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ABSTRACT **It is well established that developmental maturity is a key factor regulating the response of lower motor neurons to injury. The influence of age on the survival of motor neuron cell somata following axotomy is well documented, but it remains unclear whether maturity also influences the degeneration of axonal and synaptic compartments at the neuromuscular junction. Such information is important for our interpretation of data suggesting that neonatal neuromuscular junctions are particularly vulnerable in neurodegenerative conditions that affect the developing postnatal nervous system, such as spinal muscular atrophy. Here, we have examined the role of development in regulating the vulnerability of mouse neuromuscular junctions to two mechanistically distinct degenerative insults: hypoxia and peripheral nerve injury. We report that neuromuscular junctions in neonatal mice are significantly more resistant to both hypoxia and nerve injury than those in adult mice, with a transition from the neonatal to adult phenotype occurring at 2–3 wk of age. We also demonstrate that the reduced vulnerability of neuromuscular junctions observed in neonatal mice is not determined by the maturity of the synapse *per se*, suggesting that properties associated with the neonatal environment and/or age of the neuron are responsible for modulating vulnerability. Our results are in stark contrast to previous studies showing that motor neuron cell somata are markedly more vulnerable to axotomy in neonatal mice. We conclude that neonatal neuromuscular junctions are resistant to a range of neurodegenerative insults *in vivo* and that this resistance is developmentally regulated.—Murray, L. M., Comley, L. H., Gillingwater, T. H., Parson, S. H. The response of neuromuscular junctions to injury is developmentally regulated. *FASEB J.* 25, 1306–1313 (2011). www.fasebj.org**

Key Words: hypoxia · vulnerability · motor neuron · Wallerian degeneration

Defining the factors that modulate the vulnerability of neurons to a range of neurodegenerative stimuli remains one of the most important challenges in neurobiology research and is essential for our understanding of the nervous system in health and disease. Developmental maturity is one significant biological factor known to regulate neuronal vulnerability *in vivo*, as it is established that the neonatal and adult nervous systems respond differently when injured. This process has been extensively studied in lower motor neurons, where nerve injury induces rapid death of motor neuron cell somata in neonates, while those in the adult are spared (1–4). These observations suggest that motor neuron vulnerability to injury *in vivo* is developmentally regulated.

Whether developmental status has a similar role to play in regulating the degeneration and loss of axonal and synaptic compartments of motor neurons following injury is unknown. This is important, as mounting evidence shows that axonal and synaptic compartments of motor neurons are particularly vulnerable to a range of degenerative insults, including genetically induced forms of motor neuron disease, such as amyotrophic lateral sclerosis and spinal muscular atrophy (5–7), autoimmune disorders, such as Guillain–Barré syndrome (8, 9), and hypoxic damage (10). In many of these conditions, motor neuron cell somata are affected only subsequent to axonal and synaptic pathology (11).

Several groups have used the neonatal neuromuscular junction to study processes of reinnervation, but the initial responses of axons and synapses to injury have been overlooked (12, 13). In this study, we have specifically examined the role of development in regulating the vulnerability of mouse neuromuscular junctions to two different degenerative insults known to trigger the breakdown of neuromuscular junctions *via* distinct cellular mechanisms (10): hypoxia and peripheral nerve injury. We report that neuromuscular junctions in neonatal mice are significantly resistant to both hypoxia and peripheral nerve injury compared to adult mice, with the transition from the neonatal to adult phenotype occurring at 2–3 wk of age. Experiments employing a preconditioning lesion to generate new synapses in old mice showed that the resistance to injury observed in neonatal mice was regulated by the neonatal environment and/or neuronal age, and not by the age of the synapse. This study reveals that neonatal neuromuscular junctions are resistant to a

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range of neurodegenerative insults and demonstrates a clear developmental regulation of neuromuscular junction vulnerability *in vivo*.

MATERIALS AND METHODS

Mouse maintenance and surgery

Breeding pairs of C57/BL6J mice were purchased from Harlan-Olac (Bicester, UK), housed in a semibarrier facility, and fed a standard chow diet. Mice <5 d of age were chilled on ice and decapitated, and older mice were sacrificed by an overdose of isoflurane. All surgical procedures were performed under general anesthesia (inhalation of isoflurane; 2% in 1:1 N_2O:O_2), as described previously (14). Briefly, for tibial nerve lesions, a 2-mm incision in the skin was made just above the heel to expose the nerve. A 1- to 2-mm section of the nerve was then removed, the incision was closed with a single suture, and mice were allowed to recover from anesthetic before being returned to standard cages. For experiments investigating the effect of synaptic age in older animals, a preconditioning lesion was performed (15). One tibial nerve was exposed above the heel and crushed with number 3 fine forceps for 15 s. The wound was sutured, and the mouse was allowed to recover. After 4 wk, the animals were again anesthetized as above, but this time bilateral nerve cuts at the same site were carried out, and the animals again were allowed to recover. All breeding and surgical procedures were carried out with the licensed authority of the UK Home Office.

