Motor Neurons Exhibit Sustained Loss of Atrophy Reversal in Immunodeficient Mice

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Abstract
Our lab showed previously that whereas a substantial portion of chronically resected facial motor neurons reside in an atrophied state that can be reversed at 14 days following reinjury in wild-type (WT) mice, atrophy reversal was altered in immunodeficient mice. It was unclear, however, if the abnormal response at day 14 post-reinjury in immunodeficient mice might be due to differences in the kinetics of the reversal response or impaired regeneration. We sought to address this question, and test our working hypothesis that the normal regeneration of atrophied motor neurons is dependent on normal adaptive immunity, by comparing WT and immunodeficient recombination activating gene-2 knockout (RAG2-KO) mice that lack a mature T and B lymphocytes, at 3 and 28 days following reinjury. In WT mice, facial motor neurons that were resected for 10 weeks and subsequently reinjured for 3 days were able to regain fully an apparent 40% loss of countable neurons, and nearly 45% of that robust increase in neurons was sustained at 28 days post-reinjury in the WT mice. By contrast, at both 3 and 28 days post-reinjury RAG2-KO mice failed to show any increase in neuronal number. Size measurements showed that the surviving neurons of WT and RAG2-KO mice exhibited substantial motor neuron hypertrophy at 3 days post-reinjury, and similar levels of normal size motor neurons by 28 days post-reinjury. Among the WT mice, small numbers of T lymphocytes were found in the reinjured facial motor nucleus (FMN), and were significantly higher at 3 days, but not 28 days, in the reinjury compared to sham-reinjury groups. No differences were seen between the WT and RAG2-KO mice in overall microglial cell activity using CD11b expression following reinjury. These data suggest that many resected motor neurons did not survive the initial resection in RAG2-KO mice, whereas in WT mice they atrophied and could be restimulated by reinjury to regenerate their phenotype. Moreover, they indicate that normal T cell function, or some yet unknown function of the RAG2 gene in the brain, is essential for activating regeneration programs of atrophied motor neurons - programs with therapeutic potential for modifying neuroplasticity.

Keywords: Facial nerve axotomy; T Cells; Adaptive immunity; Immunodeficiency; Atrophy; Regeneration

Introduction
Identifying the processes by which some neurons in the central nervous system (CNS) atrophy rather than die has important implications for the discovery new treatment strategies [1-5]. Whereas infection and autoimmune diseases such multiple sclerosis can have detrimental effects on the brain, emerging evidence has established that in other contexts T cells may promote neuroprotection and survival. The adaptive immune system has been shown to have beneficial effects on neuronal outcomes in various models of trauma (e.g., mechanical, toxic, ischemic, hemorrhagic) [6]. Immune surveillance of the CNS occurs by small numbers T lymphocytes trafficking in and out of the brain, and it is now recognized that under normal physiological conditions T lymphocytes have important effects on neuronal integrity and function [7,8]. The facial nerve axotomy model is one of the best examples of the neuroprotective role of adaptive immunity, where T cells have been found to slow the rate of neurodegeneration and neuronal loss after axons are disconnected from their target muscle [9,10]. Following facial nerve axotomy in mice, T cells cross the blood–brain-barrier (BBB) and traffic to the neuronal cell bodies in the facial motor nucleus (FMN) [11]. Severe combined immunodeficient (SCID) and recombination activating gene-2 knockout (RAG2-KO) mice, which both lack functionally mature T and B lymphocytes, exhibit a faster rate of neuronal death than wild-type (WT) mice [9,10]. This literature has established that the neuroprotective activity of the adaptive arm of the immune system resides clearly within the T cell population, as B cells appear to have no effect and are not found within the facial motor nucleus following facial nerve axotomy [9,12-15].

