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Transgenic Mice With Enhanced Neuronal Major Histocompatibility Complex Class I Expression Recover Locomotor Function Better After Spinal Cord Injury

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Abstract

Mice that are deficient in classical major histocompatibility complex class I (MHCI) have abnormalities in synaptic plasticity and neurodevelopment and have more extensive loss of synapses and reduced axon regeneration after sciatic nerve transection, suggesting that MHCI participates in maintaining synapses and axon regeneration. Little is known about the biological consequences of up-regulating MHCI's expression on neurons. To understand MHCI's neurobiological activity better, and in particular its role in neurorepair after injury, we have studied neurorepair in a transgenic mouse model in which classical MHCI expression is up-regulated only on neurons. Using a well-established spinal cord injury (SCI) model, we observed that transgenic mice with elevated neuronal MHCI expression had significantly better recovery of locomotor abilities after SCI than wild-type mice. Although previous studies have implicated inflammation as both deleterious and beneficial for recovery after SCI, our results point directly to enhanced neuronal MHCI expression as a beneficial factor for promoting recovery of locomotor function after SCI.

Keywords

major histocompatibility complex; spinal cord injury; neuron; neurorepair

Classical major histocompatibility complex class I (MHI) (Ia) is comprised of a highly polymorphic heavy chain, β 2 microglobulin (β 2M), and a peptide from a degraded cytosolic protein (Heemels and Ploegh, 1995; Natarajan et al., 1999). On the cell surface, MHCI is screened by CD8⁺ T cells using receptors generated through gene rearrangement (Heemels

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and Ploegh, 1995; Natarajan et al., 1999) and by cells of the innate immune system using germline-encoded receptors (Lanier, 2005; Raulet et al., 2001).

It was long thought that neurons express little, or no, MHCI (Joly et al., 1991; Lampson, 1995; Neumann et al., 1995, 1997; Lidman et al., 1999; Kimura and Griffin, 2000). Shatz and colleagues, however, have shown that MHCI genes are expressed in neurons, especially those that undergo activity-dependent remodeling (Corriveau et al., 1998; Huh et al., 2000). Mice lacking $\beta 2M$ and the transporter associated with antigen processing (TAP), which are functionally MHCI deficient, have abnormal retinogeniculate connections and electrophysiology (Huh et al., 2000). Additionally, mice lacking PirB (an MHCI receptor of the innate immune system) have abnormalities in synaptic plasticity in their visual cortex (Syken et al., 2006), and mice lacking MHCI heavy chains (K^bD^b mice) have altered synaptic plasticity and motor learning (McConnell et al., 2009). Thus, studies using MHCI-deficient mice have revealed that MHCI plays an important role in stabilizing active synapses and eliminating inappropriate synaptic connections. Using wild-type neuronal cultures, we recently showed that recombinant soluble self-MHCI molecules can modulate axon outgrowth (Escande-Beillard et al., 2010).

Many animal studies have examined the effects of inflammatory and antiinflammatory drugs, as well as transfused immune cells and induced immune responses, on neurorepair after SCI (for review see Thuret et al., 2006; Donnelly and Popovich, 2008). These studies have both implicated inflammation as a pathogenic component of SCI, and have pointed to inflammation as beneficial for neurorepair. Oliveira et al. used MHCI-deficient mice to study the role of MHCI in synaptic plasticity and regeneration after axotomy. They found that after sciatic nerve transection, lesioned motor neurons of MHCI-deficient mice had more extensive synaptic detachments than motor neurons of wild-type mice, particularly of GABAergic neurons, and hampered axon regeneration (Thams et al., 2008; Zanon and Oliveira, 2006). These observations in the periphery are somewhat paradoxical in light of results using MHCI-deficient mice which suggest that MHCI plays a role in synaptic pruning in the CNS (Huh et al., 2000), and our observation that recombinant MHCI can inhibit neurite outgrowth from wild-type neurons in vitro (Escande-Beillard et al., 2010).