Ex vivo hypoxia-reperfusion system

Hypoxia-reperfusion experiments were performed according to protocols previously described (10). Briefly, HEPES-buffered saline (250 ml) was vigorously sparged with 95%:5% N_2:CO_2 for a minimum of 1.5 h in a 250-ml conical flask prior to the start of experimentation, which ensures that a stable and reliably low level of O_2 (0.23%) is established and maintained (10). Muscle groups were selected in order to reflect a diverse range of muscle phenotypes and properties, while being thin enough to allow efficient gas exchange to ensure homogenous exposure to the hypoxic insult and good immunohistochemical staining without the need for sectioning. Gross dissection of muscle groups was performed, and muscles were subject to hypoxia (immersion in the hypoxic HEPES-buffered saline) followed by reperfusion in 95%:5% O_2:CO_2-sparged Krebs solution. Control muscles were maintained in 95%:5% O_2:CO_2-sparged Krebs solution for the duration of the experiment. All muscles were left free floating in the conical flask at 22°C, with sufficient flux so as not to become stagnant but to remain below the surface during the course of the experiment.

Immunohistochemistry

Muscles were immunohistochemically labeled to allow quantification of neuromuscular innervation, as described previously (6). Briefly, muscles were immediately dissected from recently killed mice into oxygenated mammalian physiological saline before labeling postsynaptic ACh receptors with α-bungarotoxin (αBTX) conjugated to tetramethylrhodamine isothiocyanate (5 μg/ml for 10 min; Molecular Probes, Eugene, OR, USA). Muscles were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 15 min. Muscles were then blocked in 4% BSA and 1% TritonX in 0.1 M PBS for 30 min before incubation overnight in primary antibodies (165-kDa neurofilament, 1:250; Developmental Studies Hybridoma Bank, Iowa City, IA, USA; synaptic vesicle protein SV2, 1:250; Developmental Studies Hybridoma Bank; 150-kDa neurofilament, 1:300; Chemicon, Temecula, CA, USA) and visualized with FITC-conjugated secondary antibodies (rabbit anti-mouse or swine anti-rabbit; 1:50; Dako, Carpinteria, CA, USA). Muscles were then whole-mounted in Mowoil (Calbiochem, San Diego, CA, USA).

FM1-43FX labeling of neuromuscular synaptic function

Freshly dissected muscles were loaded with the styryl dye FM1-43FX using a high K^+ stimulus. Muscle preparations were exposed to a fixable form of the styryl dye FM1-43 (FM1-43FX; 2 mg/ml; Molecular Probes) in 95%:5% O_2:CO_2-sparged, high-K^+ Krebs solution (102 mM Na^+, 50 mM K^+, 2 mM Ca^{2+}, 2 mM Mg^{2+}, 132 mM Cl^-, 23.8 mM HCO^{3-}, 0.4 mM $H_2PO_4^{2-}$, 5 mM D-glucose, and 5.5 mM HEPES, pH 7.2–7.4) for 10 min. Following rigorous washing, muscles were fixed in 4% formaldehyde/PBS solution (Electron Microscopy Sciences), and postsynaptic ACh receptors were labeled with αBTX conjugated to Alexa-647 (5 μg/ml for 10 min; Molecular Probes). Muscles were whole-mounted in Mowoil (Calbiochem).

Imaging and quantification

Immunohistochemically labeled neuromuscular junctions were imaged using a laser scanning confocal microscope (×63 objective; 1.4 NA; Zeiss, Oberkochen, Germany). At least 100 endplates, reflecting between 50 and 80% of the total number of neuromuscular junctions in each muscle, from ≥3 different regions of each muscle, were quantified from each muscle preparation. Wherever possible, all analysis was performed without the operator knowing the status of the material. For occupancy counts, the occupancy of individual neuromuscular junctions was evaluated by categorizing endplates as either fully occupied (neurofilament/SV2 entirely overlies endplate), partially occupied (neurofilament/SV2 partially covers endplate), or vacant (no neurofilament/SV2 overlies endplate).

Statistics

All data were collected using Microsoft Excel (Microsoft, Redmond, WA, USA) and analyzed and graphed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Mann Whitney *U* and ANOVA tests were carried out on data, and values of $P < 0.05$ were considered to be significant for statistical analyses.

