th>me/me</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>80</td>
<td>120</td>
</tr>
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</table>

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Figure 9. Differential cytokine expression detected by ELISA in uninjured wild-type vs. me/me brain at P14. Motheaten mice show significantly reduced levels of the anti-inflammatory cytokine IL-10 (A₂, p < 0.05) but not IL-4 (A₁) in the brainstem-cerebellum compared to wild-type, although IL-4 levels show a downward trend in these mutants. In contrast, of the three pro-inflammatory cytokines measured, only IL-1β (B₁, p < 0.01) expression is significantly increased in the me/me hindbrain. Data are generated from three independent preparations and presented as pg cytokine per mg total protein. Error bars indicate standard error of the mean. Asterisk, significant difference by ANOVA unpaired t-test.
uninjured (contralateral) sides (data not shown) of the hindbrain were processed for cytokine expression. Significant decreases in IL-4 and IL-10 expression are found in the injured me/me hindbrain compared to wild-type 1 day after afferent deprivation but not at later times (Fig. 10; p < 0.05). Specifically, me/me mice demonstrate a reduced level of IL-4 expression that is approximately 85% of the wild-type uninjured controls at 1 day (Fig. 10A). However, by 2 and 4 days following deafferentation, me/me mice are producing similar levels of IL-4 as wild-type. In contrast, cochlear ablation in wild-type mice results in an increase of IL-4 expression that is approximately 115% of the uninjured wild-type 1 day after the injury (Fig. 10A).

IL-10 levels follow a similar pattern as that seen for IL-4. Following cochlear ablation, IL-10 production in the me/me hindbrain is approximately 85% of the uninjured wild-type and is significantly less than that observed in the deafferented wild-type brainstem (Fig. 10B; p < 0.05). IL-10 expression in me/me mice then increases at 2-4 days post-injury to similar levels as that observed in wild-type mice (Fig. 10B). IL-10 expression in the wild-type hindbrain remains relatively stable over the 1-4 days post-injury at about 100-110% of the uninjured controls (Fig. 10B). It should be noted that IL-4 and IL-10 expression levels in the me/me hindbrain at 1 day following deafferentation are similar to the levels seen in the uninjured me/me brain. Our results demonstrate that following cochlear ablation in me/me mice, not only are the levels of the anti-inflammatory cytokines IL-4 and IL-10 less than wild-type at 1 day, but their induction is delayed.
Figure 10. Decreased IL-4 & IL-10 in the injured me/me hindbrain

**A**

**IL-4**

![Graph showing IL-4 levels over time after Cochlear Ablation](image)

**B**

**IL-10**

![Graph showing IL-10 levels over time after Cochlear Ablation](image)

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Figure 10. *Me/me* mice display decreased anti-inflammatory cytokine expression 1 day after unilateral cochlear ablation. A. IL-4 expression in the injured ipsilateral side of wild-type and *me/me* hindbrain post-surgery. Levels of the anti-inflammatory cytokine IL-4 in *me/me* mice are significantly lower than wild-type at 1 day post-ablation (p < 0.05) but are similar to wild-type at days 2 and 4. B. IL-10 expression in the injured ipsilateral side of wild-type vs. *me/me* hindbrain post-surgery. Expression of the anti-inflammatory cytokine IL-10 in *me/me* mice is significantly decreased compared to wild-type mice at 1 day (p < 0.05) but not at 2 or 4 days following the cochlear ablation. Data are presented as percentage of wild-type uninjured control. Error bars indicate standard error of the mean. Asterisk, significance determined by non-parametric Mann-Whitney U-test.
3.3. **Me/me mice show enhanced levels of the pro-inflammatory cytokine IL-1β 1 day following deafferentation**

At 1 day after cochlear ablation, significantly elevated levels of IL-1β (~120% of control) are observed in the me/me hindbrain compared to the wild-type animals (90% of control) (Fig. 11; p < 0.05). There is no difference in IL-1β expression between the two genotypes of mice at 2 days. However, both genotypes display an up-regulation of IL-1β levels at 4 days as shown in Figure 11. Although IL-1β expression appears to be increased in me/me mice compared to wild-type mice at this later time, it is not statistically significant due to the large variability in the me/me groups.

3.4. **TNF-α and IFN-γ decrease in the me/me hindbrain 4 days after cochlear ablation**

Unlike the cytokine levels presented above, expression of the pro-inflammatory cytokines TNF-α and IFN-γ are similar between the two genotypes at 1 and 2 days post-injury at 100-120% of the wild-type controls (Fig. 12). However, by 4 days after the injury, both TNF-α and IFN-γ levels in the me/me hindbrain are significantly decreased compared to wild-type (Fig. 12; p < 0.05).

4. Discussion
Figure 11. Increased IL-1β in the injured *me/me* hindbrain

![Graph showing increased IL-1β levels over time after Cochlear Ablation](image-url)

