

# EFFECT OF SIALODACRYOADENITIS VIRUS INFECTION ON AXONAL REGENERATION

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The effect of sialodacryoadenitis virus (SDAV) infection on axonal regeneration and functional recovery was investigated in male Lewis rats. Animals underwent unilateral tibial nerve transection, immediate repair, and treatment with either FK506 (treated) or control vehicle (untreated). Serial walking track analyses were performed to assess functional recovery. Nerves were harvested for morphometric analysis on postoperative day 18 after an SDAV outbreak occurred that affected the 12 experimental animals. Histomorphometry and walking track data were compared against 36 historical controls. Rats infected with SDAV demonstrated severely impaired axonal regeneration and diminished functional recovery. Total fiber counts, nerve density, and percent neural tissue were all significantly reduced in infected animals ( $P < 0.05$ ). Active SDAV infection severely impaired nerve regeneration and negated the positive effect of FK506 on nerve regeneration in rats. Immunosuppressive risks must be weighed carefully against the potential neuroregenerative benefits in the treatment of peripheral nerve injuries. ©2011 Wiley-Liss, Inc. *Microsurgery* 31:458–464, 2011.

**D**isability following peripheral nerve injuries is common, resulting in impaired quality of life and decreased productivity. Accelerating nerve regeneration improves the speed and extent of recovery achieved.<sup>1</sup> Daily administration of FK506 (tacrolimus) immediately following nerve injury has been shown to accelerate nerve regeneration in crush,<sup>2–5</sup> transaction,<sup>6–10</sup> allograft,<sup>11–14</sup> and isograft<sup>15,16</sup> nerve injury animal models. But, the neuroenhancing effect of FK506 is optimal at doses that suppress the immune system.<sup>10,17</sup> Therefore, improved regeneration must be weighed against the increased risks for infection, malignancy, and systemic toxicity.<sup>18</sup>

An unanticipated sialodacryoadenitis virus (SDAV) outbreak occurred in our animal care facility, prompting us to investigate effects of viral infection on nerve regeneration in affected animals. Previous studies suggest that remyelination of the central nervous system can occur in the context of viral infection, but systemic illness may still adversely affect regeneration.<sup>19</sup> The effects of a systemic viral infection on peripheral nerve regeneration are not well studied, however. Such data may have bearing on the care of patients with peripheral nerve injury.

SDAV is a single-stranded positive-sense RNA coronavirus which commonly infects laboratory rats throughout the world.<sup>20</sup> Rats infected with SDAV demonstrate pathological, though usually temporary, changes in the

salivary glands, the lacrimal glands, upper and lower respiratory tract, the reproductive system, and general behavior.<sup>21,22</sup> Although the effect of active SDAV infection on regenerating nerves is unknown, viruses can have profound effects on nerve. For example, virus-induced axonal injury and demyelination has been used to model multiple sclerosis.<sup>23,24</sup> During the severe acute respiratory distress (SARS) epidemic, there were isolated reports proposing a link between coronavirus infection and neuromuscular dysfunction,<sup>25–27</sup> But, the effects of SDAV on peripheral nerve are unknown. This study examined the effect of SDAV infection on peripheral nerve regeneration in rats with a nerve transection injury, where animals were treated with FK506 or inert vehicle.

## MATERIALS AND METHODS

### Animal Studies Approval

All surgical procedures, experimental manipulations, and perioperative care measures were carried out in strict accordance with National Institutes of Health guidelines and were approved by the Washington University institutional Animal Studies Committee. The intended project involved study of effects of tacrolimus (FK506) on nerve regeneration. Animals were given a rodent diet (PicoLab Rodent Diet 20 #5053, PMI Nutrition International) and water *ad libitum*. Animals were promptly returned to the animal facility following surgical procedures and during the course of the experiment were monitored for weight loss, infection, or impairment.

### Operative Procedure

At the beginning of the experiment, the rats were anesthetized with medetomidine hydrochloride (Orion, NY) and ketamine hydrochloride (Fort Dodge Animal Health, Fort Dodge, IA). The tibial nerve was exposed, transected 4 mm distal to the sciatic trifurcation, and

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repaired using microsurgical techniques under microscope with four 10-0 nylon epineurial sutures. Muscle and skin were closed with 4-0 vicryl and nylon sutures respectively. On postoperative day 18, left tibial nerves were harvested for morphometric analysis and the rats were euthanized with an intracardiac injection of pentobarbital sodium (Diamond Animal Health, Des Moines, IA). The 18-day time point was selected based on prior data on optimal timing for assessment of nerve regeneration.<sup>28</sup>