RESULTS

Response of neuromuscular junctions to hypoxia-reperfusion injury is developmentally regulated

Neuronal cell body vulnerability following injury in the peripheral nervous system has a significant age-dependent component (1–4); however, it remains unclear whether the vulnerability of distal axonal and synaptic compartments of neurons are similarly developmentally regulated. To investigate the influence of development on axonal and synaptic responses to injury in the

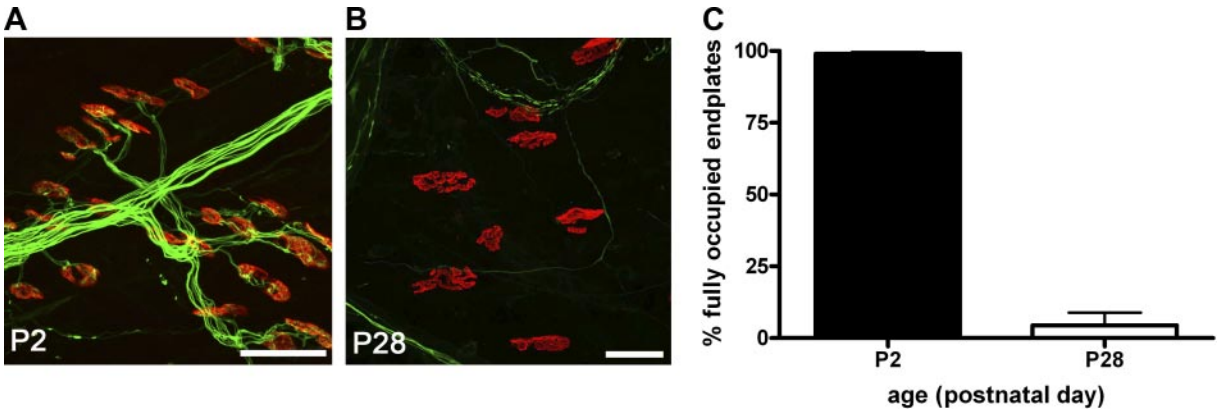

Figure 1. Neonatal neuromuscular junctions in the TVA muscle remain structurally preserved following hypoxia-reperfusion injury. *A*, *B*) Representative confocal micrographs showing neuromuscular junctions in the TVA muscle from neonatal [postnatal day 2 (P2); *A*] and adult (P28; *B*) mice following 2 h of hypoxia plus 2 h of reperfusion (green, NF/SV2 to label axon and presynaptic motor nerve terminal; red, αBTX to label postsynaptic acetylcholine receptors). At P28, most postsynaptic endplates had lost their presynaptic innervation (*B*), but at P2, all endplates remained fully occupied (*A*). *C*) Percentage of fully occupied endplates remaining in the TVA muscle from P2 and P28 muscles following 2 h of hypoxia plus 2 h of reperfusion. Values are means ± SE; $n = 6$ muscles, 3 mice/time point. Scale bars = 20 μm.

peripheral nervous system, we turned to an established *ex vivo* nerve/muscle hypoxia model, in which preparations are exposed to a 2-h hypoxic insult followed by 2 h of reperfusion (10). In adult mice, this injury induces a rapid and significant loss of distal axons and motor nerve terminals from neuromuscular junctions while leaving postsynaptic muscle fibers and motor endplates intact (10).

Analysis of muscles from 4-wk-old mice revealed that neuromuscular junctions responded similarly to those from older adult mice (10), with only 4% of transversus abdominis (TVA) neuromuscular junctions maintaining structural integrity following injury (**Fig. 1**). The remaining endplates were either devoid of presynaptic staining (vacant; 54% of endplates) or associated with highly fragmented and degenerating presynaptic terminals (partial; 42% of endplates). However, identical treatment of the TVA muscle from neonatal [postnatal day 2 (P2)] mice revealed a remarkable preservation of neuromuscular junctions, with over 99% of neuromuscular junctions retaining structural integrity (Fig. 1). To test the extent of neuromuscular resistance to hypoxic injury in neonatal mice, we repeated these experiments but subjected muscles from P2 mice to prolonged periods of hypoxia. Surprisingly, neuromuscular junction structural integrity was preserved even after 4 or 6 h of hypoxia, appearing indistinguishable from control preparations with greater than 99% of endplates fully occupied at each time point (**Fig. 2**). Neuromuscular junctions from neonatal animals are, therefore, markedly structurally resistant to severe hypoxic injury, in contrast to the rapid degenerative processes observed in mature animals.

As there was no loss of structural integrity following severe hypoxic insult at P2, we next examined whether neuromuscular junctions from neonatal mice retained function as well as structure using the vital, styryl dye FM1-43FX. This dye is taken up by nerve terminals that are actively releasing and recycling synaptic vesicles and is, therefore, a robust measure of functional synaptic integrity (16). Application of FM1-43FX using a high-potassium stimulus resulted in robust labeling of motor nerve terminals in both control and hypoxic muscles from P10 mice (**Fig. 3**). This illustrates that neonatal motor nerve terminals retain the ability to recycle vesicles and are thus likely to be functionally intact following a hypoxic insult.