Though some neurons die, many adult facial motor neurons undergo a protracted period of atrophy following peripheral resection of the facial nerve in mice, and exist in a lower energy state with decreased ability to uptake dyes such as Nissl [12]. This chronic resection-induced atrophy of facial motor neurons can be reversed by both GDNF delivery [3] and by reinjuring the same resected facial nerve [12]. Our lab has shown previously that the adaptive arm of the immune system appears to be required to reverse the atrophic status of these injured motor neurons [13]. Specifically, we used the facial nerve reinjury model to test the hypothesis that the reversal of motor neuron atrophy (i.e., increase in cell number and size) elicited by nerve reinjury would be abnormal in RAG2-KO mice. We found that whereas a substantial portion of chronically resected facial motor neurons reside in an atrophied state that can be reversed at 14 days following reinjury in wild-type (WT) mice, atrophy reversal was abnormal in immunodeficient RAG2-KO mice. It was unclear, however, if the abnormal response at day 14 post-reinjury in immunodeficient mice might be due to differences in the kinetics of the reversal response or an impaired regeneration response. Although our initial study provided the first data in the literature to suggest that a functional adaptive immune system may be required to regenerate the normal phenotype of atrophied facial motor neurons, it was possible, for example, that RAG2-KO mice...
have a less sustained reinjury response than WT mice; an attenuated response that could occur earlier, in closer proximity to the time of the reinjury stimulus. Specifically, as it has been shown that a larger response occurs at day 7 than day 14 following reinjury in WT mice, it was possible that the altered reinjury-induced reversal response that we saw in RAG2-KO mice at day 14 post-reinjury might be due to timing. As microglial proliferation is most pronounced 3 days after facial nerve axotomy, we sought to compare WT and RAG2-KO mice in the facial nerve reinjury paradigm at days 3, and later in time at day 28 post-reinjury to determine if the atrophy reversal response was sustainable out to 1-month duration post-reinjury in WT mice, and compare immunodeficient and WT mice across time. Thus, in the present study we sought to determine if there were differences in the kinetics of the reversal response between the groups, and test our working hypothesis that the normal regeneration of atrophied motor neurons is dependent on normal adaptive immunity. To this end, we compared motor neuron survival and size between WT and RAG2-KO mice in the facial nerve reinjury paradigm at 3 and 28 days post-reinjury. Given the neuroprotective actions of T cells in response to a single axotomy (without reinjury), we also assessed T cell levels and measures of microglial responsiveness in the reinjured FMN [9-13].

Materials and Methods

Animals

WT and RAG2-KO mice on the C57BL/6 background where breed in our colony. The original breeders were obtained from Taconic (Germantown, NY). Mice used in this study were cared for in compliance with the NIH Guide for the Care and Use of Laboratory Animals, and were housed under specific pathogen-free conditions in individual microisolator cages.

Facial nerve resection and reinjury surgery

Mice were 8-9 weeks old at the time of the first surgery. Animals were anesthetized with 4% isoflurane induction and maintained under 2% isoflurane and surgery was performed as described previously [13,15]. The right facial nerve was exposed and resected to prevent reconnection of the nerve to its target, as described previously [12]. At 10 weeks post-injury, the nerve was re-exposed and reinjured by removing the neuroma that formed near the proximal nerve stump (“chronic resection+reinjury”). In a second group of mice, we re-exposed the nerve but left the neuroma intact (“chronic resection+sham”). We compared the effects of reinjury between the groups at two time points following reinjury-day 3 and day 28. Thus, one cohort of mice was euthanized 3 days following the reinjury (second) surgery, and the second cohort of mice was euthanized 28 days following the reinjury (second) surgery.

For euthanasia, mice were anesthetized by intraperitoneal injection of a 0.5 mg/ml ketamine cocktail (ketamine/xylazine/acepromazine given in a 3:1:3 ratio) and were perfused with 4% paraformaldehyde (PF). Brains were dissected, post-fixed in 4% PF for 2 hrs at room temperature, and cryoprotected for 2 days in a PBS solution containing 30% sucrose at 4°C. Tissue was snap frozen in isopentane and stored at -80°C. Using the ambiguous nucleus and the facial nerve as the starting and ending points, respectively, 15-µm coronal sections were cut throughout the caudal–rostral extent of the facial motor nucleus. Sections were collected on Superfrost/Plus slides (Fisher Scientific) and stored at -80°C.