- **IL-1β**
- **% of Control (Wt Uninjured)**
- **Time after Cochlear Ablation**
- **Wild-type**
- **me/me**
Figure 11. Increased expression of the pro-inflammatory cytokine IL-1β in the me/me hindbrain following unilateral cochlear ablation. IL-1β levels in response to cochlear ablation are significantly elevated in the deafferented me/me hindbrain 1 day after the injury. By 4 days post-treatment, IL-1β levels appear to be enhanced in both wild-type and me/me mice, but there is no significant difference between the two genotypes due to the large variability in the me/me mice. Data are presented as percentage of wild-type uninjured control. Error bars indicate standard error of the mean. Asterisk, significance determined by non-parametric Mann-Whitney $U$-test.
Figure 12. Expressions of TNF-α & IFN-γ following deafferentation
Figure 12. *Me/me* mice demonstrate decreased levels of TNF-α and IFN-γ expression in the injured hindbrain 4 days following cochlear ablation. A. TNF-α expression in the injured ipsilateral side of wild-type and *me/me* hindbrain is similar between the 2 genotypes at 1 and 2 days post-injury. At 4 days post-ablation, TNF-α expression is significantly decreased in the *me/me* brain compared to wild-type. B. Similar to the pattern of TNF-α expression, IFN-γ levels are similar between the two genotypes at 1 and 2 days post-ablation. However, there is a significant decrease in IFN-γ levels in *me/me* brain at 4 days post-injury compared to wild-type. Data are presented as percentage of wild-type uninjured control. Error bars indicate standard error of the mean. Asterisk, significance determined by non-parametric Mann-Whitney *U*-test.
Our results demonstrate that lack of SHP-1 leads to significantly decreased levels of the anti-inflammatory cytokine IL-10 and increased levels of the pro-inflammatory cytokine IL-1β in the intact *me/me* hindbrain. Unilateral cochlear ablation results in decreased levels of the anti-inflammatory cytokines IL-4 and IL-10 in conjunction with increased levels of the pro-inflammatory cytokine IL-1β in *me/me* mutants compared to wild-type at 1 day post-injury. However, the cytokine levels in the injured *me/me* mice at this time point are similar to uninjured *me/me* mice, indicating that cytokine induction is delayed in mice lacking SHP-1. Nonetheless, the fact that *me/me* mice have decreased levels of anti-inflammatory cytokines and increased levels of the pro-inflammatory cytokine IL-1β at 1 day after cochlear ablation, creates a cytokine environment that is favorable for excessive microglial activation, and this is what is observed in deafferented *me/me* mice (Zhao and Lurie, submitted). The expression of the pro-inflammatory cytokines TNF-α and IFN-γ is reduced 4 days following deafferentation in *me/me* mice compared to wild-type, well after microglial activation has occurred (Zhao and Lurie, submitted).

4.1. Regulation of cytokine expression by SHP-1 in the intact brains

SHP-1 has been demonstrated to modulate cytokine production in the immune system. Stanton et al. (1985) has reported that splenocytes from SHP-1 deficient motheaten mice lack the ability to utilize exogenous cytokines IL-1 or IL-2, and produce less IL-2 upon alloantigen stimulation. In addition, motheaten viable (Mev) mice which contain SHP-1 that is only 20% active, express elevated levels of IL-6, IL-10, TNF, and
IFN-γ in sera as well as in cultured B and T cells, and decreased levels of IL-2 in sera and activated T cells (Khaled et al., 1998).

The present study extends our understanding of the regulation by SHP-1 of cytokine production in the CNS. In contrast to the finding of increased IL-10 expression in the Mev mouse sera and B and T cells (Khaled et al., 1998), me/me mice show reduced levels of this anti-inflammatory cytokine in the uninjured me/me mouse hindbrain compared to the age-matched wild-type animals at P14. Among all cell types in the brain, microglia, and possibly astrocytes, are the major sources of IL-10 (Benveniste, 1997; Aloisi, 2001; Dong and Benveniste, 2001; Ledeboer et al., 2002). The numbers of immunolabeled glia in the intact me/me brain have been shown to be significantly less than that in wild-type mice (Wishcamper et al., 2001), which may explain the decreases in IL-10 expression in these animals. Besides synthesis of cytokines by resident microglia, circulating blood as well as invading peripheral macrophages and monocytes into the brain could also contribute to the total quantity of cytokines measured in brain tissue. Because IL-10 has been shown to inhibit macrophage costimulatory activity and macrophage activation and intracellular signaling of a number of different systems (Oswald et al., 1992; Ding et al., 1993), it is not surprising to see reduced expression and function of IL-10 in the me/me hindbrain as motheaten mice possess hematopoietic abnormalities including overproduction of neutrophils and macrophages (Shultz and Green, 1976; Shultz et al., 1997).

Our data also show an increased expression of IL-1β in uninjured me/me mice, which is interesting because to date, there has been no indication that SHP-1 is involved in the regulation of IL-1β. Since both astrocytes and microglia can produce IL-1β, it
would be expected that decreased levels of IL-1β would be found in me/me mice who have reduced numbers of glial cells. However, the anti-inflammatory cytokine IL-10, previously designated as cytokine synthesis inhibitory factor (CSIF) (O'Garra et al., 1990; de Waal Malefyt et al., 1991), has the capacity to inhibit the production and signaling of a wide range of pro-inflammatory cytokines and chemokines including IL-1α, IL-1β, IL-6, TNF-α, IFN-γ and MCP-1 (Frei et al., 1994; Balashov et al., 2000; Szczepanik et al., 2001; Ledeboer et al., 2002). With reduced levels of IL-10, the inhibition of IL-1β production is therefore diminished. Furthermore, since monocytes and macrophages can both produce IL-1β (Kiefer et al., 2001; Peracoli et al., 2003), the up-regulation of IL-1β expression seen in the me/me brains could be partly due to its secretion from the larger numbers of monocytes/macrophages that occur as a result of SHP-1 deficiency.

It is intriguing to note that loss of SHP-1 does not affect expression of the two other pro-inflammatory cytokines, TNF-α and IFN-γ. This could be the outcome of balancing between enhanced production of TNF-α and IFN-γ due to decreased inhibition by IL-10, and the reduced numbers of natural killer cells, the primary producer of IFN-γ, in motheaten mice (Clark et al., 1981). These results demonstrate the complexity of the cytokine network in the CNS and highlight the fact that SHP-1 appears to modulate cytokine levels both directly and indirectly. Nevertheless, the combination of low levels of IL-10 and high levels of IL-1β within the me/me hindbrain appears to set up an environment that is conducive to microglial activation upon injury.

4.2. Cytokine induction of microglial activation
One of the prominent properties of microglia is the ability to respond promptly to signals like cytokines/chemokines and to gain antigen-presenting capacity by expressing major histocompatibility complex (MHC) I or II molecules, allowing initiation of antigen-specific immune responses of the damaged CNS (Neumann, 2001). Under infectious and inflammatory conditions, a variety of inducible cytokine receptors can be constitutively expressed on the surface of microglia, ranging from receptors for the pro-inflammatory cytokines IL-1, TNF, IFN-γ, IL-12 to receptors for the anti-inflammatory cytokines IL-4, IL-10, TGF-β (Aloisi, 2001). Binding of these cytokines to their respective receptors then triggers or amplifies innate immune responses of microglia (pro-inflammatory) or diminishes immune mediated reactions elicited by microglia (anti-inflammatory).