Critical period for loss of developmental resistance to injury at the neuromuscular junction occurs at 2–3 wk of age

We next sought to establish the critical developmental period during which the neonatal resistant phenotype undergoes the transition into the adult, vulnerable phenotype. We therefore exposed TVA muscles from mice at different ages between P2 and P28 to the same 2-h hypoxia–2-h reperfusion injury (**Fig. 4**). The neo-

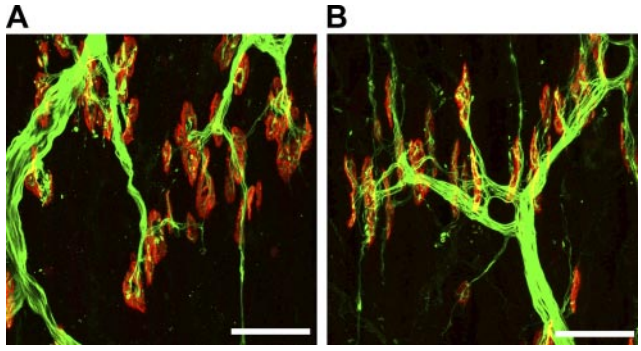

Figure 2. Neonatal neuromuscular junctions remain structurally intact following a prolonged hypoxic insult. Representative confocal micrographs showing neonatal (P2) neuromuscular junctions from the TVA muscle following 4 h of hypoxia plus 2 h of reperfusion (*A*) and 6 h hypoxia (*B*). Even after this prolonged hypoxic insult, no evidence of structural degeneration could be identified. $n = 2$ mice, 4 muscles/time point. Scale bars = 20 μm.

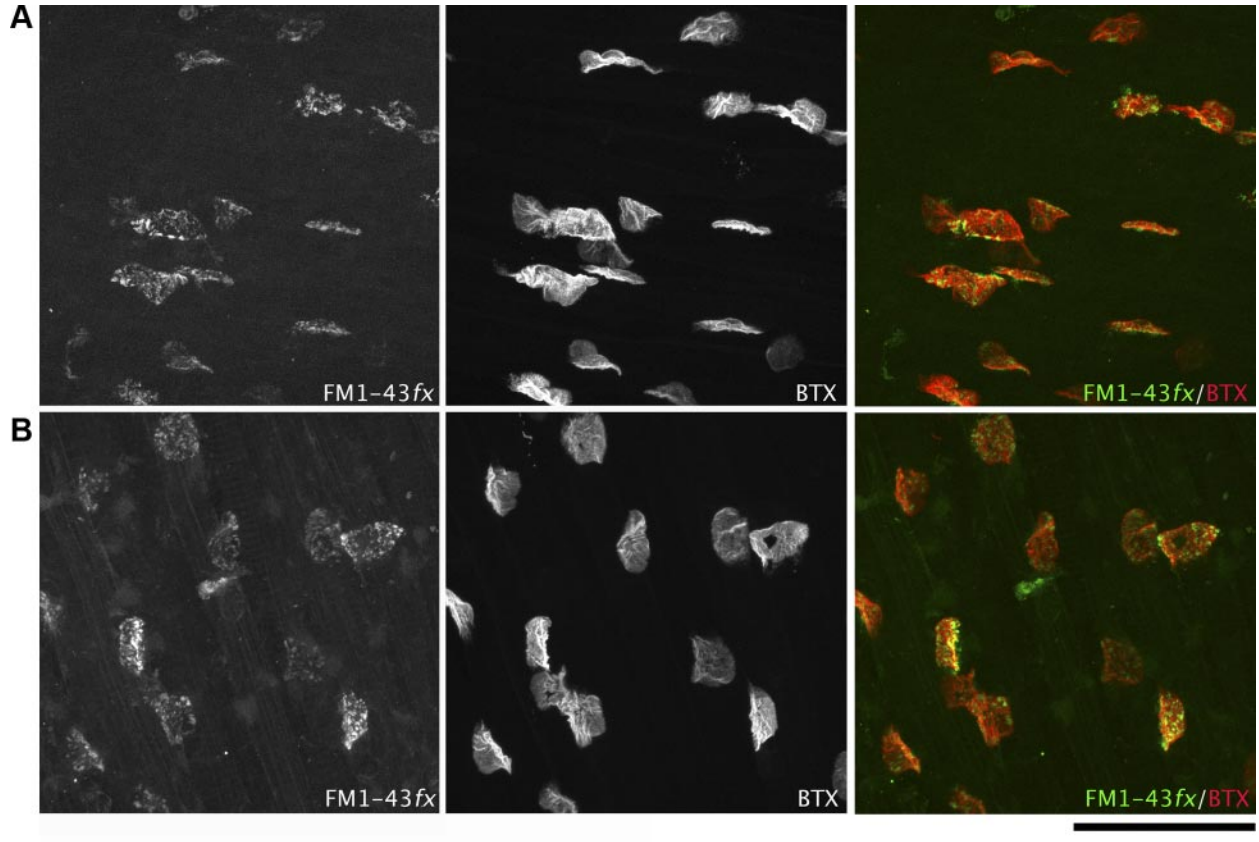

Figure 3. Neonatal neuromuscular junctions remain functionally preserved following hypoxia-reperfusion injury. Representative confocal micrographs of neuromuscular junctions loaded with the styrl-dye FM1-43FX using a high-potassium stimulus from control muscles (4 h in oxygenated solution; *A*) and experimental muscles (2 h of hypoxia plus 2 h of reperfusion; *B*). Robust FM1-43FX labeling could be observed at all neuromuscular junctions in both control and hypoxic muscles, indicating that neonatal neuromuscular junctions remained functionally intact after a hypoxic insult. $n = 3$ mice, 6 muscles/group. Scale bar = 50 μm.

