ABSTRACT

Jacquet, Benoit Victor. A comparative study of terminal disruption after partial denervation in slow- and fast-twitch muscles of neonatal CFP mice and Wistar rats. (Under the direction of Dr. Jane L. Lubischer)

After partial denervation of mature muscle, the remaining motoneurons grow terminal sprouts, which reinnervate denervated muscle fibers. In neonatal rat soleus muscle however, not only do the remaining motoneurons fail to reinnervate nearby muscle fibers, but they also display clear morphological and physiological evidence of terminal disruption. My ultimate aim is to attain a better understanding of this disruption by performing repeated in vivo observations of individually identified terminals in transgenic mice expressing fluorescent proteins in their motoneurons. For technical reasons, these in vivo imaging studies would ideally be conducted on CFP mouse sternomastoid (SM) muscle. I therefore tested whether neonatal partial denervation of CFP mouse SM muscle also causes terminal disruption. Two to five days after neonatal partial denervation, I fluorescently labeled nerve terminals and acetylcholine receptors (AChRs) to assess the effect on terminal morphology. Terminal disruption after partial denervation did not occur as extensively in CFP mouse SM muscle (a fast-twitch muscle) as it did in rat soleus muscle (a slow-twitch muscle). This relative absence of terminal disruption in CFP mouse SM suggested that the extent/occurrence of terminal disruption after neonatal partial denervation could depend on either the species being studied or muscle fiber type. To distinguish between these possibilities, I investigated terminal disruption after partial denervation of rat SM muscle. In both soleus and sternomastoid muscles of rats, neonatal partial denervation resulted in disruption of about half of the terminals, whereas in SM muscles from CFP mice only 8% of terminals showed evidence of disruption. Therefore, it appears that the nature of terminal disruption that takes place after partial denervation of neonatal muscles is not related to muscle fiber type but may be species-specific.
A comparative study of terminal disruption after partial denervation in slow- and fast-twitch muscles of neonatal CFP mice and Wistar rats.

by

BENOIT V. JACQUET

A thesis submitted to the Graduate Faculty of
North Carolina State University
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Requirements for the degree of
Master of Science
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Approved by:

______________________________ , ______________________________
Professor John R. Godwin                 Professor Robert M. Grossfeld

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Professor Jane L. Lubischer
BIOGRAPHY

Benoit Jacquet was born in Ougrée, Belgium in 1969. He got his degree in car mechanics in 1990. After a year of military draft, he worked as an auto repair mechanic for four years. In 1995, he moved to North Carolina and started college at North Carolina State University. He graduated from the Department of Zoology in 1999. He then left the United States for a year before coming back to North Carolina State University in order to pursue a Master’s degree of science in the Department of Zoology.
I want to express my deepest appreciation to Dr. Jane Lubischer for her guidance, patience and unlimited amount of help that she offered any time I needed it. She taught me a great deal more than the nuts and bolts of neuroscience. She represents an example of respect and wisdom that I have admired immensely and will try to follow for the rest of my life.

My deepest appreciation also goes to Dr. Godwin and Dr. Grossfeld, whose help, guidance and knowledge in the field of neuroscience have opened my eyes and directed me throughout this research. I will be looking forward to learning even more from them in the years to come.

I am also very grateful for all the support that was provided to me by the department of Zoology at North Carolina State University. Dr. James Rice, our director of graduate Program has shown an unparalleled support and interest in me, and I have appreciated his help, as well as that of all the staff and professors in the department of zoology very deeply.

I also greatly appreciated James Barnes’ assistance in processing the tissues used for the synapse elimination study. James was a very helpful and reliable student, and I am very glad that we got an opportunity to work together for a few months.

And finally, I want to thank my immediate family: my mother and Jerry for their unconditional love and support, as well as for making me believe in my dreams, and also Katja, my partner in life for being so kind and encouraging even when my attitude was at its lowest.
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Slow- and fast-twitch muscle fibers display similar amounts of disrupted terminals after partial denervation.

The time allowed for recovery after surgery cannot account for the difference in the amount of terminal disruption present after partial denervation.

CFP mouse muscles did not show any significant amount of terminal disruption after partial denervation.

The lack of nodal sprouting coincides with the lack of terminal disruption in CFP mice.

A timeline of synapse elimination should be established for each muscle of each species.

Terminal disruption after partial denervation might be linked to Species-specific molecular characteristics.

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INTRODUCTION

The neuromuscular junction is composed of three major structures: the motoneuron terminal, a specialized region of the muscle fiber membrane that includes a high density of acetylcholine receptors, and terminal Schwann cells. Motoneuron terminals release the neurotransmitter acetylcholine in response to action potentials coming from the central nervous system. Acetylcholine binds acetylcholine receptors to trigger muscle contractions. Terminal Schwann cells completely cover the terminal branches and have a variety of roles essential to normal physiological function as well as responses to nerve injury. Typically, neuromuscular junctions in control animals display a close apposition of nerve terminal branches and their corresponding AChRs, meaning that the surface area covered by nerve terminals matches almost exactly the surface area of their corresponding AChRs (Rich and Lichtman, 1989; Balice-Gordon and Lichtman, 1993).