To understand MHCI's neurobiological activity better, and in particular its role in neurorepair after injury, we have taken the approach of studying neurorepair in a transgenic mouse model in which classical MHCI expression is up-regulated only on neurons. This differs from previous immunomodulatory studies using inflammatory or antiinflammatory drugs in which immune cell and glia function were broadly affected. It also differs from previous studies of mice lacking $\beta 2M$ and/or TAP, which were functionally MHCI deficient. $\beta 2M$ is required not only for MHCI function but also for the function of nonclassical MHCI (Ib) molecules CD1, Qa, H2-M3, and perhaps other proteins (Peterson et al., 1978; Goodenow et al., 1982; Soloski et al., 1995; Brutkiewicz et al., 1995; Bauer et al., 1997; Lindahl et al., 1997), as well as for the maturation of CD8⁺ T cells and natural killer (NK) cells (Zijlstra et al., 1990; Viret and Janeway, 1999; Raulet et al., 2001; Kim et al., 2005). In addition to TAP's role in MHCI assembly, it is also required for the assembly of H2-M3 and Qa-1b molecules (Bai et al., 1998; Chun et al., 2001). Accordingly, both classical and nonclassical MHCI molecules are affected in $\beta 2M$ and TAP-deficient mice, as well as the immune responses to neuronal injury.

Using transgenic mice that are engineered to express higher levels of MHCI on neurons (NSE-D^b mice) and a well-established spinal cord hemisection injury model (Courtine et al., 2008), we observed that the up-regulation of MHCI specifically on neurons led to significantly better recovery of locomotor abilities after spinal cord injury (SCI). This points directly to enhanced neuronal MHCI expression as having a beneficial effect after SCI.

MATERIALS AND METHODS

NSE-D^b Mice

C57Bl/6 mice express two classical MHC I alleles, K^b and D^b. NSE-D^b mice carry a transgene consisting of a neuron-specific enolase promoter (NSE) linked to a D^b heavy-chain cDNA, matching the endogenous MHC I allele of C57Bl/6 mice (Rall et al., 1995). We bred NSE-D^b mice with wild-type C57Bl/6 mice for 10 generations to ensure that the transgene was fully on the C57Bl/6 background and then made them homozygous for the transgene.

Immunohistochemistry

We immunostained D^b in 20-μm transverse cryosections from spinal cord lumbar region using rat D^b-specific monoclonal antibody ER-HR52 (Abcam, Cambridge, MA), as recently described (Thams et al., 2009), with minor modifications. After blocking of nonspecific binding, endogenous peroxidase activity, and biotin, sections were incubated with rat ER-HR52 (2 μg/ml) or rat IgG2a isotype control (eBR2a, 2 μg/ml; eBioscience), followed by biotinylated anti-rat IgG (2 μg/ml; Sigma, St. Louis, MO) and a streptavidin-HRP detection system (Dako, Carpinteria, CA). Images were taken using a Nikon microscope (Microphot-FXA) with a ×20 objective and a Spot digital camera (1400Color). Sections from wild-type and NSE-D^b mice were stained side by side and imaged at the same light intensity. The staining intensity (integrated optical density) of MHC I immunoreactivity in motoneurons was measured using Image Pro Plus software (Media Cybernetics Inc., Bethesda, MD).

Hemisection of the Spinal Cord

Surgical procedures were the same as those used by Fong et al. (2005). The surgeon was blinded to the genotype of the mice. All animal procedures used in this study were conducted in accordance with the Animal Care Guidelines of the American Physiological Society and were reviewed and approved by the Animal Research Committee at the University of California, Los Angeles.

Briefly, male C57Bl/6 and NSE-D^b mice (10–12 weeks in age) were maintained in a deep anesthetic state throughout the surgery with isoflurane gas (1–2.5%) via face mask. The mice were placed on a heating pad maintained at 37°C to prevent hypothermia. Under aseptic conditions, a longitudinal dorsal midline skin incision was made from T7 to T10, and the musculature covering the dorsal vertebral column was retracted to expose the vertebral column. Laminectomy was performed from T8 to T9 to expose the spinal cord. The dural mater was incised longitudinally. The midline of the spinal cord was identified, and the right half of the cord was completely transected using microdissection scissors and fine surgical forceps. Small cotton balls were used to separate the cut ends of the hemisected cord to ensure a complete hemisection. The paravertebral musculature and fascia surrounding the spinal column were sutured using 5-0 Dexon, and the skin incision was closed using 5-0 Ethilon. The mice were placed in an incubator maintained at 37°C and observed until fully recovered from anesthesia after surgery. The mice were returned to their cages and given Baytril (40 μg/g body weight), a broad-spectrum antibiotic, via drinking water for 14 d. Postsurgical care and maintenance procedures were similar to those described previously for SCI rats and cats (Roy et al., 1992; Ellegala et al., 1996). The bladders of all animals were expressed manually twice daily until they self-expressed. Surgeries were performed on 14 mice/group, and 12 mice/group survived.