The altered cytokine environment in injured me/me mice appears to push the microglia into a pro-inflammatory pathway. These abnormally activated microglia may in turn, produce significant amount of pro-inflammatory cytokines such as IL-1β, IL-6, and/or neurotoxic substances like nitric oxide that are dangerous to the already impaired CNS, potentiating the degeneration of adjacent auditory nucleus neurons.

Interestingly, the expression of the pro-inflammatory cytokines TNF-α and IFN-γ, two common stimulators of monocytes/macrophages/microglia, is reduced at 4 days post-injury in the me/me brain. It should be noted that these cytokines have a similar expression in both me/me and wild-type mice before and at early times after the injury. Increased expression of IL-4 and IL-10 at later times post-injury may exert an inhibition on the induction of these two pro-inflammatory cytokines in me/me mice, leading to their
decreased expression at 4 days post-injury. Thus, TNF-α and IFN-γ appear to not be involved in the deafferentation-induced microglial response in mice lacking SHP-1.

5. Conclusion

The current study provides insight into the modulation of the CNS cytokine network by SHP-1 following deafferentation. Lack of SHP-1 results in altered expression of cytokines in the mouse hindbrain before and after injury, with reduced levels of the anti-inflammatory cytokines IL-4 and IL-10 in combination with elevated levels of the pro-inflammatory cytokine IL-1β. This cytokine profile may contribute to the excessive activation of me/me microglia that is observed following deafferentation.
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CHAPTER THREE

Lipopolysaccharide-activated SHP-1 deficient motheaten microglia release increased nitric oxide, TNF-α and IL-1β

Abstract

Accumulating evidence suggests a deleterious role for activated microglia in facilitating neuronal death by producing neurocytotoxic substances during injury, infection or neurodegenerative diseases. Following cochlear ablation, abnormal microglial activation accompanied by increased neuronal loss within the auditory brainstem occurs in motheaten (me/me) mice deficient in the protein tyrosine phosphatase SHP-1. To determine whether abnormally activated microglia contribute to neuronal death in me/me mice, primary microglial cultures from me/me and wild-type mouse cortices were stimulated by the bacterial endotoxin lipopolysaccharide (LPS) to evaluate the secretion of the neurotoxic mediators nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Me/me microglia release significantly greater amounts of all three mediators compared to wild-type microglia. However, the increased release of these compounds in microglia lacking SHP-1 does not appear to occur through activation of extracellular signal-regulated kinase (ERK) or p38 kinase subgroups of mitogen-activated protein (MAP) kinases. These results suggest that abnormal microglial activation can be detrimental to neurons that are already weakened by deafferentation and can thus potentiate neurodegeneration in the me/me brainstem. Our data also indicates that SHP-1 is engaged in signaling pathways in LPS-activated microglia but not through regulation of the ERK and p38 MAP kinases.
1. Introduction

Microglia are considered to be the important immune effector cells residing in the central nervous system (CNS). They serve the role of immune surveillance of damaged tissue and become activated in a broad range of CNS disorders, from acute injuries like ischemia (Gehrmann et al., 1992; Gehrmann et al., 1995) to chronic neurodegenerative conditions such as Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis (Kreutzberg, 1996; Nelson et al., 2002; Liu and Hong, 2003). Increasing evidence, however, has indicated that microglia can become hyperactivated during injury or disease, and can be contributors to secondary damage following CNS injury (Dickson et al., 1993; Kalla et al., 2001; Eyupoglu et al., 2003). Activated microglia (Medana et al., 1997) can secrete a host of soluble factors that are neurotoxic, including reactive oxygen species (ROS), nitric oxide (NO), proteinase, glutamate, and inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), which in turn, can exacerbate the degeneration of neurons (Barger and Basile, 2001; Liu and Hong, 2003). In addition, the bacterial endotoxin lipopolysaccharide (LPS) is a potent activator of microglial cells in vitrō and the release of NO and TNF-α from LPS-stimulated microglia has been demonstrated to be regulated by extracellular signal-regulated kinase (ERK) and p38 kinase subgroups of mitogen-activated protein (MAP) kinases (Bhat et al., 1998).

However, little is known regarding the role of microglial activation during neuronal death induced by deafferentation. We have previously shown that following deafferentation of the auditory brainstem, abnormal microglial activation accompanied by an increase in neuronal death occurs in motheaten (me/me) mice deficient in the protein R reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
tyrosine phosphatase SHP-1 (Zhao and Lurie, submitted). While cochlear ablation in mice has been shown to result in loss of neurons within the auditory nucleus of the brainstem during a limited early stage of postnatal development known as the "critical period" (Mostafapour et al., 2000), most of the studies examining the underlying physiological machinery that controls whether neurons survive or die following afferent removal have focused on the intrinsic properties of neurons (Mostafapour et al., 2000; Mostafapour et al., 2002). But glial cells might also participate in this process. Our demonstration of a direct correlation between an extended critical period of neuronal loss and abnormal microglial activation after unilateral cochlear ablation in mice lacking SHP-1 suggests that microglia may play a role in defining the fate of neurons following deafferentation. The current in vitro study is designed to examine whether activated SHP-1 deficient microglia secrete higher levels of neurotoxic substances NO, TNF-α and IL-1β than wild-type.

SHP-1 is a cytoplasmic phosphatase that acts to dephosphorylate a variety of intercellular signaling molecules on tyrosine residues. It is expressed predominantly in hematopoietic cells (Yi et al., 1992; Shultz et al., 1993) and SHP-1 deficient me/me mice display severe hematopoietic abnormalities and immunodeficiencies (Shultz and Green, 1976). In the CNS, SHP-1 has been implicated in the negative regulation of proliferation and differentiation of glial cells after injury (Lurie et al., 2000; Massa et al., 2000). It remains to be elucidated whether SHP-1 functions to negatively modulate the production of neurotoxic factors by activated microglia, and if so, whether SHP-1 provides this negative regulation via the ERK and p38 MAP kinase signaling cascades.