natal resistance phenotype was maintained until P10, but at P15, the first signs of neuromuscular junction vulnerability were apparent. This trend increased rapidly over the subsequent 10 d, with 50% of junctions damaged at P17, and adult levels of vulnerability established by P28.

As synapse elimination (the process of pruning of supernumary axonal inputs at individual neuromuscular junctions) is ongoing in TVA muscle over the first 2 wk of life (17), it was possible that we had underestimated the loss of nerve terminals by not counting the number of inputs to each muscle endplate. Data collected from control and TVA muscles exposed to hypoxia-reperfusion injury showed that there was no significant difference in the percentage of polyneuronally innervated neuromuscular junctions (3 mice/group, $P>0.05$, *t* test), and therefore that we had not underestimated the loss of nerve terminals in previous experiments.

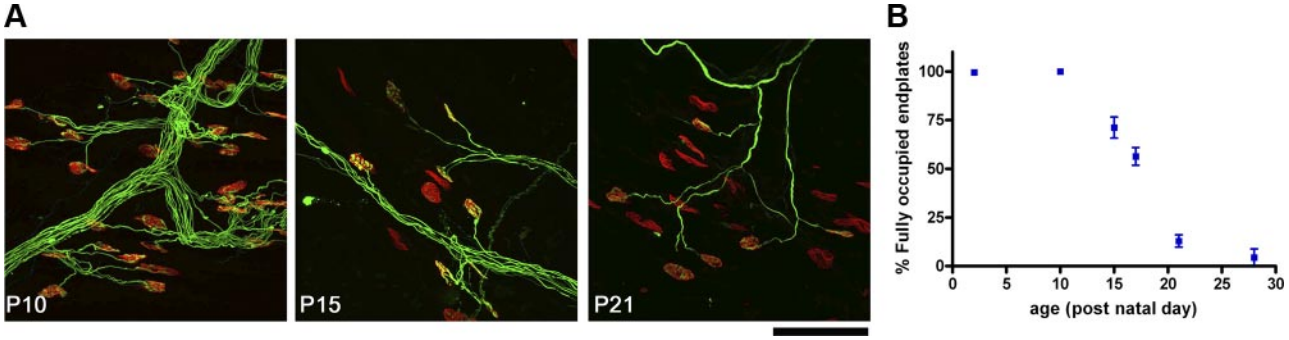

Figure 4. Resistance of neonatal neuromuscular junctions to injury is gradually lost between 2 and 3 wk of age. *A*) Representative confocal micrographs of neuromuscular junctions from the TVA muscle following 2 h hypoxia plus 2 h reperfusion from P10, P15, and P21 mice. Structural resistance to injury was progressively lost between P10, where no degeneration could be observed, and P21, when the adult rapid-degeneration phenotype was apparent. Scale bar = 50 μm. *B*) Time course of the loss of neonatal resistance between P2 and P28. Values are means ± SE; $n = 6$ muscles, 3 mice/time point.

Taken together, these data show that the susceptibility of neuromuscular junctions to hypoxia-reperfusion injury is developmentally regulated, with the critical period for transition between neonatal and adult phenotype occurring between P10 and P28 in TVA muscle.

Muscle phenotype subtly modifies neonatal neuromuscular junction resistance to hypoxia

We next sought to determine whether specific characteristics of individual muscles, such as fiber type and developmental plasticity phenotype (6, 18), influenced the resistance to injury observed in neonatal animals. We therefore extended our study to include a group of superficial cranial muscles with diverse phenotypes (19): namely, levator auris longus (LAL), auricularis superior (AS), and adductor auris longus (AAL). These muscles offer the opportunity to study a range of muscle phenotypes, while eliminating variables, such as nerve stump length and anatomical position. The LAL muscle is a homogeneous fast-twitch muscle, which is anatomically split into two bands, caudal and rostral, with the rostral band possessing differential plasticity of neuromuscular junctions compared to the caudal band (6, 18). The AS and AAL comprise predominantly slow-twitch and fast-twitch fibers, respectively.