Histological Analysis of the Lesion

One day after the last behavioral test (8 weeks postsurgery), animals were terminated by transcardial perfusion of 4% paraformaldehyde, and their spinal cords were dissected,

impregnated with 30% sucrose, frozen, and prepared for histological analysis. The lesion site and surrounding spinal cord, both rostral and caudal, were cryosectioned (30- μ m-thick sections), and alternate sections were thaw mounted on slides in a sequential fashion for histological staining.

Since the extent of the spinal cord lesion can vary between mice and may affect the extent to which the mice recover, we assessed the percentage damage of the hemisection in each mouse based on cresyl violet-stained sections. The lesion epicenter was identified in stained serial cross-sections of spinal cord. Two investigators, blind to animal groups, measured the total cross-sectional areas of the nonhemisected side and the spared tissue of the hemisected side by outlining digital images of spinal cord cross-sections from the epicenter, immediately above and below the lesion, using C-imaging software (C-imaging; Compix). The area of spared tissue was measured by outlining the tissue that contained normally staining neurons. The extent of the spared tissue was expressed as a percentage of the nonhemisected side and then subtracted from 100 to derive the percentage of the damage.

Treadmill Step Testing

Behavioral testing was performed 1 and 8 weeks after surgery by investigators who were blinded to the mouse's genotype, using treadmill protocols established in our laboratory (Courtine et al., 2008). In brief, treadmill stepping ability was measured utilizing video motion capture analysis tools. Retroreflective markers were placed on the shoulder, iliac crest, hip, knee, ankle, and foot bilaterally. Video was collected using the Simi system while the mice stepped quadrupedally on a treadmill. The speed of the treadmill was increased by approx 3 cm/sec once the animal was able to complete at least 10 consecutive steps. Speeds ranging from 3 to 36 cm/sec (or until the mouse could not keep up with the treadmill) were tested. For animals that could maintain stepping at 10.9 cm/sec, the markers were digitized using the Simi system to create 3D coordinates of all markers. Kinematic analysis was done by individuals blinded to the animal's genotype. The analysis included counting the number incidence of toe drags at toe-off during initiation of the swing phase of step, the number of plantar placed steps, cycle/step duration, step length, and step height.

Statistical Analysis

One-way ANOVA followed by Tukey's posttest was used to compare extent of the lesion across the experimental groups (GraphPad Prism version 4; GraphPad Software, San Diego, CA). A repeated-measures ANOVA was used to determine differences among the testing periods (weeks 1 and 8). A one-way ANOVA was used to determine differences between the wild-type and the NSE-D^b groups at each time point. Bonferroni post hoc tests were used to determine significant differences between testing periods and experimental groups. Significance was determined at $P < 0.05$.

RESULTS

Increased D^b Expression in NSE-D^b Mouse Spinal Cord Neurons

Elevated expression of D^b on the cell surface of NSE-D^b mouse neurons was originally demonstrated by 1) the increased adherence of primary hippocampal neurons to anti-D^b-coated slides and 2) the increased susceptibility of their neurons, after loading with a lymphocytic choriomeningitis virus (LCMV) peptide or infection with LCMV, to killing by CD8⁺ T cells in vitro and in vivo (Rall et al., 1995). To verify that NSE-D^b mouse spinal cord neurons have increased MHCI expression, we immunostained spinal cord cross-sections from 10-week-old wild-type and NSE-D^b mice with an anti-D^b monoclonal antibody (Thams et al., 2009). We observed that wild-type spinal motor neurons express low levels of MHCI, as previously reported (Linda et al., 1998; Edstrom et al., 2004; Thams et