The combination of a single motoneuron and the muscle fibers that it innervates is referred to as a motor unit. During prenatal and very early postnatal development, motor units tend to be substantially larger than later in life. This difference in motor unit size is due to the fact that in early postnatal development, rat and mouse muscle fibers are typically innervated by 2 or more motoneurons. During the first two weeks after birth, all but one axon innervating a given muscle fiber is withdrawn in a process known as synapse elimination, and each motor unit becomes smaller (Redfern, 1970; Brown et al., 1976; Jansen and Fladby, 1990).

In some instances, following either trauma or the effects of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS or Lou Gehrig’s Disease), a subset of
motoneurons can degenerate and die, leaving their corresponding muscle fibers denervated and the muscle partially denervated. To experimentally study the effects of the loss of a subset of motoneurons, partial denervations can be performed by transecting some of the axons leading to a particular muscle. By doing so, one can observe what the remaining motoneurons do when their neighbors disappear. Typically, after partial denervation of adult muscle, the remaining motoneurons extend sprouts, some of which contact and reinnervate the denervated muscle fibers, thereby increasing motor unit size (Hoffman, 1950; Rafuse et al., 1992). Through this process, muscle functionality can be maintained despite loss of axons caused by injury or disease. Fast- and slow-twitch muscle fibers are reported to differ in the type of sprouting that they display after partial denervation of mature muscle (Brown et al., 1980). Terminal sprouts are the projections extended by nerve terminals after partial denervation, and nodal sprouts are sprouts that take place at the nodes of Ranvier (gaps between myelinated sections of axon), upstream from the nerve terminals. The soleus muscle of rats shows more terminal than nodal sprouting (Brown et al., 1980).

After partial denervation during the period of synapse elimination, full muscle functionality is not regained very efficiently, and motor units fail to increase in size (Brown et al., 1976; Thompson and Jansen, 1977; Brown and Ironton, 1978; Gordon et al., 1993). Early investigators suggested that the failure of motoneurons to reinnervate denervated muscle fibers after partial denervation in neonates was due to the fact that at that stage of development, motoneurons were already at their maximum capacity in terms of the number of synapses formed, and the motor units could not increase in size any further (Brown et al., 1976; Thompson and Jansen, 1977). It was demonstrated later by Lubischer and Thompson (1999), however, that very soon after the transition from multiple to single innervation, i.e.
after motor units had reached their smaller adult size, motoneurons are still deficient in their response to partial denervation compared to adult motoneurons. In fact, in early postnatal development of the rat, not only do the remaining motoneurons fail to extend terminal sprouts, but a significant fraction of them display terminal disruption. Terminal disruption is characterized by a failure of terminals to fully cover AChRs, by a decreased tetanic tension, and by a reduction in safety factor (Lubischer and Thompson, 1999).

Today, neuroscientists have come to realize that the role of glial cells all throughout the nervous system has been largely neglected in the past. In 1999, Lubischer and Thompson suggested that the reason motoneurons fail to expand their arbors after partial denervation in neonates is not because of saturation of motor unit size, but rather because of the apoptotic death of terminal Schwann cells at denervated endplates (Trachtenberg and Thompson, 1996). Schwann cells are known to play an important role in the process of motor unit expansion after partial denervation by serving as guides for terminal sprouts (Son and Thompson, 1995a, b).

The precise cause of nerve terminal disruption is not known, but a connection to glial cells is likely. Consistent with the view that Schwann cells could be mediating the fate of axon terminals after partial denervation, Trachtenberg and Thompson (1997) showed that upon application of a neuregulin isoform known as glial growth factor II (GGF 2) to developing rat muscles, Schwann cells extend processes and migrate away from the synapse, drawing nerve terminals along their path. Neuregulins are expressed by embryonic and adult motoneurons and muscle fibers (Marchionni et al., 1993; Moscoso et al., 1995), are concentrated at neuromuscular junctions during the early postnatal period (Goodearl et al., 1995; Jo et al., 1995), and mediate the survival of terminal Schwann cells at developing
neuromuscular junctions (Trachtenberg and Thompson, 1996). Trachtenberg and Thompson (1997) also demonstrated that nerve terminals in adult rats could be disrupted by the transplantation of exogenous reactive Schwann cells in their proximity. Reactive Schwann cells are Schwann cells that have lost contact with the neuron, and which have extended processes thought to provide guidance for regenerating axons and newly grown sprouts (Son and Thompson, 1995). In light of all this evidence, attributing terminal disruption after partial denervation to apoptotic Schwann cells is a very plausible argument.

Originally, fluorescent antibodies targeting axons, terminals or Schwann cells were utilized to label the neuromuscular junctions of dissected muscles. However, there are several limitations to this approach. The first limitation is that this technique rendered difficult the interpretation of whether the disruption that was observed after partial denervation was an actual loss of terminal branches or just a loss or relocation of synaptic vesicles. The primary antibody used to visualize nerve terminals (SV2) binds specifically to a synaptic vesicle protein. Therefore, an absence of labeling could possibly be interpreted as a loss of nerve terminal, but also as a loss of synaptic vesicles within the terminal. The second important limitation to this immunocytochemical method is that it is limited to a single observation per animal, which prevents investigators from following cells at one neuromuscular junction over time, making it difficult to determine the ultimate fate of the disrupted nerve terminal. Following the observed terminal disruption after partial denervation, nerve terminals could (1) completely retract and lose contact with their muscle target, (2) re-grow and completely re-cover the AChR area, or (3) remain static and later be matched in size by a decrease in the AChR area. The creation of transgenic animals that express fluorescent proteins throughout their axons and terminals will allow us to address
this question. In recent years, many lines of such transgenic mice expressing various fluorescent proteins in their axons, terminals and/or Schwann cells have been created (Feng et al., 2000; Zuo et al., 2004). These animals are ideal for repeated in vivo observations, because their motoneuron terminals and Schwann cells can be visualized without applying dyes of any sort.