### Pharmacological Regimen

A 10-mg/mL solution of dissolved crystalline FK506 (Fujisawa USA, Deerfield, IL) in 20% cremaphor (Sigma, St. Louis, MO) and 80% ethanol (Quantum Chemical, Tuscola, IL) was diluted with an aqueous solution of 75% 1,2-propanediol (Sigma, St. Louis, MO) to a 2.5-mg/mL working solution. The rats received daily subcutaneous injections of 2-mg/kg FK506 or control vehicle after nerve transection and were redosed weekly according to weight. Because of loss of weight in animals in the setting of infection, dosing was proportionately reduced.

### SDAV Outbreak and Veterinary Oversight

The SDAV outbreak became apparent approximately 1 week into the experiment. The symptoms and signs were observed first in animals immunosuppressed with FK506, but were similar among all of the animals, including bulging eyes, facial swelling, squinting, and production of porphyrin around the eyes and nose. Other associated findings included sneezing, swollen salivary glands and lymphadenopathy in the cervical lymph node chain. A few animals exhibited early keratoconjunctivitis. Diagnosis of SDAV was confirmed with ELISA laboratory assay. With the approval of the veterinary staff, animals were maintained in the facility through the course of the 3-week experiment. Supportive measures included quarantine and standard feeding and hydration. Three rats treated with FK506 showed decreased oral intake for roughly 72 hours, and these animals were dropper fed, with soluble ibuprofen in the drinking water to alleviate discomfort during recovery.

### Experimental Design

Twelve inbred adult male Lewis rats (Charles River Laboratories) were housed in a central animal care facility at Washington University. All 12 animals underwent right tibial nerve transection, immediate microsurgical repair, and administration of either FK506 (treated,  $n = 6$ ) or control vehicle (untreated,  $n = 6$ ). Walking track analysis was performed at scheduled intervals until postoperative day 18, when rats were sacrificed. The left tibial nerves were harvested for morphometric analysis, and blood from animals representing each group was collected to verify serum FK506 levels.

The primary data endpoints included peripheral nerve morphometry (using four quantitative parameters to assess nerve regeneration)<sup>29</sup> and serial walking track analysis (using print length factor, a validated assessment of postoperative hindlimb function).<sup>30</sup> Three prior studies with corresponding experimental groups and comparable methods were used to provide historical controls.<sup>7,8,31</sup> These three prior studies were conducted in the same laboratory as used for this study and involved the same animal strain (male Lewis rats) undergoing the same experimental procedures, and comparable timing endpoints. The original raw morphometry data from these three prior studies was pooled as a collective analysis to avoid bias from any individual animal cohort (Given that SDAV infection tends to spread rapidly and uncontrollably through animal facilities, having a true contemporaneous uninfected control group was not a logistical possibility in this study). The 12 SDAV infected experimental animals from the present study, either treated with inert vehicle ( $n = 6$ ) or treated with FK506 ( $n = 6$ ), were compared with the historical healthy control animals treated with inert vehicle ( $n = 17$ ) or FK506 ( $n = 19$ ).

### Functional Assessment

Functional recovery was assessed with walking track analysis performed before transection and on postoperative days 7, 13, 15, and 17. The 7-day assessment serves as a baseline for hindlimb impairment, with days 13, 15, and 17 selected to capture the typical window for improvement in recovery of hindlimb function. Hind feet were dipped in X-ray developer, the rat walked down a 14 × 56 cm corridor lined with exposed undeveloped X-ray film, and the prints were used to derive quantitative measure of hindlimb function. The length of the normal right footprint (NPL) and the length of the experimental left footprint (EPL) were measured with a digital pen linked to morphometry software and used to calculate the print length factor using the following equation:  $PLF = (EPL - NPL)/NPL$ .

### Histomorphometric Analysis

Tibial nerve segments were fixed in glutaraldehyde, dehydrated with ethanol, postfixed with osmium tetroxide, and embedded in Araldite 502. One micrometer thick cross-sections obtained 3–5 μm distal to the repair site were stained and examined by light microscopy. Microscopic images were examined with an automated digital image analysis system linked to morphometry software. At 1000× magnification, six randomly selected fields per nerve were measured to determine axon width, fiber diameter, and myelin width. These measurements were then used to calculate percentage of neural tissue ( $100 \times$  neural area/intrafascicular area), percentage of neural debris ( $100 \times$  neural debris/intrafascicular area), total

number of myelinated fibers, and nerve fiber density (fibers/mm<sup>2</sup>). Light microscopy was used to evaluate 1- $\mu$ m thick toluidine blue-stained cross-sections for the quality and quantity of regenerated nerve fibers, preservation of nerve architecture, degree of myelination, and presence of Wallerian degeneration.