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In the present study, primary microglial cell cultures isolated from wild-type and me/me neonatal (P2) mouse cortices were stimulated with LPS, and the secretion of NO, TNF-α and IL-1β from activated microglia compared between the two genotypes. The phosphorylation state of ERK and p38 MAP kinases induced by LPS activation in the SHP-1 deficient microglia was also examined. Our results demonstrate that LPS-activated me/me microglia release significantly greater amounts of the neurotoxic substances NO, TNF-α and IL-1β compared to wild-type, but this increase does not appear to occur through regulation of ERK or p38 MAP kinases. The increased release of these neurotoxic substances by me/me microglia may contribute to the increased neuronal death observed within the auditory brainstem.

2. Materials and methods

2.3. Animals

The wild-type (+/+) and mutant me/me neonatal mice used for the primary microglial cultures were propagated by mating heterozygous +/me mice on a C3HeB/FeJLe background obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the University of Montana Laboratory Animal Resources facilities. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Montana Institutional Animal Care and Use Committee.
2.2. Mouse Genotyping

Polymerase chain reaction (PCR) amplification of a 426-bp fragment, which encompasses the site of the me/me mutation, was utilized to genotype the mice as previously described (Zhao and Lurie, submitted). The PCR reaction was carried out using forward (5'-CGCTGCTGCTCATGTATCTC-3') and reverse (5'-GCCTACAGAGGAAGAGGCT-3') primers (Operon Technologies, Alameda, CA) and the PCR Master Mix (Promega Corporation, Madison, WI). Briefly, 2-3 mm tail samples were digested in 100 mM NaOH at 65°C for 3 hours. Digestion was terminated by adding 0.75 M Tris-HCl (pH 7.5) prior to centrifugation. DNA was subjected to 35 cycles consisting of: 30 seconds at 95°C, 1 minute at 56°C, and 1 minute at 72°C. The amplified PCR products were digested for 1 hour with Taq I enzyme (New England BioLabs, Beverly, MA) at 65°C and separated by 1.2% agarose gel electrophoresis at 120 V for 100 minutes. PCR products were stained with ethidium bromide and visualized by UV light.

2.3. Primary microglial cultures

Primary microglial cultures were prepared from the cortex of neonatal mice (n = 2-10 per culture) as previously described by Suo (2002) with some modifications. In brief, postnatal day 2 (P2) wild-type and me/me mice were sacrificed by decapitation and the cerebral cortices were dissected in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with F12 containing 15 mM HEPES (Gibco BRL) media mixed with 1%
antibiotic-antimycotic mixture Penicillin-Streptomycin-FUNGIZONE (PSF, Gibco BRL), and meninges and blood vessels were removed. Cortices were then minced with a sterile pipet tip and the tissue was dissociated in 0.25% trypsin with 1 mM EDTA (Gibco BRL) for 30 minutes at room temperature. Dissociation was terminated by adding an equal volume of growth media [DMEM/F12 medium containing 10% heat-inactivated Fetal Bovine Serum (FBS, Gibco BRL) and 1% PSF], and dissociated cells were triturated to obtain a single-cell suspension. Cells were pelleted by centrifugation at 210 x g for 5 minutes and washed one more time with fresh growth media. Cells were finally seeded at a density of 1 cortex per flask in 75 cm² T-flasks pre-coated with 0.01 mg/ml of poly-L (or D)-lysine (Sigma) overnight. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and growth media was replaced every third day and to generate mixed glial cultures.

By 12 days in vitro (DIV), microglia that were loosely attached to the top of the mixed glial monolayer were harvested by shaking on a gyratory shaker at 275 rpm for 45 minutes. Collected supernatants containing detached microglia were centrifuged at 860 x g for 5 minutes, and cells were re-suspended and plated in DMEM/F12 medium containing 10% FBS and 1% PSF, either in 12-well culture plates at a density of 4.5 x 10⁵ cells/well (for measuring secreted compounds) or in 30 mm culture dishes at a density of 2.0 x 10⁶ cells/dish (for Western blotting analyses). Non-adherent substances were removed 30 minutes later by changing with fresh media.

Purity of microglial cultures was assessed using fluorescence immunocytochemistry by staining cells seeded on poly-L-lysine-coated coverslips (in 12-well culture plates) with antibodies against glial fibrillary acidic protein (GFAP; astrocyte
marker) or F4/80 (microglial marker). This procedure produced pure primary microglial cultures with > 95% of microglia (data not shown).

2.4. LPS treatment of microglia

The endotoxin lipopolysaccharide (LPS) from Salmonella typhimurium was obtained from Sigma and the lyophilized product was reconstituted in growth media. Cultures of microglia growing in 12-well culture plates were activated by exposure to LPS at 1 μg/ml for 1, 9, 18, 24, or 48 hours and then assessed for NO, TNF-α, and IL-1β. Microglial cells growing in 30 mm culture dishes were stimulated by LPS at 1 μg/ml for 30 minutes and western blots used to assess the phosphorylation state of ERK and p38 MAPKs. Media with or without LPS treatment was collected, aliquoted, and stored at −80°C until use.