Light microscopic immunohistochemistry

Tissue sections were incubated in normal goat serum (NGS) (Vector, 1:30 NGS/PBS) for 1 h at room temperature followed by overnight incubation with the following primary antibodies at 4°C; for labeling T cells with the pan-T marker anti-mouse CD3 (17A.2; 1:1000; PharMingen), for microglial cells using the pan-microglial cell marker CD11b (5C6; 1:500; Serotec), or for identifying activated microglial cells using MHC2 I-A/I-E (M5/114.15.2; 1:400; PharMingen). Sections were washed in 1 x phosphate buffered saline (PBS). Visualization of the primary antibodies was performed by incubation of sections in goat anti-antibody secondary antibody (1:2000, Vector Labs) for 1 h at room temperature followed by incubation in avidin-peroxidase conjugates (1:500, Sigma) for 1 h. No signal was obtained with each of the primary or secondary antibodies alone. The chromagen reaction was revealed by incubation in 3,3-diaminobenzidine (DAB)-H2O2 solution (Sigma; 0.07% DAB/0.004% H2O2). Sections were counterstained with cresyl violet, dehydrated in ascending alcohol washes, cleared in xylenes, and coverslipped.

Quantification of neurons, T cells, measures of microglial responsiveness, and statistical analyses

Sections throughout the FMN were assessed by an experimenter under blind conditions. Sections were Nissl stained as described previously [13-16]. The number of Nissl stained motor neurons was quantified by counting neuronal profiles that contained a visible nucleolus and neuronal cell sizes were also measured using ImageJ software (National Institutes of Health) in 4 representative sections (approximately 1/10 of the entire facial motor nucleus), every other section (each spaced 120 µm apart) throughout the extent of the FMN [13]. The number of neurons in the injured FMN was expressed as a percentage of the number of neurons in the uninjured, contralateral FMN. For CD3+ T cells counts, an average of seven sections (approximately 1/6 of the entire facial motor nucleus) per animal were used to assess the number of CD3+ T cells/section. Quantification of MHC2+ activated microglial cells/section and overall microglial cell activity using CD11b expression in the FMN, were performed as described previously [15]. Using the ImageJ image analysis software, the density (Mean of Gray Value) of CD11b staining in the reinjured FMN was compared to the opposite uninjured FMN. The average ratio of CD11b staining intensity of the reinjured to the uninjured FMN was calculated for each animal and the subject groups (day 3 WT reinnjury vs. RAG2-KO reinnjury and, day 28 WT reinnjury vs RAG2-KO reinnjury) were compared. Analysis of variance (ANOVA) was used to assess main effects, and Fishers least-significant difference tests were used to make post-hoc comparisons between groups.

Results

Immunodeficiency impairs the reversal of neuronal atrophy

We compared the effect of treatment condition (chronic resection+reinjury vs. chronic resection+sham reinjury) on motor neuron survival between RAG2-KO and WT mice. As seen in figure 1A, for motor neuron survival at day 3 post-reinjury, whereas reinjury induced a robust increase in the chronic resection+reinjury WT group compared to the chronic resection+sham reinjury WT group [F(1,9)=12.34, p<0.01], the chronic resection+reinjury RAG2-KO group did not differ from chronic resection+sham reinjury RAG2-KO group. As seen in figure 1B, for motor neuron survival at day 28 post-reinjury, again, whereas reinjury induced a significant increase in the chronic resection+reinjury WT mice compared to the chronic resection+sham reinjury WT mice [F(1,9)=8.41, p<0.05], motor neuron survival did not differ between chronically resected RAG2-KO mice that received reinjury or sham reinjury.
We also compared the effect of reinjury on the cross-sectional area of motor neurons of RAG2-KO and WT mice. As seen in figure 2A, at day 3 post-reinjury, average neuronal size was significantly increased in chronically resected WT mice that received reinjury compared to chronically resected WT mice that received sham reinjury [F(1,9)=20.23, p<0.005]. Similarly, at day 3, average neuronal size was also significantly greater in chronically resected RAG2-KO mice that received reinjury compared to the RAG2-KO sham reinjury group [F(1,8)=64.03, p<0.001]. As depicted in figure 2B, at day 28 post-reinjury, average neuronal size was significantly increased in chronically resected WT mice that received reinjury compared to chronically resected WT mice that received sham reinjury [F(1,9)=53.61, p<0.001]. At day 28, average neuronal size was also significantly greater in chronically resected RAG2-KO mice that received reinjury compared to the RAG2-KO sham reinjury group [F(1,8)=31.40, p<0.005]. Figure 3 is comprised of photomicrographs of representative sections that illustrate these differences in neuronal survival and size between the subject groups.