### Statistical Analysis

All results are reported as mean  $\pm$  standard deviation in results and accompanying figures. Statistica version 6 (Stat-Soft, Tulsa, OK) was used for statistical analysis of the histomorphometric data. Historical control data were analyzed in comparisons against the experimental data using the same statistical methods as would be applied if an uninfected cohort of animals were enrolled at the time of the original experiment. Raw histomorphometric data on total number of fibers, density of fibers, percent nerve fiber, and fiber width was included for all historical control and experimental animals. Data were compared using Kruskal–Wallis's one way analysis of variance on Ranks for nonparametric data distribution. Statistically significant differences were found in values among the treatment groups ( $P = <0.001$ ) and post hoc analysis was performed to isolate groups that significantly differed from the others using Dunn's method for pairwise multiple comparison procedure. The alpha level was set at  $P = 0.05$ .

### RESULTS

Infected rats suffering from SDAV exhibited periorbital and perioral red–brown discoloration, decreased activity, audible rhonchi and labored respiratory effort. One infected rat in the FK506 treatment group died on postoperative day 13 and was excluded from analysis. Infected rats receiving FK506 demonstrated a mean serum level of 23 ng/mL, which is an immunosuppressive level. The serum levels of two randomly selected animals receiving only vehicle were undetectable. Infected rats on FK506 demonstrated weight loss after the first week of the study, with subsequent slow recovery over the next 2 weeks. At nadir, weight loss averaged 10% of body weight. Untreated infected rats did not demonstrate weight loss until the second week, with average of 6% weight loss and gradual recovery toward the conclusion of the experiment on day 18. Weight loss was not a primary endpoint in this study, and the data available from the veterinary division regarding weight loss and precise relation to timing of onset of infection was fragmented, although are similar to previously reported experience with weight loss following SDAV infection.<sup>22</sup> The magnitude of weight loss was greater in immunosuppressed animals treated with FK506, and the duration required for recovery from illness tended to be longer.

The functional assessment with serial walking track analysis confirmed that recovery of hindlimb function

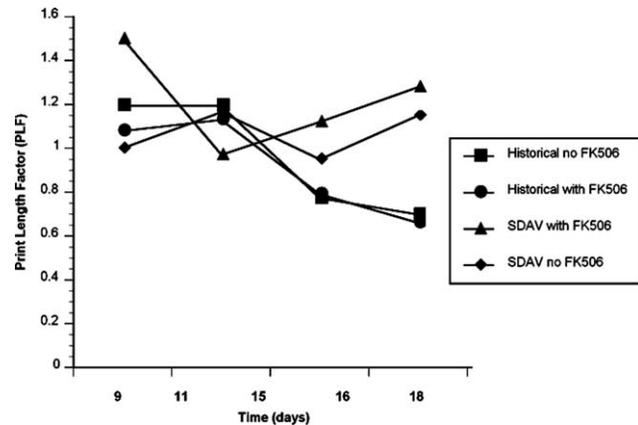
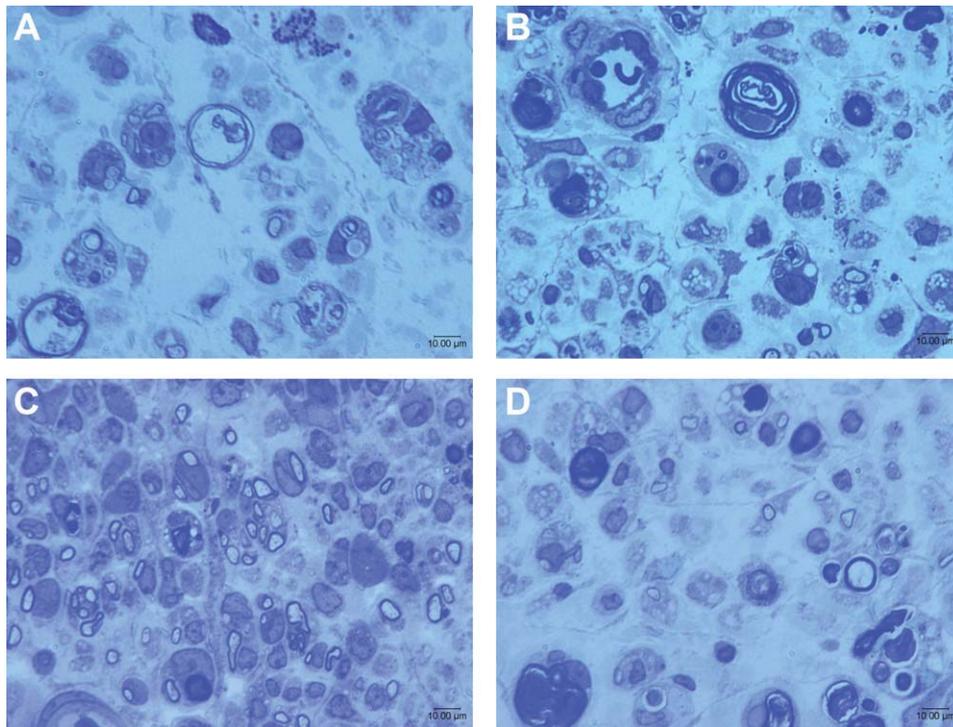