2.5. Measurement of nitric oxide generation

NO production in the microglial culture supernatants was determined by Griess reaction using the Nitrate/Nitrite Colorimetric Assay kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Nitrite, the stable derivative of NO in the media, was measured. Briefly, 100 μl of the media were mixed with an equal volume of 1:1 mixture of sulfanilamide and N-(1-Naphthyl)ethylenediamine in 96-well plates. Nitrite contents were converted into a colored azo compound that was measured using the colorimetric method by reading the absorbance at 540 nm. A nitrite standard curve was generated using the nitrite standard reagent provided in the kit.
2.6. Measurement of TNF-α and IL-1β release

TNF-α and IL-1β released by microglia with or without LPS activation was measured using the respective mouse cytokine ELISA kit according to the manufacturer’s instructions (BD Pharmingen, San Diego, CA). Microglial supernatants were added to Nunc-Immuno™ Maxisorp™ 96-well plates (VWR International) coated with anti-mouse TNF-α or IL-1β monoclonal antibodies. Cytokines were detected by treating the plates with respective biotinylated mouse monoclonal antibodies with avidin-horseradish peroxidase conjugate. Enzyme substrate solution (BD Pharmingen) containing tetramethylbenzidine (TMB) was added to generate a color product and the reactions were terminated by adding stop solution (1 M H₃PO₄) immediately before the absorbance was read at 450 nm. A recombinant mouse antibody specific to TNF-α or IL-1β included in the kit was utilized to produce a standard curve and included in each assay.

2.7. Cell protein isolation

Microglial cells were washed with ice-cold PBS (Gibco BRL), then either modified RIPA buffer (Basu et al., 2002) containing a final concentration of 1% Triton X-100, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 40 μg/ml PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin (for using to normalize microglial secretions), or 1× Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) supplemented with 1 mM PMSF (for Western blot analyses) was added to lyse the cells.
Cells were immediately scraped off the plates or dishes and sonicated for 15 seconds to shear DNA and reduce viscosity. The lysates were incubated on ice for 30 minutes prior to centrifugation at 10,000 x g for 10 minutes at 4°C. Protein lysates were aliquoted and stored at -80°C until needed. Total protein concentrations in these supernatants were analyzed using the BCA Protein Assay Reagent (Pierce, Rockford, IL).

2.8. Western Blot Analysis

Western blot analysis was performed for ERK and p38 MAPK activation using PhosphoPlus p44/42 MAP Kinase (Thr202/Tyr204) (ERK 1/2) and PhosphoPlus p38 MAP Kinase (Thr180/Tyr182) Antibody Kits (Cell Signaling Technology) according to the manufacturer's instructions with some modifications. Ten μg of protein extracts from microglial cell preparations were denatured by boiling for 5 minutes in Laemmlli buffer containing 5% 2-mercaptoethanol (2-ME), separated on 10% precast polyacrylamide gels (Cambrex Bio Science Rockland, Inc., Rockland, ME), electrophoresed, and transferred onto Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech Ltd., Little Chalfont, England). The membranes were blocked for 1 hour at room temperature with 5% dry milk in 20 mM Tris base containing 137 mM NaCl and 0.1% Tween 20 (TBST, pH 7.6) and incubated overnight at 4°C with primary antibodies (1:1000; phospho-p44/p42 MAP kinase antibody or phospho-p38 MAP kinase antibody). After extensive washing with TBST, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (1:2000), followed by development with LumiGLO chemiluminescent reagent.
provided in the kit, and the blots were visualized using the VersaDoc 3000 imaging system (Bio-Rad) using the Quantity One software. To normalize the phosphorylation results, membranes were processed in stripping buffer containing 62.5 mM Tris base, 2% SDS and 100 mM 2-ME, pH 6.8 at 65°C for 30 minutes and reprobed with antibodies specific for total p44/42 or p38 MAP kinase, respectively.

2.9. Statistical analyses

Data were produced from three or more independent microglial culture preparations. Differences in NO, TNF-α and IL-1β production between groups were compared by two-way ANOVA. Phosphorylation of ERK and p38 MAP kinases within microglia was evaluated using ANOVA unpaired t-test. The data are presented as the mean ± standard error of the mean (SEM), p < 0.05 was considered significant.

3. Results

3.1. Increased NO release from LPS-activated me/me microglia

Nitric oxide has been demonstrated to be released by cultured murine microglia and substantially involved in the mechanisms of neurodegeneration (Chao et al., 1992; Peterson et al., 1994). As demonstrated by Figure 13, LPS stimulation induces significantly more NO production from me/me microglia compared to wild-type microglia at all times (p < 0.001). It should be noted that, although me/me microglia
Figure 13. Nitric oxide release from wild-type vs. me/me microglia
Figure 13. LPS induced NO release from cultured wild-type vs. me/me microglia. Microglia of each genotype were treated with 1 µg/ml of LPS for the indicated time periods. Media was applied without LPS exposure at 1 and 48 hr, the two control time points. NO release was determined by the Griess reaction, and normalized to the total protein concentrations of the cultured microglia. Results are taken from three independent cultures per time point. Significant difference between the two genotypes for the entire time course of NO release is analyzed by two-way ANOVA (p < 0.001). Data are expressed as the mean ± SEM (standard error of the mean).
secrete considerably more NO compared to wild-type, the time course of release is
similar between the two genotypes, with the upregulation of NO production starting at 18
hrs and peaking at 48 hrs following LPS exposure.

3.2. Enhanced TNF-α production from LPS-activated me/me microglia

The pro-inflammatory cytokine TNF-α is another potent neurotoxic mediator that
could be produced by microglial cells in response to injury or stimuli such as LPS
(Nicholas et al., 2001; Sriram et al., 2002; Eskes et al., 2003). Therefore, the secretion of
TNF-α from LPS-activated me/me and wild-type microglia was determined at 1, 9, 18,
24, and 48 hrs of exposure (Fig. 14). Both wild-type and me/me mouse microglia release
TNF-α into the media soon after stimulation by LPS (1 hr) and continue through 48 hr,
the last measured time point. However, LPS treatment results in significant increases in
the amount of TNF-α production by me/me microglia compared to wild-type for the
whole time course of activation (p < 0.0001). This is particularly obvious, from 9
through 48 hrs of LPS exposure with peak secretion occurring at 9 hr (70 ng/mg of cell
protein). In contrast, maximal TNF-α release by wild-type microglia is also observed at
9 hrs of LPS treatment, but is approximately 30 ng/mg of cell protein (Fig. 14). TNF-α
release diminishes in both wild-type and me/me microglia by 48 hr post-activation.
Figure 14. TNF-α production by wild-type vs. me/me microglia
Figure 14. TNF-α production induced by LPS activation of cultured wild-type vs. me/me microglia. Microglia of each genotype were treated with 1 μg/ml of LPS for the indicated time periods. At two control time points, 1 and 48 hr, media was applied without LPS exposure. TNF-α production was determined by ELISA assay, and normalized to the total protein concentrations of the cultured microglia. Results are taken from three independent cultures per time point. Significant difference between the two genotypes for the entire time course of TNF-α release is analyzed by two-way ANOVA (p < 0.0001). Data are expressed as the mean ± SEM (standard error of the mean).
3.3. Elevated levels of IL-1β secretion from LPS-activated me/me microglia