In order to provide the best kind of support to neuromuscular systems disrupted by trauma or disease, it is imperative that we understand the sequence, origin, and destination of signals between affected Schwann cells, neurons, and muscles. Therefore, with the aim of describing how the key cellular players interact after partial denervation, I prepared for repeated in vivo observations of selected nerve terminals. Such in vivo observations involve the repeated photographing of individually identified neuromuscular junctions before and at several times after partial denervation. These photographs would then be compared and analyzed for any observable changes in neuromuscular junction anatomy.

My long-term goal is to describe in better detail the phenomenon of terminal disruption following partial denervation by doing repeated in vivo observations of selected motoneuron terminals in transgenic mice expressing Cyan Fluorescent Protein (CFP) in their axons and terminals, as well as Green Fluorescent Protein (GFP) in their Schwann cells. The original work performed by Lubischer and Thompson (1999) was done on rat soleus muscle. However, the soleus muscle is not very suitable for repeated in vivo imaging, as its location deep inside the leg can be an obstacle for successful repeated observations. Also, transgenic lines of rats are not currently available, which explains the switch from rats to CFP mice for my proposed experiments. I therefore decided to perform in vivo imaging on the sternomastoid of CFP mice, as this muscle has been characterized in great detail in the
literature, and its access for imaging is close to ideal (Lichtman et al., 1987; Balice-Gordon and Lichtman, 1990). However, before I could start my \textit{in vivo} investigations, I first needed to confirm that the type of terminal disruption that was originally observed in the soleus muscle of rat neonates was also displayed in the sternomastoid muscle of our transgenic CFP mice. Preliminary observations suggested that the sternomastoid muscle of CFP mice may not exhibit the same kind of terminal disruption as the soleus muscle of rats. This apparent lack of terminal disruption after partial denervation of CFP mouse sternomastoid muscle could be explained in three ways. The first possible explanation is that I partially denervated muscles at the wrong age and missed the window during which neuromuscular junctions have become singly innervated, but still do not possess the characteristics of adult neuromuscular junctions in terms of their response to injury. The second explanation could be that the muscle fiber composition differences between the two muscles could affect terminal behavior after partial denervation. The sternomastoid muscle is mostly fast-twitch fibers, whereas the muscle fiber composition of soleus is mostly slow-twitch. The third plausible explanation is that terminal disruption after partial denervation is a species-specific phenomenon whose effects are more pronounced in Wistar rats than in CFP transgenic mice.

I therefore determined the ideal age for partial denervations of the sternomastoid muscle in CFP transgenic mice, and I tested the hypotheses that (1) terminal disruption after partial denervation occurs only in muscles composed of mostly slow-twitch fibers, and (2) terminal disruption is a species-specific phenomenon that occurs in Wistar rats but not in CFP transgenic mice. My results indicate that after partial denervation of neonatal muscle, terminal disruption is more common in Wistar rat than in CFP mouse, and is not limited to slow-twitch muscles. These findings establish an intriguing and unexpected species
difference in the ability of motoneurons to compensate for the loss of neighboring motoneurons in early postnatal development.

MATERIAL AND METHODS

All experiments used CFP mice and rats bred in the Biological Resources Facility at North Carolina State University. Four animals were included in each group (24 animals total). Data are presented as mean ± SEM.

**Surgical procedures:** All surgical procedures were performed under antiseptic conditions. Animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (ketamine 0.018mg/g body weight, xylazine 0.001mg/g body weight). Surgeries were performed on a rodent heating pad (Fine Science Tools) kept at 37°C. Supplemental oxygen was provided during the time of surgery to counter the hypoxia-inducing effects of the anesthetics. After surgery, animals were given yohimbine (1 mg/g body weight) to reverse the effects of the anesthetic. Yohimbine was injected half intraperitoneally and half subcutaneously. All animals also received a subcutaneous injection of lactated Ringers solution (0.1ml per 10 g body weight) to prevent dehydration. Animals were left to recover on a heating pad until they reached a satisfactory level of consciousness and motility. Animals were monitored daily after surgery.
Time course of synapse elimination: Sternomastoid muscles were collected from 2-3 CFP mice at each of 4 ages [postnatal day 6 (P6), P8, P10, and P12], and the muscles were immunostained and mounted as described below. The muscles were then screened from one edge to the other, along the endplate band, and all the flat, easy-to-see terminals were categorized as being either singly (one axon) or multiply (more than one axon) innervated. The total percentage of multiple innervation (i.e., the percentage of muscle fibers innervated by two or more axons) was then calculated for each muscle.

Sternomastoid partial denervation: Partial denervations of the sternomastoid muscle were performed on postnatal day 10 (P10). A small incision was made on the ventral surface of the neck. The sternomastoid muscle was exposed by retracting the glandular and fatty tissues. A hand-made glass probe was then used to expose and isolate the sternomastoid nerve where it approaches the lateral edge of the sternomastoid muscle and part of this axon bundle was cut, using micro-scissors (Fine Science Tools). Exposure of the sternomastoid muscle and nerve was kept to a minimum (approximately 3 minutes). Sham-operated animals received the same treatment as their counterparts, except the nerve was not cut.