Significant neonatal neuromuscular junction resistance to injury was observed in all muscles examined, although subtle differences were observed between muscles. In particular, the onset of the critical period for transition from neonatal to adult phenotype was postponed by ~5 d in the cranial muscles compared to the TVA, with >95% of neuromuscular junctions maintaining structural integrity after insult until P15 in all of the cranial muscles tested (**Fig. 5**). Subsequent to P15, the kinetics of resistance loss progressed in a similar fashion in all muscles analyzed, with a marked decrease in resistance/increase in vulnerability between P15 and P21. AS and AAL had remarkably similar time course curves, irrespective of their differing muscle fiber type composition. LAL, however, was different: vulnerability began to appear at P15, and increased at a much slower rate than the other muscles; in fact, a degree of resistance was maintained beyond P28. Furthermore, the rostral band, which displays a greater degree of plasticity, maintained a consistently greater level of resistance to the insult. These data demonstrate that neonatal resistance to hypoxia-reperfusion injury is a developmentally regulated phenomenon intrinsic to a wide variety of skeletal muscle phenotypes, but also suggest that individual muscles and muscle groups may exhibit subtly different developmental schedules.

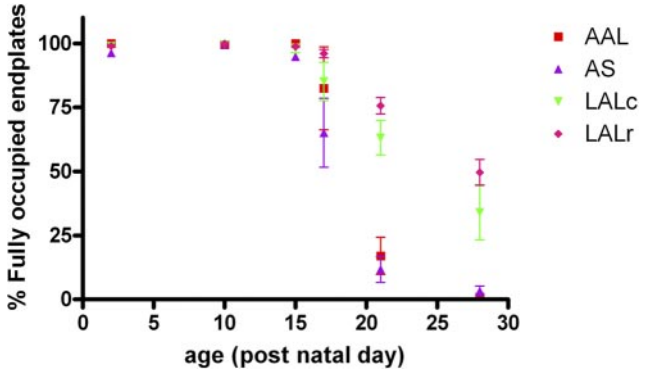

Figure 5. Muscle phenotype has a subtle modifying effect on the loss of neonatal resistance to injury. Graph shows the percentage of fully occupied endplates remaining after 2 h of hypoxia plus 2 h of reperfusion at increasing postnatal ages in the LAL, AS, and AAL muscles. A similar trend was observed in all muscles, with protection being progressively lost from P15 until P28. Subtle differences in resistance to injury were observed between muscles, with the transition to adult phenotype being delayed in the rostral band of the LAL compared to the caudal band, while both bands of LAL underwent a slower transition than that observed in AS and AAL. Values are means ± SE; n = 6 muscles, 3 mice/time point.

Neuromuscular junctions from neonatal mice are resistant to Wallerian degeneration following nerve injury

The observation of neonatal resistance to hypoxia-reperfusion injury lead us to question whether neonates might also be resistant to neurodegenerative stimuli, such as traumatic nerve injury (20). In adults, nerve section triggers rapid onset of Wallerian degeneration in the distal axon and motor nerve terminal at the neuromuscular junction (21), but surprisingly, there are no data on the immediate effects of nerve section in neonates. To investigate neonatal responses to Wallerian degeneration, we moved to a well-characterized hind-limb system, as experimental denervation of the small cranial muscle is technically unreliable. We performed tibial nerve cuts and subsequently analyzed neuromuscular junction morphology in 1st–3rd deep lumbrical muscles. In young adult mice (P25), 24 h following a tibial nerve cut, almost all neuromuscular junctions had undergone Wallerian degeneration, with 3% remaining fully occupied, 8% partially occupied, and 89% denervated (**Fig. 6**). In contrast, the identical procedure in neonatal mice at an age known to be resistant to hypoxic stimuli (P17) resulted in minimal denervation, with 86% of endplates innervated, 2% partially occupied, and 12% denervated 24 h after nerve section (Fig. 6). Furthermore, an identical procedure performed at an intermediate time point at P20, with muscle analysis at P21, indicated that this striking preservation of morphology after insult was maintained, with 92% fully occupied, 6% partially occupied, and 2% vacant. This result suggests that the developmental switch in the vulnerability to Wallerian degeneration occurs very rapidly, between P21 and P25. Together, the data demonstrate that neonatal neuromuscular junctions are resistant to diverse, physiologically relevant injury stimuli, and that this characteristic is developmentally regulated.