Activated microglial cells also release the cytokine IL-1β and increasing evidence has suggested a detrimental function for this pro-inflammatory cytokine following CNS injury and in several inflammatory neurological disorders (Basu et al., 2002; Griffin and Mrak, 2002; Ma et al., 2002; Pinteaux et al., 2002). Figure 15 demonstrates that activated microglia do produce IL-1β with detectable levels observed from both wild-type and me/me microglia at 9, 18, 24 and 48 hours of LPS stimulation. Notably, our data show me/me microglia release significantly higher levels of IL-1β compared to wild-type following 18-48 hrs of LPS stimulation (Fig. 15, p < 0.01). Again, the pattern of IL-1β secretion between me/me and wild-type mouse microglia is similar, with peak expression occurring at 24 hours of LPS activation.

3.4 Increased production of NO, TNF-α and IL-1β from LPS-activated me/me microglia does not occur through activation of ERK or p38 MAP kinases

Activation of the ERK and p38 kinase subgroups of MAP kinase has been shown to be associated with the production of NO and TNF-α from LPS-activated cultured microglia (Bhat et al., 1998). In order to evaluate whether the loss of SHP-1 results in increased activation of ERK or p38 following LPS stimulation, the phosphorylation (activation) state of ERK 1/2 and p38 MAPKs was evaluated in wild-type and me/me microglia.
Figure 15. IL-1β secretion from wild-type vs. me/me microglia
Figure 15. IL-1β secretion induced by LPS activation of cultured wild-type vs. me/me microglia. Microglia of each genotype were treated with or without 1 µg/ml of LPS for 1, 9, 18, 24, and 48 hrs. IL-1β levels were determined by ELISA assay, and normalized to the total protein concentrations of the cultured microglia. Only the time periods with detectable IL-1β production are shown. Results are taken from three independent cultures per time point. Significant difference between the two genotypes for the entire time course of IL-1β release is analyzed by two-way ANOVA (p < 0.01). Data are mean ± SEM (standard error of the mean).
As seen in Figure 16, LPS potentiates strong phosphorylation of ERK 1 and 2 (p44 and p42) 30 min after stimulation in both genotypes of microglia as demonstrated by Western blot (Fig. 16A). Densitometric analysis of the double bands of ERK reveals that there is statistically significant (p < 0.01) induction of ERK phosphorylation after LPS treatment in wild-type as well as in me/me mouse microglial cells (Fig. 16B). However, lack of SHP-1 in me/me microglia fails to result in an up-regulation of ERK phosphorylation compared to wild-type.

Similarly, phosphorylation of p38 MAP kinase is evident in both wild-type and me/me microglia primed by LPS exposure for 30 minutes as shown by Figure 17A. Densitometric analysis demonstrates significant (p < 0.01) phosphorylation of p38 MAPK by LPS stimulation in both cultured wild-type and me/me microglia (Fig. 17B). However, this LPS-induced phosphorylation of p38 kinase does not appear to be affected by loss of SHP-1, as there are no significant differences in phosphorylation of p38 kinase between the two genotypes (Fig. 17B). Thus, the increased production of TNF-α and NO from activated me/me microglia does not appear to occur via activation of ERK and/or p38 MAP kinases.

4. Discussion

The current study demonstrates that LPS-activated me/me microglia secrete increased amounts of the cytotoxic substances NO, TNF-α and IL-1β compared to wild-type microglia. These results confirm that, in vitro, activated me/me microglia are
Figure 16. LPS-induced ERK phosphorylation in microglia

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Phospho-p44

Phospho-p42

p44

p42

B

[Graph showing ERK phosphorylation levels in wild-type and me/me microglia with and without LPS treatment.]

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Figure 16. LPS activation induces similar amounts of ERK phosphorylation in cultured wild-type and me/me microglia. A: Wild-type and me/me microglial cells growing in 30 mm culture dishes (2 x 10^6 cells/dish) were treated with or without LPS (1 µg/ml) for 30 minutes and lysed in Cell Lysis Buffer for Western blot analysis of ERK 1/2 (p44/42) phosphorylation as described in Materials and Methods. Blots were incubated with antibody to phospho-p44/42, stripped and reprobed with antibody to total p44/42 to verify equal protein loading. Note that LPS activation induces similar amounts of phosphorylated ERK in the two genotypes. B: Quantification of ERK phosphorylation with densitometric analysis of Western blot using Quantity One software. Values are obtained as a ratio of the density of phosphor-p44/42 to the density of total p44/42. Data are expressed as the mean ± SEM from three to six independent culture preparations. Asterisks indicate significant differences compared to untreated groups within each genotype (***p < 0.01).
Figure 17. LPS-induced p38 MAPK phosphorylation in microglia