Soleus partial denervation: Partial denervations of the soleus muscle were performed on postnatal day 14 (P14). The soleus muscle is innervated by axonal projections branching from both the L4 and L5 spinal nerves (Greene, 1956). Hence, completely sectioning either one of these nerves results in a partial denervation of the soleus muscle. A longitudinal incision was made in the skin and underlying muscles along the lumbar area of the spine. The L4 spinal nerve was exposed between the transverse processes of the L4 and L5
vertebrae and cut. Sham-operated animals received the same treatment as their counterparts, except the nerve was not cut.

**Muscle removal, immunostaining and mounting:** Two days (sternomastoid) or five days (soleus) after surgery, animals were deeply anesthetized and the muscles rapidly dissected into oxygenated Ringer’s solution (in mM: 137 NaCl, 4 KCl, 1MgCl2 z 6H2O, 1 KH2PO4, 12 NaHCO3, 2 CaCl2 z 2H2O, 11 D-glucose; Liley, 1956) for a few minutes until they could be fixed in 4% paraformaldehyde buffered solution (pH=7.4) for 10 minutes. After fixation, the muscles were rinsed 3 times (10 min) in phosphate buffered saline (PBS). Muscles were then permeabilized in absolute MeOH cooled to -20°C (7 min), rinsed in PBS, blocked for 30 min (0.3% Triton X-100, 0.2% BSA, 0.1% sodium azide in PBS), and incubated overnight in the primary antibodies of interest, diluted in blocker. The following primary antibodies were used: a monoclonal antibody to neurofilament (2H3) to visualize axons (1:200; Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences) and a monoclonal antibody to the synaptic vesicle protein SV2 (1:400; Developmental Studies Hybridoma Bank) to visualize nerve terminals. Primary antibodies were visualized with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:200, Sigma, F2266). Acetylcholine receptors (AChRs) were labeled using Alexafluor 594 α-bungarotoxin (Molecular Probes Inc., Eugene, OR). Once stained, the muscles were “butterflied” and thinned before being mounted in Vectashield hard set mounting medium for fluorescence imaging (Vector Laboratories, Inc. Burlingame, CA).
**Analysis of NMJ morphology:** Images were captured using a 100X, 1.40-0.7 NA oil-immersion objective, a cooled CCD camera (Hamamatsu Orca-ER), and Openlab software (Improvision, Inc. Lexington, MA). The endplate band was screened from one edge of the muscle to the other, and each neuromuscular junction that met criteria was categorized as “normal” or “disrupted,” based on a subjective assessment of how thoroughly the terminal branches covered the AChR area. A neuromuscular junction was included in analysis only if it was located near the surface of the muscle and could be visualized in its full extent (i.e., it did not curl around the edge of the muscle fiber and was not obscured by overlying axons). Control neuromuscular junctions display a close correspondence between nerve terminals and AChRs, whereas disrupted ones are defined by substantial regions of AChR labeling without overlying terminal branches (Lubischer and Thompson, 1999). Approximately 100-140 neuromuscular junctions were categorized in each rat muscle, and 40-50 in each mouse muscle. The degree of correspondence between nerve terminal branches and AChRs was then quantified more precisely for a subset of neuromuscular junctions. This was done using an image thresholding method in the ImageJ program (NIH).

**RESULTS**

**Terminal disruption is observed in rat soleus muscle after partial denervation.**

The soleus muscle was partially denervated on P14, and 5 days later soleus muscles were removed and immunofluorescently labeled for analysis. Approximately 100 nerve terminals in each muscle were analyzed and categorized as being either “normal” or
“disrupted”. The disrupted terminals were those in which the nerve terminal was clearly not covering the entire acetylcholine receptor area of its corresponding muscle fiber. Examples of normal and disrupted terminals are shown in Figure 1.

Control P19 soleus muscles (n=4), taken from sham-operated rats, displayed an average of 3.8 ± 1.1 % of disrupted terminals, whereas partially denervated soleus muscles (n=4) displayed an average of 54 ± 1.5% of terminal disruption (Table 1, Figure 2). These results are consistent with the work of Lubischer and Thompson (1999), who reported between 50 and 55 percent of disrupted terminals in partially denervated soleus muscles of rats.

One of the ways in which my work diverged from that of Lubischer and Thompson, however, is in the method used for partial denervation of the soleus muscle. Lubischer and Thompson utilized a line of rats that possessed two axon bundles leading to the soleus muscle. Therefore, by cutting only one of those two branches (the soleus nerve), they could perform a clean partial denervation without exposing a whole subset of axons (the aberrant nerve). For this work, however, I used regular Wistar rats, and I performed my soleus partial denervations by transecting the fourth lumbar spinal nerve between the transverse processes of the L4 and L5 vertebrae. The soleus muscle gets its innervation from the L4 and L5 ventral roots (Greene, 1955), and therefore, a clean partial denervation can be performed by transecting one of these two nerves. Interestingly, the ambulatory behavior of animals after cutting the L4 or L5 root was hardly affected at all. Because the nerve transection in my experiments was done farther from the muscle, I extended the survival time to five days. Despite the difference in partial denervation method between this work and that of Lubischer and Thompson, the amount of terminal disruption after partial denervation is remarkably
consistent. This suggests that the five day survival time after L4 nerve cut in the present study was equivalent to the shorter survival time after nerve cut closer to the soleus muscle in terms of the response of the remaining motor terminals.