Figure 1. Walking track analysis. SDAV-infected animals demonstrated no significant improvement in hindlimb function over the course of the experiment, whereas reduction in print length factor (corresponding to recovery of hindlimb function) was observed at 14 days in controls.

was delayed in animals that suffered SDAV infection (Fig. 1). Whereas walking tracks began to normalize briskly at 14 days in healthy animals, no improvement was observed in either of the groups with SDAV infection at the experimental endpoint of 18 days. Some fluctuation in walking tracks is seen over time for all groups, but hindlimb performance was essentially flat for infected animals in contrast to the recovery of function observed after day 14 in the pooled data from animals enrolled in the three prior historical studies.

The histological assessment of nerve specimens demonstrated marked decrease in nerve regeneration with SDAV infection. Representative sections from the four comparison groups are shown in Figure 2. Only scant nerve fibers are visible amidst Wallerian degeneration in infected animals. In healthy animals, robust nerve regeneration was observed at the same time point. Cross-sections in healthy animals demonstrate densely packed and well myelinated nerve fibers, with restored neural architecture. Regenerating fibers appear as many small annulae with central clearing. The impaired regeneration in the infected animals was evident in both the paucity of fibers as well as dramatically increased myelin debris, and degenerative changes.

Quantitative histomorphometry confirmed reduced total fiber counts, decreased neural tissue, and lower fiber density in the infected animals. The complete data on total number of fibers, density of fibers, percent nerve fiber, and fiber width is reported in Figure 3. Among SDAV infected animals, the mean fiber counts were  $718 \pm 381$  and  $462 \pm 346$  for the FK506 and control animals, respectively. In contrast, healthy animals from historical controls had mean fiber counts that were roughly fivefold higher,  $3496 \pm 1189$ , and  $2242 \pm 1050$



**Figure 2.** Photomicrographs of representative histological sections of tibial nerves distal to site of nerve transection at 18 days post injury (Toluene blue stain). **(A)** Nerves of SDAV-infected animals treated with FK506, demonstrating scant nerve fibers and Wallerian degeneration, **(B)** Nerve from SDAV-infected animals treated with inert vehicle, showing similar impairment of nerve regeneration, **(C)** Healthy historical controls treated with FK506, demonstrating densely packed and maturing nerve fibers, and **(D)** Nerve sections from healthy historical controls treated with inert vehicle, also showing substantial regeneration, although less than with FK506. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

for the FK506 treated and control animals, respectively. Similar trends were observed for nerve density, with  $1181 \pm 641$  and  $643 \pm 428$  fibers/mm<sup>2</sup> for FK506 treated and vehicle infected animals, respectively versus  $7606 \pm 2389$  and  $47681 \pm 305$  fibers/mm<sup>2</sup> for the FK506 treated and control healthy animals. Percent neural tissue was  $0.923 \pm 0.5\%$  and  $0.495 \pm 0.4\%$  for FK506 treated and vehicle, respectively versus  $5.925 \pm 2.0\%$  and  $3.375 \pm 1.6\%$  for the FK506 treated and control animals. Among infected animals no significant differences were detectable between groups due to substantial interanimal variability and the overall frail nature of nerve regeneration. Nerve fiber widths were similar across all four groups ( $P > 0.05$ ).

## DISCUSSION

Studies of peripheral nerve regeneration are routinely performed in rat models, and this study demonstrated a profound impairment of axonal regeneration, with lack of functional recovery in all animals infected with SDAV. This study suggests that experiments on nerve regeneration are unreliable in the setting of an SDAV outbreak.