**Figure 1:** Quantification of mean cell counts in chronically resected (70 days) WT and RAG2-KO mice that received nerve reinjury (3 days) or sham reinjury (3 days) in panel A, and nerve reinjury (28 days) or sham reinjury (28 days) in panel B. Each bar in panel A and B represent the mean ± S.E.M. of 5 WT and RAG2-KO mice/group except for RAG2-KO chronic resection+sham at 3 days which was 4 mice/group. *p<0.05, **p<0.01.

**Figure 2:** Quantification of mean cell size in chronically resected (70 days) WT and RAG2-KO mice that received nerve reinjury (3 days) or sham reinjury (3 days) in panel A, and nerve reinjury (28 days) or sham reinjury (28 days) in panel B. Each bar in panel A and B represent the mean ± S.E.M. of 5 WT and RAG2-KO mice/group except for RAG2-KO chronic resection+sham at day 3 which was 4 mice/group. *p<0.05, **p<0.01.

T cell counts and microglial responsiveness in the reinjured FMN

We also compared the effect of treatment condition (chronic resection+reinjury vs. chronic resection+sham reinjury) on T cell levels in the reinjured FMN between RAG2-KO and WT mice. T cells were not detectable in the reinjured FMN in the RAG2-KO subject groups, thus comparisons were only performed between the WT subject groups. As seen in figure 4, overall, low levels of T cell were detectable in the reinjured FMN of the WT mice. As depicted in Figure 4, at day 3 post-reinjury, there were significantly higher levels of CD3+ T cells/section in the reinjury WT group compared to the sham-reinjury WT group [F(1,8)=33.32, p<0.001]. By contrast, at day 28 post-reinjury between these groups were not different. No differences in overall CD11b staining intensity of the reinjured FMN were found between the groups. Levels of MHC2+ were close to zero per section, and thus were not quantified.
thought that the response would be impaired or show an altered kinetic profile compared to WT mice. As noted earlier, we speculated that the RAG2-KO mice may have had a less sustained reinjury response than WT mice, where an attenuated response might be seen in closer proximity to the time of the reinjury stimulus (i.e., we needed to determine if the apparent lack of a reinjury reversal response in the RAG2-KO mice that we found initially at day 14 was due to timing) [13]. The fact that there was no atrophy reversal response whatsoever at both days 3 and 28 in the RAG2-KO mice, despite a robust response in WT mice (Figure 1), coupled with the finding that the size of the surviving motor neurons of RAG2-KO and WT mice were similar in size (Figure 2), indicates that the chronically axotomized motor neurons of RAG2-KO mice likely died from the first resection surgery. Future research will need to confirm this loss of neurons with retrograde tracing. To this end, we are currently working on a retrograde tracing procedure to accomplish this goal, as fluorogold tracing attempted previously with this model was unsuccessful, possibly due to the potentially toxic effects of the tracer on chronically axotomized facial motor neurons [12].

Together, our data show that many resected motor neurons did not survive the initial resection in RAG2-KO mice, whereas in WT mice they atrophied and could be restimulated by reinjury to robustly regenerate their phenotype. In fact, the duration of the atrophy reversal response of these resected WT neurons-disconnected from their target tissue—was sustained at 1 month following reinjury where they surprisingly maintained nearly half the gain in neurons that they exhibited at day 3 post-reinjury. The most parsimonious explanation for this finding is that normal T cell function is essential for activating regeneration programs of atrophied motor neurons. The literature has established that the neuroprotective activity of the adaptive arm of the immune system resides clearly within the T cell population in the facial nerve axotomy model [9,13,15]. Despite the low levels of T cells in the reinjured FMN, the lack of reinjury-induced regeneration suggests that normal T cell function in the CNS and/or the periphery could be essential for activating regeneration programs of atrophied motor neurons. It remains to be determined if the lack of an atrophy reversal response in RAG2-KO mice is generalizable to other strains of mice with deficits in adaptive immunity, such as severe combined immunodeficient (SCID) mice, and if immune reconstitution with WT T cells can rescue this loss of reinjury-induced motor neuron regeneration in RAG2-KO mice.