al., 2008). Anti-D^b more intensely stained neurons in sections from NSE-D^b mouse spinal cords compared with that in sections from wild-type mice, confirming that D^b expression is up-regulated in NSE-D^b mouse spinal cord neurons (Fig. 1). Analysis of staining intensities of anti-MHCI-labeled cell bodies in lamina IX of the ventral horn showed about a 1.4-fold greater optical density in NSE-D^b vs. C57Bl/6 mice. Topographic location and immunoreactivity with heat shock protein 27 antibody (data not shown) strongly suggest these neurons to be motor neurons. Previous studies of classical MHCI expression in the spinal cord of wild-type mice using in situ hybridization found that the MHCI heavy chain was expressed at low levels by neurons throughout the spinal cord, but β 2M expression was prevalent only in motor neurons (Linda et al., 1998). Since MHCI heavy chain must associate with β 2M to form a stable complex on the cell surface, motor neurons are the most intensely stained by anti-MHCI in unpermeabilized spinal cord sections. Accordingly, we believe that the stronger immunohistological staining of motor neurons in NSE-D^b mouse sections reflects primarily the availability of β 2M.

NSE-D^b Mice Display Enhanced Functional Recovery After SCI

To assess the role of neuronal MHCI in neurorepair, we performed a spinal cord hemisection of wild-type C57Bl/6 and NSE-D^b mice. We chose the hemisection model because, unlike complete transection, there is significant regeneration and/or sprouting of axons in this model. Concurrently, there is a significant level of recovery of locomotion over a period of 7–8 weeks (Courtine et al., 2008). Given that there is an extensive amount of plasticity following a hemisection, this model provides an excellent opportunity to determine whether this traumatic process is affected by neuronal MHCI levels.

Prior to SCI and 1 week after SCI, there were no significant differences in the stepping ability parameters between wild-type and NSE-D^b mice (Fig. 2). Eight weeks after SCI, wild-type mice displayed a doubling in their plantar placement ratio and a 35% increase in their total number of consecutive steps; however, these improvements were not significant compared with the first week. In contrast, the NSE-D^b mice significantly improved from 1 to 8 weeks in their total number of steps (Fig. 2A), plantar placement (Fig. 2B), maximum step length (Fig. 2C), and maximum step height (Fig. 2D). Moreover, the NSE-D^b mice displayed significantly better locomotor abilities in all measured parameters relative to wild-type mice 8 weeks postlesion (Fig. 2).

Representative tracings of the step trajectories of wild-type and NSE-D^b mice for each time point are shown in Figure 3. Horizontal and vertical trajectories of the ankle 1 week after the injury show that the right hind limb (hemisected side) of both wild-type and NSE-D^b mice produced inconsistent step trajectories compared with the left hind limb (Fig. 3A–D). By 8 weeks postinjury, however, the step trajectories of the hemisected side leg had improved more in the NSE-D^b mice than in the wild-type mice (Fig. 3F,H).

One week postsurgery, 63% of wild-type and 67% of NSE-D^b mice had normal step trajectories on the nonhemisected leg, suggesting that the hemisections were of similar magnitude in both groups. Eight weeks later, 88% of wild-type and 92% of NSE-D^b mice had normal trajectories on their nonhemisected side. On the hemisected side, 13% of wild-type, and 8% of NSE-D^b mouse right hind limb's had a consistent step trajectory 1 week postinjury. Eight weeks later, 38% of wild-type and 58% of NSE-D^b mice showed normal trajectories on the hemisected side.

To determine whether there was any bias attributed to lesion severity, the spinal cord sections from the epicenter and immediately above and below the lesion were stained with cresyl violet (a representative section is shown in Fig. 4A,B) to estimate the size of the injury in individual wild-type and NSE-D^b mice. No significant differences in the extent of

the lesion (% damage compared with the nonlesioned side) between the experimental and the control groups were observed (Fig. 4C).