Figure 1: Comparison of control versus disrupted terminals in rat soleus muscles. A: Control nerve terminal and its corresponding AChR. B: Disrupted terminal showing an incomplete covering of the AChR area.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Partially denervated</th>
<th>Sham-operated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal 1</td>
<td>53%</td>
<td>Animal 5: 6%</td>
</tr>
<tr>
<td>Animal 2</td>
<td>54%</td>
<td>Animal 6: 5%</td>
</tr>
<tr>
<td>Animal 3</td>
<td>51%</td>
<td>Animal 7: 1%</td>
</tr>
<tr>
<td>Animal 4</td>
<td>58%</td>
<td>Animal 8: 3%</td>
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Table 1. Percentage of disrupted terminals in rat soleus muscle after partial denervation or sham surgery.
Figure 2. After neonatal partial denervation of Wistar rat soleus muscle, an average of over 50% of terminals no longer fully covered the underlying AChRs, significantly more than in sham-operated muscles (p < .001). Muscles were partially denervated five days earlier by sectioning the L4 spinal nerve.

**Single innervation is achieved at P10 in the sternomastoid muscle of CFP mice.**

In order to be consistent with the previous work that was done on soleus muscles, I performed my first CFP mouse sternomastoid partial denervations on P14 animals. However, the very low amount of terminal disruption that could be observed at that point prompted me to question whether P14 in the CFP mouse sternomastoid really corresponded to P14 in the Wistar rat soleus. With the help of James Barnes, an undergraduate student in our lab, I therefore established a timeline of synapse elimination in the sternomastoid muscle of our transgenic CFP mice.

In early postnatal life, mammalian muscle fibers are innervated by more than one axon (Figure 3). The first two weeks of postnatal development are the approximate time
during which muscle fibers transition from being multiply innervated to being singly innervated (Balice-Gordon and Lichtman, 1993). For studies of terminal disruption after partial denervation, it is important to not perform the partial denervation at a time when muscles are multiply innervated, because it could result in the partial denervation of individual muscle fibers, leaving AChRs uncovered by terminal branches simply because that terminal was removed by the partial denervation. Conversely, performing the partial denervation too late in development does not result in terminal disruption (Lubischer and Thompson, 1999).

Figure 4 demonstrates that most sternomastoid muscle fibers in our line of CFP transgenic mice are singly innervated by P10. The same curve drawn for rat soleus muscle shows a similar, low level of multiple innervation at P14 as is seen in this curve at P10. This suggests that P10 is the appropriate time for partial denervation on transgenic CFP mice, comparable to P14 in Wistar rat soleus muscle.

Figure 3: Comparison of multiple versus single innervation in CFP mouse sternomastoid muscles. A: The arrowheads show two separate axons leading to a single acetylcholine receptor area. B: Examples of singly innervated neuromuscular junctions.
Figure 4. Synapse elimination is essentially complete by P10 in CFP mouse sternomastoid muscle. Muscles were labeled immunohistochemically, and the number of axons innervating each endplate was counted for at least 40 endplates in each muscle (n = 2-3 animals at each age).

Terminal disruption is not observed in CFP mouse sternomastoid muscles after partial denervation

Partial denervations of CFP mouse sternomastoid muscle were performed on P10. After surgery, animals were allowed to recover for two days, as opposed to the five days allowed for soleus muscle partial denervation. This shorter recovery time is due to the fact that, since the nerve cut for the sternomastoid muscle partial denervation was done much closer to the muscle, the time needed for the dead axons to be reabsorbed and be cleared away was considerably shorter. In addition, nerve terminals and terminal Schwann cells are influenced by the partial denervation earlier when the nerve is cut closer to the muscle than when the site of nerve transection is farther from the muscle. Approximately 40 to 50 nerve terminals in each muscle were analyzed and classified as being “normal” or “disrupted”. The
lower number of terminals analyzed compared to Wistar rat soleus muscles is simply a function of muscle size. Interestingly, very little terminal disruption could be observed after partial denervation of CFP mouse sternomastoid muscle at this age (Table 2, Figure 4). As a result, I performed additional partial denervations of CFP mouse sternomastoid muscle as early as P8 (data not shown), but the amount of terminal disruption that was induced at that age was not significantly different from P10, and certainly much lower than the 54% displayed by Wistar rat soleus muscles after partial denervation on P14.

This finding raised the question of whether the lack of disruption observed in CFP mouse sternomastoid muscle was due to a difference in muscle fiber composition between soleus (predominantly slow-twitch) and sternomastoid (predominantly fast-twitch) muscles, or if this phenomenon was attributable to some species difference.