Because transmission of SDAV from rat to mice via direct contact has been documented, these results may also be relevant for study in mouse models, the other commonly used model in studies of peripheral nerve.

The study also underscores the potential risks of immunosuppressive agents from an infectious standpoint. The mechanism for immunosuppression by FK506 involves ligation to FK506-Binding Protein 12 (FKBP-12) to form a complex which binds and inhibits calcineurin from binding nuclear factor of activated T cells (NF-AT). The phosphatase activity of calcineurin dephosphorylates NF-AT, which joins its nuclear subunit to modulate gene transcription and T-cell activation. Calcineurin also masks the nuclear export signal of NF-AT. The neuroregenerative effects of FK506, however, may be the result of a separate mechanism mediated by FKBP-52 and involving c-jun expression.<sup>32</sup> Yoo et al. have identified genes in the SDAV genome coding for a spike protein, a small membrane protein, a membrane-associated protein, a nucleocapsid protein, and an esterase protein.<sup>33</sup> Further molecular studies may answer the question of whether viral structural proteins interact with the neurogenic and/or immunosuppressive mechanisms of FK506.

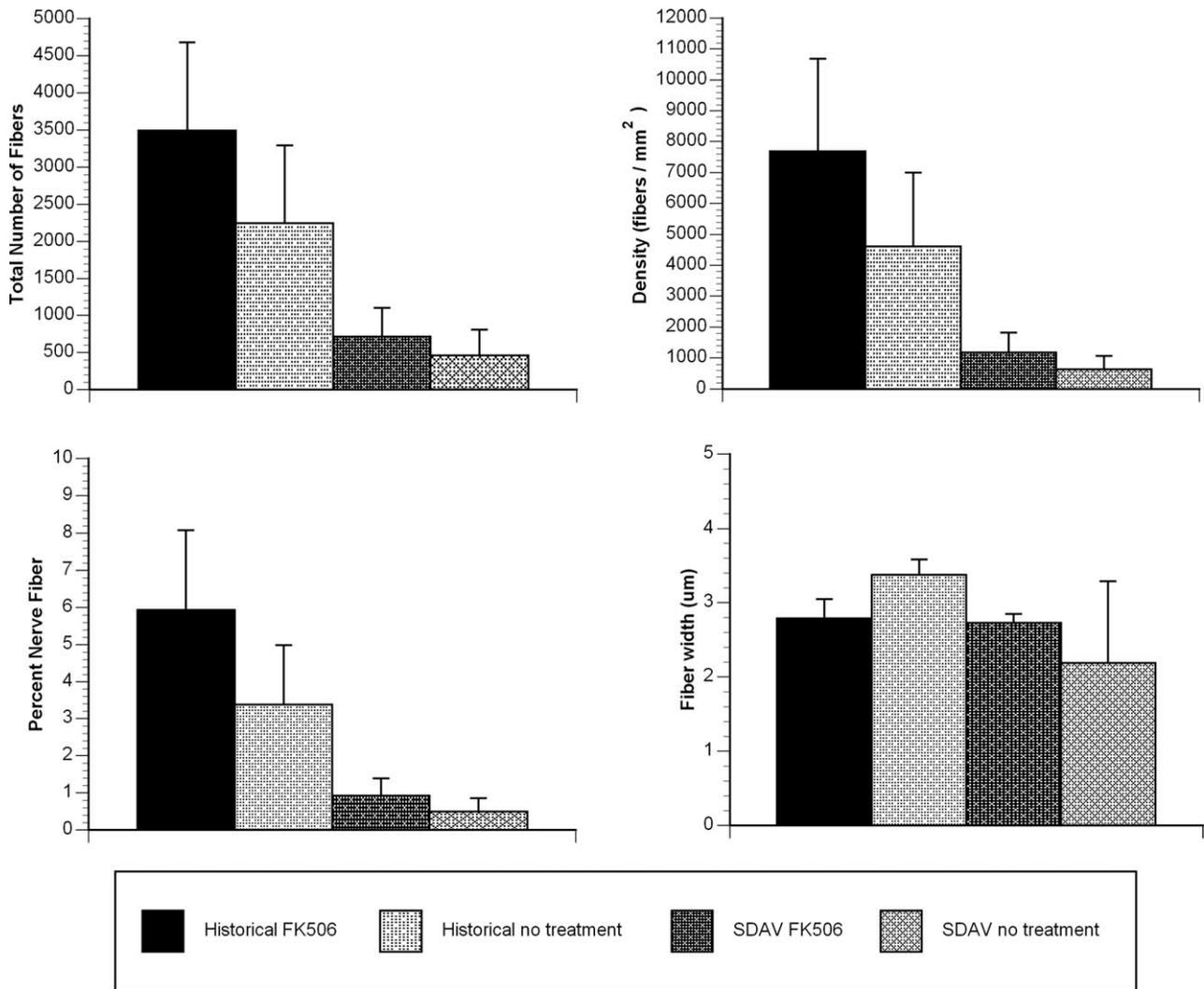


Figure 3. Quantitative histomorphometric analysis of tibial nerve fiber regeneration after nerve transection at 18 days. Comparisons of nerves from SDAV infected and healthy animals showed statistically significant reduction in fiber counts, percent nerve tissue, and nerve density was observed with SDAV infection ( $P < 0.05$ ). Mean fiber width did not differ significantly between groups. Error bars reflect standard deviation.

The earlier onset of weight loss in FK506 treated animals versus untreated animals likely relates to FK506 induced immunosuppression, which is associated with increased susceptibility to viral infection. Compared to untreated rats, immunosuppressed rats had a more fulminant course with earlier onset and more severe illness, likely due to higher viral titers in the context of compromised lymphocyte function. There also exists the possibility that secondary bacterial infection developed in some animals, although the clinical findings were pathognomonic for SDAV infection in affected animals. The positive effect of FK506 on neuroregeneration documented in numerous studies was negated by infection. The premature death in one of the infected animals receiving FK506 supports the hypothesis that mortality

and morbidity due to SDAV and other infectious agents is intensified by the burden of immunosuppression.<sup>18</sup>

The walking track analysis of both groups of rats infected with SDAV demonstrated a significant delay in functional recovery. This was an expected finding, given the very limited regeneration (and therefore minimal muscle reinnervation) observed in infected animals. Although walking track analysis is less precise than morphometry, it is an important outcome measure because it actually evaluates recovery of function, rather than regeneration (which is necessary, but not sufficient for recovery of function). The erratic pattern of the walking track data is typical of this assay, given inherent imprecision of this technique. Nonetheless, the particularly high baseline print length factor day 7 of the SDAV infected group