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Phospho-p38

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Figure 17. LPS-activated p38 MAPK phosphorylation appears similar in cultured wild-type and me/me microglia. A: Wild-type and me/me microglial cells growing in 30 mm culture dishes (2 \times 10^6 cells/dish) were treated with LPS (1 \mu g/ml) for 30 minutes, and untreated control and treated cells were lysed in Cell Lysis Buffer for Western blot analysis of p38 phosphorylation as described in Materials and Methods. Blots were incubated with an antibody to phospho-p38 MAPK, stripped and reprobed with an antibody to total p38 MAPK to verify equal protein loading. Note that LPS activation results in a similar induction of phosphorylated p38 MAPK in both genotypes. B: Quantification of p38 MAPK phosphorylation with densitometric analysis of the Western blot using Quantity One software. Values are obtained as a ratio of the density of phosphor-p38 to the density of total p38, and the data are expressed as the mean ± SEM from three to six independent culture preparations (n = 3-6 per culture). Asterisks indicate significant differences compared to untreated groups within each genotype (**p < 0.01).
potentially more neurotoxic than wild-type microglia and might explain our previous in vivo findings where excessive microglial activation is correlated with increased neuronal death within the auditory brainstem of me/me mice following cochlear ablation (Zhao and Lurie, submitted). In addition, LPS activation was found to cause a similar phosphorylation of ERK and p38 MAP kinases within wild-type and me/me microglia. This suggests that the increased production of NO, TNF-α and IL-1β by activated me/me microglia is regulated either through a pathway other than the ERK and p38 kinase signaling cascades, or via signaling molecules down-stream of these two MAPKs.

4.1. SHP-1 and microglial activation

SHP-1 has been implicated as a negative regulator in cell growth, proliferation and/or differentiation in various types of cells, including T lymphocytes (Saxena et al., 1998), myeloid cells (Dong et al., 1999), astrocytes (Lurie et al., 2000; Wishcamper et al., 2003), oligodendrocytes (Massa et al., 2000), and epithelial cells (Duchesne et al., 2003). In the present study, we demonstrate a negative role for SHP-1 in mediating mouse microglial activation and differentiation, as revealed by increased NO, TNF-α and IL-1β release from LPS-activated me/me microglia compared to wild-type microglia. The production of NO and TNF-α (or the expression of mRNAs encoding these substances) by LPS or IFN-γ-stimulated microglia is a hallmark of microglial activation in vitro (Chao et al., 1992; Gazzinelli et al., 1993; Paris et al., 2000). The present finding that activated me/me microglia secrete substantially more NO, TNF-α and IL-1β in a 9-48 hr period of LPS activation, indicates a non-transient negative regulation by SHP-1 in
modulating microglial activation and production of these cytotoxic mediators. It should also be noted that me/me microglia in vitro appear to grow at a faster rate than wild-type cells (Zhao and Lurie, personal observation), suggesting a negative role for SHP-1 in controlling microglial proliferation. Because microglia are macrophage-like cells, this observation is consistent with the finding that me/me peripheral macrophages have a very high proliferative capacity in vitro (Miyamoto et al., 1998).

A number of studies have elucidated the function of SHP-1 in controlling cytokine signaling and the cellular immune response in various systems. The involvement of SHP-1 in regulating the cytokine initiated JAK/STAT (Janus kinase/signal transducers and activators of transcription) and transcription factor NF-κB (nuclear factor-kappa B) signaling pathways has been demonstrated in macrophages and astrocytes, respectively (David et al., 1995; Haque et al., 1998; Massa and Wu, 1998). However, the role of SHP-1 in modulating cytokine production from specific CNS glial cell types and the resultant consequence of neuron-glia crosstalk during brain injury is not well understood. The present study is the first to describe a role for SHP-1 in the negative regulation of TNF-α, and IL-1β, as well as the free radical NO, production from activated microglial cells.

4.2. Activated microglia secrete neurotoxic substances

Secretion of NO, TNF-α and IL-1β from activated microglia has been widely accepted to be cytotoxic, triggering neuronal death and/or degeneration (Banati et al., 1993; Hirsch et al., 2003; Liu and Hong, 2003). NO generated from activated microglia
has been shown to mediate neurodegeneration by inhibiting neuronal respiration and causing glutamate release, resulting in excitotoxicity (Bal-Price and Brown, 2001). The pro-inflammatory cytokines TNF-α and IL-1β that are released by activated microglia are thought to stimulate NO production in glial cells, and may exert a more direct deleterious effect on neurons by activating receptors that contain the intracytoplasmic death domains involved in apoptosis (Hirsch et al., 2003). It has also been demonstrated that TNF-α and IL-1β can work synergistically to induce degeneration of cortical neurons (Chao et al., 1995; Jeohn et al., 1998). The present study confirms that activated me/me microglia are potentially more toxic to neurons than activated wild-type microglia, particularly to those neurons that are already vulnerable due to afferent deprivation.

4.3. SHP-1 and ERK and p38 MAPKs

Two of the three common MAPKs, ERK and p38 kinases, have been demonstrated to be activated by bacterial LPS or the non-replicating adenovirus vectors (AdV) in brain microglia and are implicated as key signaling pathways in regulating microglial expression of iNOS and TNF-α, and NO production (Bhat et al., 1998; Bhat and Fan, 2002). Additional studies have documented the participation of only p38 MAPK in mediating iNOS gene expression in either LPS- or hypoxia-induced activated microglia (Han et al., 2002; Park et al., 2002) while ERK activation does not appear to be a required function of LPS-mediated signaling events in macrophage/microglial cells (Watters et al., 2002). Nevertheless, the present study shows that both ERK and p38 MAP kinases are activated (as determined by induced phosphorylation) following LPS.
activation in both wild-type and me/me microglia. Interestingly, although activated me/me microglia produce more NO, TNF-α and IL-1β, there is no change in the activation of ERK or p38 MAP kinase in these cells compared to wild-type. This is intriguing because evidence has shown that SHP-1 is able to inhibit ERK activity in angiotensin II type 2 receptor (AT$_2$)-induced apoptosis and AT$_2$-mediated EGFR (epidermal growth factor receptor) signaling (Lehtonen et al., 1999; Shibasaki et al., 2001). In contrast, loss of SHP-1 results in decreased phosphorylation of p38 kinase from spleen cells of me/me mice in response to gamma-irradiation-induced stress (Hsu et al., 2001). However, our finding that loss of SHP-1 does not affect the activation of both ERK and p38 kinases in activated microglial cells suggests that microglial secretion of NO, TNF-α, and IL-1β is not occurring exclusively through ERK or p38 MAP kinase signaling pathways. These results are consistent with those of You et al. (2001), who found that SHP-2, an analogue of SHP-1, does not work through the three types of MAPK pathways in mediating IL-1/TNF-induced IL-6 synthesis but instead, modulates the NF-kappa B pathway. Accordingly, our data suggests that over-production of NO, TNF-α and IL-1β in activated me/me microglia is either independent of the ERK and p38 MAP kinase pathways or that SHP-1 acts as a downstream regulator in MAPK signaling pathways. Future studies will address this issue.