<table>
<thead>
<tr>
<th>Partially denervated</th>
<th>Sham-operated</th>
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<tbody>
<tr>
<td>Animal 1</td>
<td>2%</td>
</tr>
<tr>
<td>Animal 2</td>
<td>5%</td>
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<tr>
<td>Animal 3</td>
<td>8%</td>
</tr>
<tr>
<td>Animal 4</td>
<td>2%</td>
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</tbody>
</table>

*Table 2.* Percentage of disrupted terminals in the sternomastoid muscle of partially denervated and sham-operated transgenic CFP mice.
Figure 5. After neonatal partial denervation of CFP mouse sternomastoid muscle, an average of 4.3 ± 1.3 % terminals no longer fully covered the underlying AChRs, which was not significantly different from the 3.8 ± 1.5% observed in sham-operated muscles (p = 0.82). Muscles were partially denervated two days earlier by sectioning part of the sternomastoid nerve.

Terminal disruption is observed in rat sternomastoid muscles after partial denervation.

To determine whether muscle type or animal species was the reason for the observed difference in terminal disruption after neonatal partial denervation, I could either perform partial denervations in the soleus muscle of CFP mice, or in the sternomastoid muscle of Wistar rats. I selected the latter for practical reasons. Sternomastoid partial denervations are much less invasive, less stressful for the animal, and with the cocktail of anesthetics used, neonatal Wistar rats are significantly more resistant to surgery than neonatal CFP mice.

In order to be consistent with the sternomastoid partial denervations that were done in CFP mice, these Wistar rat sternomastoid partial denervations were done on P10. In Wistar
rat sternomastoid muscles partially denervated on P10, an average of 43 ± 4.2 % of terminals were disrupted (n=4), which was significantly greater than the 9 ± 0.6 % seen in sham-operated animals (n=4) (Table 3, Figure 6). The 9% of incompletely covered acetylcholine receptor plaques in P10 sham-operated Wistar rats appears to be a rather large number, but considering that fact that these surgeries were done so early in postnatal development, an appreciably larger number of neuromuscular junctions might still have been in the process of synapse elimination. The present results, however, strongly suggest that terminal disruption at the neuromuscular junction after partial denervation is not limited to slow-twitch muscles, but is specific only to certain species.

<table>
<thead>
<tr>
<th>Partially denervated</th>
<th>Sham-operated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal 1</td>
<td>31%</td>
</tr>
<tr>
<td>Animal 2</td>
<td>45%</td>
</tr>
<tr>
<td>Animal 3</td>
<td>51%</td>
</tr>
<tr>
<td>Animal 4</td>
<td>45%</td>
</tr>
</tbody>
</table>

*Table 3. Percentage of disrupted terminals in Wistar rat sternomastoid muscle after partial denervation or sham surgery.*
Figure 6. After neonatal partial denervation of Wistar rat sternomastoid muscle, an average of over 40% of terminals no longer fully covered the underlying AChRs, significantly more than in sham-operated muscles (p < 0.01). Muscles were partially denervated two days earlier by sectioning part of the sternomastoid nerve.

Quantification of terminal coverage of acetylcholine receptors confirms the subjective determination of terminal disruption.

In order to bring a higher level of credibility to my non-quantitative approach of nerve terminal classification, I photographed 10 “disrupted” terminals in each experimental animal and 10 “normal” terminals in each experimental and control animal. The pictures were then imported into ImageJ (NIH) in order to precisely measure the terminal area and the AChR area and to calculate the percentage of the AChR plaque covered by terminal branches. The obtained values allowed me to quantify more objectively the level of correspondence between nerve terminals and AChR area, and to validate the non-quantitative classification of disrupted terminals. CFP mouse and Wistar rat neuromuscular junctions are morphologically
different, with the CFP mouse neuromuscular junctions being more punctate in appearance. This morphological difference rendered the comparison between the 2 species difficult and even more subjective. However, despite the presence of morphological differences in neuromuscular junctions of Wistar rats and CFP mice, the precise measurements obtained in ImageJ confirmed a significant difference between disrupted terminals and normal terminals in both animals (Table 4, Figure 7). The similar numbers obtained from the two Wistar rat muscles also confirmed the validity of the non-quantitative approach.

<table>
<thead>
<tr>
<th></th>
<th>Rat Sternomastoid</th>
<th>Rat Soleus</th>
<th>Mouse Sternomastoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disrupted terminals</td>
<td>21.4 ± 4.4</td>
<td>36.6 ± 6.8</td>
<td>30.6 ± 2.5</td>
</tr>
<tr>
<td>from partially denervated muscles.</td>
<td></td>
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<tr>
<td>Normal terminals</td>
<td>85.0 ± 3.2</td>
<td>89.2 ± 4.5</td>
<td>89.7 ± 2.6</td>
</tr>
<tr>
<td>from partially denervated muscles.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal terminals</td>
<td>84.7 ± 5.1</td>
<td>82.0 ± 6.0</td>
<td>72.6 ± 5.0</td>
</tr>
<tr>
<td>from sham-operated muscles.</td>
<td></td>
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</tr>
</tbody>
</table>

*Table 4.* Amount of AChR plaque covered by the corresponding nerve terminal (expressed in percent, 10 endplates per animal, n = 4 animals). Data are presented as mean ± SEM.
Figure 7. The percentage of AChRs covered by “disrupted” nerve terminals varies between 21 and 36%. In contrast, the coverage of AChRs by normal terminals from partially denervated and sham-operated muscles varies between 72 and 89%. These data support the validity of non-quantitative classification of disrupted vs. non-disrupted terminals.