treated with FK506 (which exceeded 1.5), is notable. Most likely, these animals were quite sick at this time point, dragging their hindlimbs even more than is normally seen with complete nerve transaction.

Several caveats must be considered in interpreting this study. First, the study relied upon historical controls rather than contemporaneous data. Although a practical necessity, this approach introduces the theoretical concern that another factor in addition to viral infection may have contributed to poor nerve regeneration. Retrieving the raw data from three separate cohorts of rats of the same species, age, and endpoint helps mitigate concerns regarding validity of the control group. The pooling of animals from different studies does explain the relatively wide standard deviation in controls. Another limitation is that the sacrifice of animals at day 18 precluded a long-term assessment of whether there would have been "catch up" regeneration long after the infection was cleared. The sacrifice of animals at day 18 was based on prior literature on optimal timing of walking track analysis and desire to minimize suffering of any animals with residual disease related to SDAV infection. Applicability of the findings reported here to other viral illnesses or other strains of rats is uncertain. All study and control animals were male Lewis rats. Last, the difficulty in correlating precise onset of infection combined with the fragmented data on weight loss precluded statistical analysis of differences in weight loss for immunosuppressed versus immunocompetent animals infected with SDAV.

The results of this study have relevance to research on nerve regeneration in rats and may also have bearing on ongoing use of FK506 when viral infection is observed. From an experimental standpoint, nerve regeneration studies conducted in animals that are suffering viral infection must be interpreted cautiously. Based on the data from this study, we would abort any future nerve regeneration study in which the animals were afflicted with viral infection. The findings in the FK506 group are particularly relevant, as these animals became sicker than their non-immunosuppressed counterparts. FK506 is, to date, arguably the most effective systemic neuroregenerative agent tested on human patients with peripheral nerve injury. This study demonstrates that the potential benefit of enhanced nerve regeneration may be negated in the setting of infection.

## CONCLUSIONS

Rats infected with SDAV suffered impaired nerve regeneration following neurotmetic injury. SDAV viral infection dramatically slows nerve regeneration and functional recovery. Immunosuppression with FK506 also intensifies systemic manifestations of viral illness, with

early onset of symptoms, increased weight loss, and more protracted recovery.

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