In conclusion, the current study demonstrates that me/me microglia lacking SHP-1 release significantly increased amounts of the neurotoxic substances NO, TNF-α and IL-1β compared to wild-type microglia in response to LPS stimulation. In addition, LPS-induced activation of ERK and p38 is not affected by the absence of SHP-1, indicating that the increased secretion of these neurotoxins occurs via regulation of alternative
signaling pathways or downstream effectors of the MAP kinase pathways. Our findings
add additional support to our previous hypothesis that abnormally activated me/me
microglia may contribute to the increased neuronal death observed within the
deafferentated auditory brainstem.
References


CONCLUSIONS

The current studies investigate the role of the protein tyrosine phosphatase SHP-1 in the cellular mechanisms of deafferentation-induced neuronal death in the murine auditory brainstem using mutant mice lacking active SHP-1 protein. Specifically, the function of activated microglia is explored as SHP-1 has been implicated in glial proliferation and differentiation in the CNS.

Termination of auditory afferent input activity by cochlear ablation results in an extended critical period of neuron loss in the me/me AVCN beyond P14 but not P21 compared to wild-type littermates, suggesting a requirement of SHP-1 in maintaining survival of post-injury neurons, especially those that may still be vulnerable to deafferentation injury. Specifically, an exaggerated microglial but not astrocyte activation corresponding with a significantly increased loss of AVCN neurons is seen in P14 me/me mice 4 days following unilateral cochlear ablation. In contrast, microglial/astrocyte activation and neuron loss at P5 or P21 is indistinguishable between wild-type and me/me mice. These results suggest that abnormally activated me/me microglia are a factor contributing to neuronal death following deafferentation at P14 as activated microglia have been demonstrated to be capable of releasing neurotoxic substances. This again suggests a role for SHP-1 in negatively regulating glial activation.

These studies also show that it is the magnitude, not the time course, of microglial activation that is altered in me/me mice. This lends more support to the hypothesis that there is a correlation between the greater magnitude of microglial activation and the increased neuronal death in AVCN of the P14 me/me mice. It should also be noted that

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there is a normal auditory startle response and normal AVCN synaptophysin
immunoreactivity in P14 me/me mice, confirming that the auditory system of me/me mice
is fully developed at P14 and that neuronal loss is not due to delayed development.

However, microglial activation due to lack of SHP-1 appears to be one of many
coeexisting contributors to AVCN neuronal degeneration following deafferentation of the
auditory system. Other factors like the maturation of synapses projecting from the
peripheral auditory eighth nerve to AVCN neurons may also play a key role in
determining the fate of neurons following afferent injury. In support of this hypothesis,
both wild-type and me/me mice show a prominent amount of neuronal loss and activated
microglia at P5, suggesting that activated microglia are capable of inducing neuronal
death when synaptic connections are very immature. At P14, me/me but not wild-type
mice display significantly more neuronal death and microglial activation, indicating that
exacerbated microglial activation can kill AVCN neurons when synaptic maturation has
just occurred and neuronal susceptibility to deafferentation may still be high. Finally,
neuronal loss in me/me mutants does not take place at P21, suggesting that activated
microglia are no longer able to induce neuronal death because functional synaptic
connections are mature and confer some protection onto AVCN neurons.

The cytokine environment also appears to play a role in the response of the me/me
brain to deafferentation. Brain inflammation in response to trauma or injury is largely
modulated by the immunoregulating function of microglial cells, and SHP-1 belongs to
one of the three major families of the negative regulators of the JAK/STAT proteins
involved in many cytokine signaling pathways (Hilton, 1999; Kile et al., 2001). The
present studies found that SHP-1 deficiency results in reduced levels of the anti-
inflammatory cytokine IL-10 and elevated levels of the pro-inflammatory cytokine IL-1β in the intact \textit{me/me} mouse hindbrain compared to wild-type, setting the stage for an inflammatory response once injured. \textit{Me/me} mutants continue to demonstrate decreases in IL-4 and IL-10 (anti-inflammatory) and increases in IL-1β (pro-inflammatory) at 1 day after cochlear ablation. Although the cytokine induction appears to be delayed in \textit{me/me} mice, the combination of early down-regulation of anti-inflammatory cytokines and up-regulation of pro-inflammatory cytokines could provide a sufficiently aberrant cytokine environment that favors inflammation and microglial activation in \textit{me/me} mice in response to deafferentation.

Once activated, \textit{me/me} microglia release more neurotoxic substances than wild-type. \textit{Me/me} microglia in culture secrete increased amounts of NO, TNF-α and IL-1β, all of which could contribute to the increased neuronal death observed in P14 mice. Interestingly, the signaling molecules ERK and p38 MAP kinases that have been shown to be associated with the activation of microglia fail to show a difference in phosphorylation (activation) between wild-type and \textit{me/me} microglial cells with LPS stimulation. This suggests that SHP-1 regulates a different signaling cascade leading to increased microglial activation in \textit{me/me} mice, or that alternatively, the modulation of SHP-1 is employed down-stream of the two MAPKs.

In summary, the current studies demonstrate that loss of SHP-1 results in an altered cytokine environment that favors microglial activation in the uninjured mouse hindbrain. Following cochlear ablation, there is a decrease in anti-inflammatory cytokines and an increase in the pro-inflammatory cytokine IL-1β in P14 \textit{me/me} mice compared to wild-type. This in turn, results in upregulated microglial activation in the
SHP-1 deficient mice. The elevated secretion of neurotoxic substances from these increased numbers of activated microglial cells could then lead to greater neuronal death within AVCN.

These studies are summarized below.
References