**DISCUSSION**

**Summary of findings**

My work confirmed the findings of Lubischer and Thompson (1999) who demonstrated that terminal disruption after partial denervation occurs in the slow-twitch soleus muscle of Wistar rats. However, contrary to my expectations, I did not observe the same kind of disruption in the muscles of transgenic CFP mice. Terminal disruption was also observed in a fast-twitch muscle of Wistar rats, which suggests that terminal disruption after partial denervation is not dependent on muscle fiber composition, but rather on the species in
which the study is conducted. It is important to mention that terminal disruption cannot be interpreted as a result of axonal injury during the partial nerve cut. Neonatal motoneurons show a striking inability to recover from axonal injury, and any kind of damage inflicted to them results in cell death (Lubischer and Arnold, 1995; Lowrie and Vrbova, 1992).

Furthermore, terminal disruption occurred after either a partial nerve cut (Wistar rat sternomastoid muscle) or after spinal nerve cut that does not risk damaging remaining axons (Wistar rat soleus muscle).

In light of these results, it appears that transgenic CFP mice would not be appropriate candidates for the *in vivo* work that I proposed. However, several options should still be tested that could open new doors to nerve regeneration studies, as I believe that repeated *in vivo* imaging of individual neuromuscular junctions has the potential to become an important tool in understanding neuromuscular junction dynamics after partial denervation. These options are described in greater detail below.

**Slow- and fast-twitch muscle fibers display similar amounts of disrupted terminals after partial denervation.**

There are a number of physiological and morphological differences between fast- and slow-twitch muscles and their motoneurons (Sanes and Lichtman, 2001). One of the differences that is relevant to this work is the fact that motoneurons innervating fast- and slow-twitch muscles differ in the type of sprouting that they display after partial denervation of mature muscle. Terminal sprouts are the projections extended by nerve terminals after partial denervation, and nodal sprouts are sprouts that take place at the nodes of Ranvier (gaps between myelinated sections of axon), upstream from the nerve terminals.
Motoneurons innervating fast-twitch muscles typically display more nodal sprouts, whereas motoneurons innervating slow-twitch muscles display a greater amount of terminal sprouting as a result of partial denervation (Brown et al., 1980). Atkin et al. (2005) also suggested that fast-twitch muscle fibers are more affected by the denervating effects of amyotrophic lateral sclerosis, but partial denervations are not a perfect model for amyotrophic lateral sclerosis, as they mimic the results of the disease but not the actual gradual process. Lubischer and Thompson also suggested that neonates show more nodal sprouting than adults do (1999). Moreover, they used this fact as a possible explanation for the reason why neonatal terminals appear to withdraw from their corresponding AChRs, suggesting that nodal sprouts might be more metabolically costly to the neurons than terminal sprouts, making axons unable to sustain adequate nerve terminals after partial denervation. This explanation is not consistent with my results, which suggest that neonatal Wistar rats show a similar number of disrupted terminals in fast-twitch and slow-twitch muscles, although I did not directly assess nodal sprouting. In future research, a slightly longer recovery time should be allowed after partial denervation, allowing more time for nerve sprouting, and the amount of nodal and terminal sprouting in fast- and slow-twitch neonatal muscles should be quantified within a single species.

The extent of terminal disruption after partial denervation might also be related to factors other than the fast- and slow-twitch parameter. Recent evidence suggests that skeletal muscles in the mouse can be subdivided into two previously unrecognized subtypes, designated FaSyn and DeSyn muscles (Pun et al., 2002; Santos and Caroni, 2003). These muscles differ in the pattern of neuromuscular synaptogenesis during embryonic development. This classification method suggests that the distinct rates of synaptogenesis in
the periphery may influence processes of circuit maturation through retrograde signals. While neuromuscular junctions on FaSyn and DeSyn muscles exhibit a comparable anatomical organization in postnatal animals, treatments that affect synaptic stability result in nerve sprouting, neuromuscular junction remodeling, and ectopic synaptogenesis only on DeSyn muscles. Unfortunately, all muscles have not been classified in CFP mice and Wistar rats, but this factor might be of great interest when selecting muscles that display terminal disruption for \textit{in vivo} imaging.

\textbf{The time allowed for recovery after surgery cannot account for the difference in the amount of terminal disruption present after partial denervation.}

One of the differences which could arguably account for the slightly greater amount of terminal disruption in the soleus muscle than in the sternomastoid muscle of rats is the survival time. Muscles were removed 5 days after PD of the soleus muscle and 2 days after PD of the SM muscle. This is because the soleus nerve cut was performed at a much greater distance from the target muscle, and therefore required more time for nerve degradation and reabsorption. The main aim in performing \textit{in vivo} imaging would be to determine the fate of specific neuromuscular junctions. At present, it is not known if the observed disruption after partial denervation is a phenomenon that is reversed over time, or if the disruption progresses until the surviving motoneurons completely lose their connection to the muscle. Although speculative at this stage, one hypothesis for the reason why more terminals were disrupted in the soleus muscle than in the sternomastoid muscle of Wistar rats is because the soleus axons had more time to get disrupted than their sternomastoid counterparts. However, several factors argue against this explanation. First, this scenario is technically impossible due to the
amount of time needed for the transected axons to clear out so that the remaining axons can be observed. The distinction between damaged and surviving axon is impossible to make as long as the damaged axons have not nearly completely disappeared. In addition, Lubischer and Thompson performed their partial denervation at close proximity to the soleus muscle, allowed 1-3 days for recovery, and still obtained a very similar amount of terminal disruption as I did. Also, when partial denervations are performed farther away from the muscle, the time needed to convey the information to the nerve terminals and terminal Schwann cells that the axon has been severed is proportionally increased. In light of this, seeing a similar amount of terminal disruption despite the difference in partial denervation technique suggests that the survival times chosen were appropriate in each case.