It is noteworthy that following a single axotomy much higher levels of T cells are present in the injured FMN than the low levels of T cells seen here and in our study of T cells in the reinjured FMN [11,13,14,16]. Levels of T cells in the reinnervated FMN of WT mice here at day 3 post-reinjury were not substantially higher than typical baseline T cell levels patrolling the unmanipulated/normal CNS of mice, levels comparable to those seen here at day 28 post-reinjury in the WT mice (Figure 4). This suggest further that the neuroprotective effects of T cells likely occurs at the time of the initial axotomy, where they may enable axotomized facial motor neurons to survive in a low energy state with abnormal phenotypic characteristics (e.g., atrophied, inability to uptake Nissl stain), but retain the potential to be restimulated to regenerate their phenotype and function [17].

In the mouse facial motor nerve axotomy model, following a single axotomy, CD4+ T cells in conjunction with antigen presenting microglia appear to play a critical role in neuroprotection [18,19]. Although we did not find differences in overall CD11b microglial activity following reinjury between WT and immunodeficient mice (and very low levels of MHC2+ microglia among both groups as well),

Figure 4: Quantification of CD3+ T cells in chronically resected (70 days) WT mice that received nerve reinjury (3 days and 28 days) or sham reinjury (3 days and 28 days). Each bar represents the mean ± S.E.M. of 5 WT mice/group except for WT chronic resection+reinjury at 3 days which was 4 mice/group. *p<0.001.

Discussion

These studies confirmed our hypothesis and support our initial finding at day 14 that atrophy reversal was abnormal in immunodeficient RAG2-KO mice. We did not expect however, that there would be no response whatsoever in the RAG2-KO, rather we

Figure 3: Photomicrographs of Nissl stained facial motor neurons in WT and RAG2-KO mice that received nerve reinjury or sham reinjury (3 days or 28 days). The facial motor nucleus is oriented so that the medial sub-nucleus is located on the right. There was a significant loss and shrinkage of neurons in WT and RAG2-KO mice that received a chronic resection + sham injury (3 days or 28 days; B, E, H and K) compared to their respective contralateral controls (A, D, G and J). Following nerve reinjury, the number and size of injured motor neurons were markedly increased in WT mice (3 days and 28 days; C and I), whereas there was an increase of neuron size but not in neuron number in RAG2-KO mice (3 days and 28 days; F and L).
approaches such as molecular profiling microglia associated with motor neurons in this reinjury paradigm may identify key microglial processes involved in this unique form of neuroplasticity [20]. The actions of T cells in neuronal recovery and function may have important implications for aging. Aging is associated with decreased adaptive immune system functioning as well as brain neuroplasticity. As mice age, for example, increased baseline levels of T cells are found in the CNS [21,22], and they exhibit higher expression of certain immune response genes following immune challenge with LPS [23]. It is noteworthy that although innate immunity is better preserved, more severe and often detrimental age-dependent changes occur in the adaptive immune system, in particular T cells. As aging is associated with decreased T cell function [24-26], older T cells may be less effective at protecting injured or aging neurons. Immunosenescence that develops in the elderly could make them more prone to trauma and may similarly be linked to the cognitive behavioral deficits associated with normal aging. Alternatively, the regeneration program used by chronically axotomized facial motor neurons to regenerate their phenotype could be associated with some yet unknown function of the RAG2 gene in the brain [27,28]. Using this reinjury model, we have not found neurons co-labeled with BrdU and doublecortin in the reinjured FMN model, confirming that the reinjury-induced reappearance of neurons are not neurostem cells (manuscript in preparation). Further research using this model of motor neuron regeneration could identify the molecular signals involved in this powerful and unique form of neuroplasticity [29], and lead to novel treatment strategies for successful neuroregeneration in patients with neurotrauma and other forms of CNS insult and disease.

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