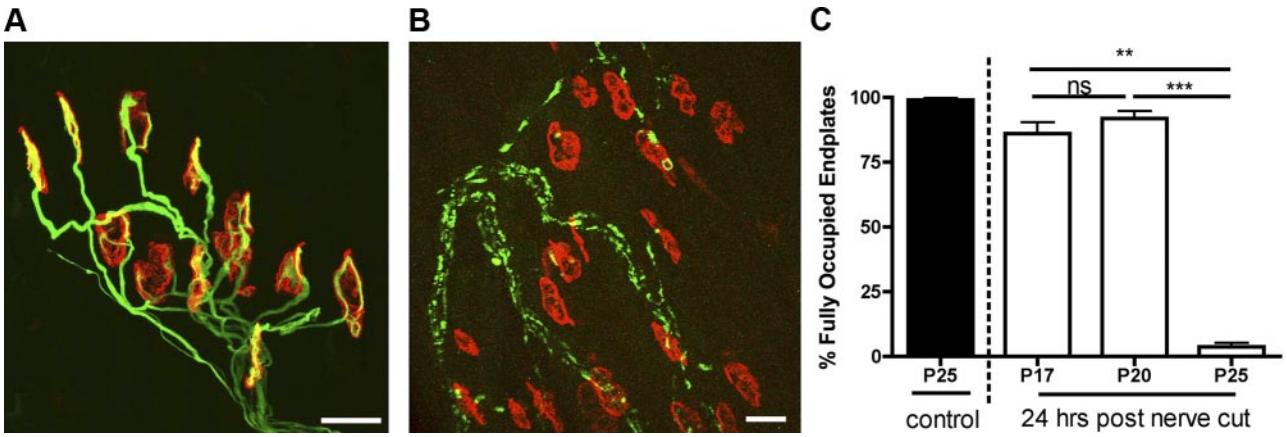

Figure 6. Neonatal neuromuscular junctions are also protected from Wallerian degeneration. *A*, *B*) Representative confocal micrographs of neuromuscular junctions in lumbrical muscles from mice at P17 (*A*) and P25 (*B*) 24 h after tibial nerve lesion. *C*) Percentage of fully occupied endplates remaining 24 h following axotomy in P17, P21, and P25 mice. Widespread degeneration, fragmentation, and denervation was observed in axotomized P25 muscles (*B*, *C*). In contrast, at P17 and P21, significant protection of neuromuscular junctions was observed, with 86 and 92% of neuromuscular junctions, respectively, remaining structurally intact 24 h following a nerve cut (*A*, *C*). Scale bars = 20 μm. Values are means ± SE; $n = 13$ muscles 5 mice at P17; $n = 18$ muscles, 3 mice at P20; $n = 47$ muscles, 16 mice at P25. $^{**}P < 0.01$, $^{***}P < 0.001$; ANOVA with Tukey *post hoc* test.

Synaptic age does not determine resistance to injury

Finally, we asked whether synaptic resistance to injury was a function of the age of the animal *per se* or of the age or developmental maturity of the synapse. We adopted a preconditioning, reinnervation paradigm to create immature neuromuscular synapses in a mature animal by crushing one tibial nerve in adult animals and allowing it to degenerate, grow, and reinnervate lumbrical muscles in the hindfoot. Four weeks later, when synapses had recently been reestablished, we cut both tibial nerves and tested whether these newly formed "immature" synapses were more resistant to Wallerian degeneration than "mature" synapses in adult mice. The data collected clearly indicate that there was no difference in the course of Wallerian degeneration between the immature and mature synapses (**Fig. 7**). The newly formed synapses did not show a recapitulation of their developmental, resistant phenotype, but rather showed an adult, vulnerable phenotype in line with the chronological age of the animal. This suggests that synaptic maturity is not the key factor in determining resistance, but rather that developmental changes occur in the neuron itself, or in the environment of the synapse, which results in the loss of resistance to injury.

DISCUSSION

Here, we show, for the first time, that neonatal neuromuscular junctions are resistant to two mechanistically distinct forms of injury (10), in sharp contrast to the vulnerability of adult neuromuscular junctions to identical insults. This demonstrates that development plays an important role in regulating the vulnerability of neuromuscular junctions *in vivo*. Time course experiments identified a transitional period occurring at around 2 or 3 wk of age in mice, during which the neonatal resistant phenotype converts to the adult vulnerable phenotype. Muscle subtype and location exerted only a subtle influence on resistance in neonatal mice. Experiments using a preconditioning nerve lesion in order to generate newly formed synapses in adult mice demonstrated that the reduced vulnerability in neonates was not determined by the age of the synapse *per se*, suggesting that the neonatal environment and/or neuronal age is a key regulator of vulnerability *in vivo*. Taken together, these findings clearly demonstrate a developmentally regulated increase in neuromuscular junction vulnerability to injury. It will

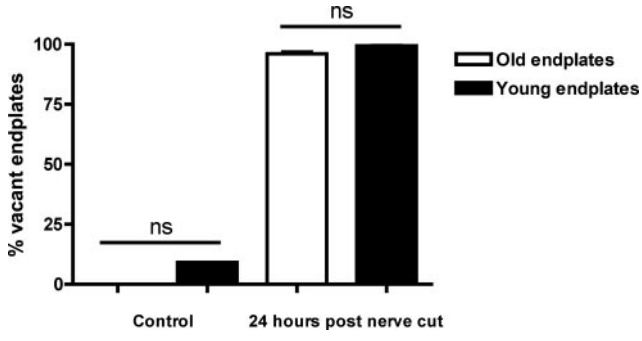

Figure 7. Immature synapses in mature mice are not resistant to Wallerian degeneration. Chart shows percentage of fully occupied (innervated) endplates in lumbrical muscles of adult (2 mo old) mice 24 h after tibial nerve lesion. Mature synapses in normal adult mice were compared with immature synapses in adult mice, generated by performing a preconditioning nerve crush 4 wk prior to experiments. An equally small number of endplates remained innervated 24 h after nerve section, irrespective of whether the synapses were mature or immature. ns, not significant, Mann Whitney *U* test. Values are means ± SE; $n = 6$ muscles, 3 mice/time point.

now be of interest to establish whether or not this phenomenon is replicated in central synapses from the brain and spinal cord.