CFP mouse muscles did not show any significant amount of terminal disruption after partial denervation.

Contrary to my expectations, Wistar rats showed a great deal of terminal disruption in both fast-twitch and slow-twitch muscles after partial denervation, whereas CFP mice did not display significant terminal disruption in a fast-twitch muscle or any significant difference between control and experimental animals in denervation studies. This suggests that the small percentage of terminal disruption that is observed in CFP mice is most likely unrelated to partial denervation. Very low survival rate did not allow the inclusion of results from partial denervations performed on CFP mouse soleus muscles, but patterns in the animals I was able to examine indicate that terminal disruption did not occur in those muscles either (data not shown). These findings suggest that the neuromuscular junction of CFP mice is not only anatomically, but also developmentally different from that of Wistar rats.
I noticed morphological differences between the neuromuscular anatomy of CFP mice and Wistar rats that made a comparison between the two species difficult. Wistar rats typically have robust neuromuscular junctions (Figure 1 A) that are shaped like pretzels. CFP mice, on the other hand, have motoneuron terminals that are typically more spread and punctate (Figure 8). This difference in anatomical features could complicate the subjective classification of neuromuscular junction morphology after partial denervation in CFP mice.

One approach to counter this problem could be to combine repeated *in vivo* observations with the method of quantification used in the present work. Individual neuromuscular junctions could be photographed repeatedly at different times after partial denervation, and a quantification of AChR covering by nerve terminals could also be done repeatedly to assess any change in the nerve terminal-to-AChR correspondence.

*Figure 8.* CFP mouse terminals are typically more elongated and punctate than Wistar rat terminals.
The lack of nodal sprouting coincides with the lack of terminal disruption in CFP mice.

Trachtenberg and Thompson (1996) reported that partial denervation induces apoptosis in terminal Schwann cells, but increases the sprouting of nodal ones (Lubischer and Thompson, 1999). Similar Schwann cell apoptosis has also been shown to take place in mouse sternomastoid muscle after partial denervation (J.L. Lubischer, unpublished observations). However, this was accompanied by very little, if any, nodal sprouting (data not shown). This paucity of nodal sprouting in CFP mouse sternomastoid muscles, which show little terminal disruption, is consistent with the idea that nodal sprouting is metabolically costly and could account for terminal disruption (1999). This hypothesis predicts that an absence of nodal sprouting in CFP mouse muscles should be correlated with an absence of terminal sprouting.

A timeline of synapse elimination should be established for each muscle of each species.

Another factor that I believe should be considered in the future before continuing this type of study would be to establish a timeline of synapse elimination for all the muscles and all the species included in the study. Nervous system development is known to take place gradually from the rostral end of the animal toward its caudal end. Therefore, neuromuscular junctions might mature several days faster in rostral muscles than in caudal muscles. I started my CFP mouse partial denervations on postnatal day 14 and later switched to an earlier time as a result of not seeing the terminal disruption that I was anticipating. Switching from P14 to P10 did not affect the amount of disruption that I observed. It appears that partial denervation may be done within a window of several days without significant
changes in the effects induced. However, eliminating the possibility that that is not the case would strengthen analyses of future work.

**Terminal disruption after partial denervation might be linked to species-specific molecular characteristics.**

Finally, despite all the evidence showing differences in fast-/slow-twitch or fasyn/desyn muscle fibers, the amount of terminal disruption that takes place after partial denervation might be more related to species differences between Wistar rats and CFP transgenic mice. Differences between rats and mice have been known to exist in various physiological systems, with the newest evidence demonstrating some of those differences through tissue proteome characterization in a variety of species (D’Ambrosio et al., 2005). As closely related as those two species appear to be, there are still many differences in muscle fiber composition (Balon and Jasman, 2001) as well as in other systems. Moreover, as is the case in all transgenic modifications, the insertion of an exogenous gene has the potential of inducing subtle behavioral and physiological variations from wild-type animals. Therefore, it could also be reasonably argued that the CFP transgene might affect the disruption of nerve terminals after partial denervation. Ideally, several strains of mice should be tested in order to determine whether the differences reported here are species- or strain-related.

The reasons why neuromuscular junctions do not behave similarly in Wistar rats and CFP mice after partial denervation could be related not only to a difference in expression of chemical messengers such as neuregulins, but also to the presence or lack of receptors for those paracrine signaling molecules. Testing for the expression of those signaling molecules
and their receptors could provide insights into the mechanisms that underlie the occurrence of terminal disruption after partial denervation.

Summary and Conclusions.

Terminal disruption after partial denervation was observed in slow- and fast-twitch muscles of Wistar rats, but not in the fast-twitch sternomastoid muscle of CFP mice. These data suggest that CFP transgenic mice are not a useful system for studies of terminal disruption after neonatal partial denervation, although some alternate approaches might still be worth investigating. It appears that transgenic CFP mice and Wistar rats are intrinsically different in their neuromuscular junction anatomy and physiology.

LITERATURE CITED