DISCUSSION

MHCI-deficient mice have reduced regeneration of axons and more extensive loss of synapses on motor neurons after sciatic nerve transection (Oliveira et al., 2004; Thams et al., 2009), suggesting that neuronal MHCI plays a beneficial role in neurorepair in wild-type mice. These mice also have broad immune deficiencies because interaction with MHCI is required for CD8⁺ T cell and NK cell maturation (Zijlstra et al., 1990; Viret and Janeway, 1999; Raulet et al., 2001; Kim et al., 2005), which might have affected their immune response to the injury. Additionally, β 2M- and TAP-deficient mice have deficiencies in nonclassical MHCI molecules CD1, Qa, and H2-M3 (Peterson et al., 1978; Brutkiewicz et al., 1995; Soloski et al., 1995; Bauer et al., 1997; Lindahl et al., 1997; Bai et al., 1998; Chun et al., 2001), which play important roles in bridging innate and adaptive immunity and are involved in immune responses to injury (Jahng et al., 2001; Faunce et al., 2003; Lu et al., 2006; Barral and Brenner, 2007).

We have for the first time assessed the impact of enhanced neuronal MHCI expression on recovery after neuronal injury using immune-competent transgenic mice and a SCI model. Our study differs from previous studies that broadly modulated immune system function by administering antiinflammatory or proinflammatory factors; in our transgenic mice, MHCI is specifically up-regulated on neurons in the absence of general immune suppression or stimulation.

Before lesioning, and 1 week after hemisection, there were no differences between wild-type and NSE-D^b locomotor abilities, indicating that, prelesion and initially after lesioning, the expression of the D^b transgene had no impact on NSE-D^b mouse motor abilities. After spinal cord hemisection, the most important locomotor function deficiencies include the inability to initiate the initial part of the swing phase of the step cycle, which causes the foot to drag forward; the inability to place the paw on the plantar surface rather than on the dorsal surface; and the lack of consistency in stepping

We observed that, 8 weeks after SCI, wild-type mice had spontaneous improvement in some kinematic parameters, although this was not statistically significant. In contrast, 8 weeks after lesioning, NSE-D^b mice displayed significantly improved locomotor function recovery in all key parameters compared with their locomotor abilities 1 week postlesion. Moreover, 8 weeks postlesion, NSE-D^b mice displayed improved locomotor abilities in all measured parameters compared with those of wild-type mice 8 weeks postlesion. There were significant improvements in the total number of steps, plantar placement, maximum step length, and maximum step height of NSE-D^b mice. The recovery of the length of the step on the injured side of the NSE-D^b mice resulted in the stepping being more symmetrical than in wild-type mice. These improvements in key kinematic parameters reflect better recovery of locomotor function after SCI.

Although we cannot be certain that there would not eventually be equal levels of performance in the two groups, this seems unlikely given the magnitude of the differences that persisted 8 weeks postlesion. This is a substantial time period for spinal injury studies in mice. In our experience with various spinal cord injury models in mice and rats, we find that, after more prolonged periods, there is a gradual decline in the performance of all animals with age, regardless of the treatment. Hence, we favor the notion that the enhanced neuronal MHCI levels promoted greater restoration and did not just increase the rate of recovery.

It is possible that enhanced neuronal MHCI expression in NSE-D^b mice affected the neurodevelopment of the spinal cord, resulting in subtle neuroanatomical differences in the spinal cord of NSE-D^b mice prior to the SCI. Even in this case, it is notable that NSE-D^b mice did not significantly differ from wild-type mice in their locomotor ability before or 1 week after lesioning but rather displayed better recovery of function 8 weeks after lesioning.

It is possible that enhanced neuronal MHCI expression helped to stabilize remaining synaptic connections, increase synapse formation, and/or limit secondary neuronal degeneration. Consistent with these possibilities, MHCI-deficient mice have been shown to have more extensive synaptic detachment on motor neurons after sciatic nerve lesion than wild-type mice (Oliveira et al., 2004). Additionally, it has been noted that wild-type mouse strains with a greater ability to up-regulate neuronal MHCI expression after injury had more effective axonal regrowth (Sabha et al., 2008).