Neuromuscular junction vulnerability *vs.* cell soma vulnerability

Whereas a number of studies have demonstrated increased vulnerability of neonatal motor neuronal cell bodies to injury (1–4), the data presented here suggest conversely that synapses are surprisingly resistant to degeneration in the neonate. In neonates, the developmental regulation of cell soma survival following nerve section, is thought to be driven by the dependence of neonatal motor neurons on trophic support from skeletal muscle. When they are disconnected from skeletal muscle by nerve section, they are unable to survive and undergo apoptosis. This apoptotic response encompasses the cell soma, its dendrites, and proximal axon stump. In the present experiments, we show that conversely the distal axon stump and synaptic connections are preserved in neonates, which is suggestive of compartmentalized mechanisms of neurodegeneration, as previously suggested (22).

What might regulate the transition from neonatal to adult phenotype at the neuromuscular junction?

We found that the transitional period during which the neonatal resistant phenotype converts to the vulnerable adult phenotype occurs during the first few weeks of life in mice. This time window coincides with a critical period at the neuromuscular junction in mice, when significant changes in neuromuscular junction morphology and physiology are occurring (23, 24). This suggests that features associated with newly formed and maturing neuromuscular junctions (*e.g.*, activity levels, polyneuronal innervation) might be responsible for conferring the resistance to injury. However, our finding that the resistance to injury was not evident in newly formed neuromuscular junctions in adult muscles implies that the maturity of the synapse itself is not regulating its vulnerability. Rather, it is likely that other factors associated with the neonatal environment, such as muscle and glial cells and/or the characteristics of neonatal motor neurons, which regulate vulnerability.

One possible environmental regulator of this developmental switch could be the nonmyelinating terminal Schwann cells that cap neuromuscular junctions. Here, a developmental regulation of the response to injury is also seen. In adults, peripheral nerve section and ensuing denervation of neuromuscular junctions induce Schwann cell sprouting followed by proliferation (25, 26). In neonates, however, denervation triggers Schwann cell apoptosis (27–29). The developmental window during which the switch between the neonatal and adult terminal Schwann cells phenotype occurs closely correlates with our observations, with significant Schwann cell apoptosis observed following nerve injury at P15, but minimal Schwann cell apoptosis at P25 (27). It is tempting to suggest that the observed neonatal resistance could be due to a delay in clearance of degenerating neuronal debris by phagocytosing Schwann cells. However, the fact that we observe significant functional protection alongside structural protection argues that any influence of terminal Schwann cells on clearing neuronal debris is likely to be minimal in conferring neonatal resistance to degeneration.

Implications for neonatal neuromuscular junction breakdown in neurodegenerative conditions

Neuromuscular junctions are early pathological targets in several neonatal neuromuscular conditions. In particular, the disruption and breakdown of neuromuscular junction connectivity is an early postnatal event in the childhood motor neuron disease, spinal muscular atrophy (6, 30, 31). Given the increased vulnerability of motor neuron cell somata in neonatal mice, the most parsimonious explanation for neuromuscular junction pathology occurring during the first few days of life in spinal muscular atrophy would be that neuromuscular junctions break down in the presence of low levels of survival of motor neuron (SMN) protein because neonatal motor neurons are, in general, highly vulnerable to neurodegenerative stimuli (*e.g.*, stress induced by low levels of SMN). However, here, we have shown that neonatal neuromuscular junctions are, in fact, "resistant", not vulnerable, to injury. If we presume that common degenerative pathways are involved, this then suggests that either the nature of the neurodegenerative insult occurring in severe forms of spinal muscular atrophy must be extreme to overcome the intrinsic resistance to degeneration, or that the loss of SMN protein in spinal muscular atrophy interferes with the molecular pathways required to confer neonatal resistance *in vivo*. Recent insights into molecular pathways altered during the early stages of spinal muscular atrophy pathogenesis (32) provide an opportunity to begin investigating these possibilities.

CONCLUSIONS

Neonatal neuromuscular junctions are resistant to various nerve injury/degenerative stimuli. This resistance is developmentally regulated and is gradually lost over the first few weeks of life until the general pattern of adult vulnerability to injury is established. The time course over which resistance to injury is lost is only subtly modified by muscle phenotype. Generating young synapses in old animals did not recreate the resistance to injury characteristic, suggesting that neuronal age or the environment of the neuromuscular junctions determines resistance to injury. FJ

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