MHCI expression has been observed to increase after CNS injury (Lampson, 1995; Corriveau et al., 1998). Conceivably, up-regulation of neuronal MHCI after injury is a beneficial adaptive response not only to injury but to promote neurorepair. Evidently, higher levels of neuronal MHCI in the NSE-D^b mice promoted a more rapid and complete recovery. While this observation complements previous studies reporting reduced axon regeneration in the periphery of MHCI-deficient mice (Zanon and Oliveira, 2006; Thams et al., 2008, 2009), it contrasts with the exuberance of synapses in the LGN of MHCI-deficient mice (Huh et al., 2000) and the observation that recombinant MHCI can inhibit neurite outgrowth from wild-type neurons in vitro (Escande-Beillard et al., 2010). We have also recently observed that NSE-D^b mice have deficiencies in compensatory neuronal sprouting responses after a CNS lesion (Wu et al., in press). The mechanisms underlying these contrasting findings are currently unclear.

There is conflicting evidence on whether administration of interferon- β (IFN β) to up-regulate neuronal MHCI expression has a beneficial effect after peripheral nerve lesion (Payes et al., 2008; Zanon et al., 2010). Systemic IFN β administration alters the expression of many genes (including classical and nonclassical MHC genes), antigen presentation on glia and neurons (Jiang et al., 1995; McLaurin et al., 1995), and immune cell function. In contrast, in our transgenic model, MHCI was specifically up-regulated on neurons in the absence of broad immune system effects and led to better locomotor recovery after SCI.

It is possible that enhanced levels of MHCI on NSE-D^b neurons affected the self-reactive CD8⁺ T-cell repertoire through peripheral tolerance mechanisms or enhanced the presentation of neuronal antigens and altered the CD8⁺ T-cell response to the injury, leading to beneficial effects. Accordingly, it will be of interest to characterize immune responses to neuronal antigens in NSE-D^b mice vs. wild-type mice.

In summary, we report for the first time a study of neurorepair in transgenic mice with enhanced levels of MHCI specifically on neurons. We found that this enhanced expression was associated with better recovery of locomotor ability after spinal cord hemisection. These data suggest that up-regulation of MHCI in spinal cord neurons after SCI, by pharmacological or gene therapy approaches, may be beneficial for long-term recovery of locomotor function.

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Fig. 1. Enhanced expression of D^b in NSE-D^b spinal cord neurons. Spinal cord cross-sections from the lumbar region of wild-type (WT) or NSE-D^b mice were immunostained with an anti-D^b-specific monoclonal antibody or an isotype control antibody, as described in Materials and Methods.

Fig. 2.

NSE-D^b mice display enhanced functional recovery after SCI. The spinal cords of wild-type C57Bl/6 and NSE-D^b mice were hemisected at the T8–T9 level. One and eight weeks after SCI, the treadmill stepping ability of the mice was videotaped and analyzed in a blinded fashion. All measures represent injured hind limb over contralateral hind limb for 10 step cycles of the uninjured hind limb at 10.9 cm/sec on the treadmill. **A:** Total number of steps regardless of incidence of plantar placement. **B:** Number of plantar placed steps during the stance phase of the step cycle. **C:** Maximum horizontal distance for each step cycle. **D:** Maximum vertical distance for each step cycle. All mice were analyzed in a blinded fashion N = 12 mice/group. * $P < 0.05$, ** $P < 0.01$.

Fig. 3.

A–H: Illustrations of wild-type and NSE-D^b foot step trajectory. Representative tracing of the foot position through 10 steps taken by the left and right (hemisected side) foot of a wild-type and NSE-D^b mouse at 1 and 8 weeks following hemisection (thin gray lines). Step trajectories were acquired by plotting the position of a reflective marker on the foot as it stepped on a treadmill. Vertical distance is the step height and the horizontal distance is the step length. The thick black line shows the average step trajectory in each panel.

Fig. 4. Histological evaluations of the lateral hemisection lesion. Representation of the spinal cord damage schematically (**A**) and in cresyl violet-stained spinal cord cross-section (**B**) from a wild-type mouse near the center of the lesion. The red line encircles nondamaged areas. Comparison of percentage spinal cord hemisection damage compared with the nonlesioned (left side) shows no significant difference between wild-type and NSE-D^b mice (**C**). Wild-type mean = 86.5 ± 3.85 SEM, NSE-D^b mean = 83.9 ± 2.87 SEM, $n = 6$ mice/group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]