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C6 deficiency does not alter intrinsic regeneration speed after peripheral nerve crush injury

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ABSTRACT

Peripheral nerve injury leads to Wallerian degeneration, followed by regeneration, in which functionality and morphology of the nerve are restored. We previously described that deficiency for complement component C6, which prevents formation of the membrane attack complex, slows down degeneration and results in an earlier recovery of sensory function after sciatic nerve injury compared to WT animals.

In this study, we determine whether $C6^{-/-}$ rats have an intrinsic trait that affects sciatic nerve regeneration after injury. To study the contribution of complement activation on degeneration and regeneration with only minimal effect of complement activation, a crush injury model with only modest complement deposition was used. We compared the morphological and functional aspects of crushed nerves during degeneration and regeneration in $C6^{-/-}$ and WT animals.

Morphological changes of myelin and axons showed similar degeneration and regeneration patterns in WT and $C6^{-/-}$ injured nerves. Functional degeneration and regeneration, recorded by *ex vivo* electrophysiology and *in vivo* foot flick test, showed that the timeline of the restoration of nerve conduction and sensory recovery also followed similar patterns in WT and $C6^{-/-}$ animals. Our findings suggest that C6 deficiency by itself does not alter the regrowth capacity of the peripheral nerve after crush injury.

1. Introduction

The peripheral nervous system has the ability to regenerate after damage. Injured peripheral nerves undergo a process called Wallerian degeneration (WD). During WD the nerve degenerates from the first node of Ranvier proximal of the damaged site toward its target muscle; myelin and axons degenerate and are removed by phagocytes (Bruck et al., 1996; Friede and Bruck, 1993; Hirata et al., 1999; Hirata and Kawabuchi, 2002; Stoll et al., 1989; van der Goes et al., 2005). WD is followed by regeneration during which Schwann cells align to form a conduit, along which sprouting axons will regrow from the proximal nerve stump toward the muscle, remyelinate and finally form a new functional nerve (Chen and Strickland, 2003; Guenard et al., 1994). The regenerative process is slow, with a maximum regrowth rate of 2 mm/day and additional problems such as hyper or hypo innervations and misdirection can also influence the functionality of the regenerated nerve (Sunderland, 1947; Wood et al., 2011).

The innate immune system, in particular the complement system, is activated within the first hours after injury (Alexander et al., 2008; Morgan et al., 1997a,b). Complement activation results in attraction and activation of blood-derived phagocytes and the formation of the membrane attack complex (MAC), a barrel shaped, pore forming, structure which consists of C5b, C6, C7, C8 and multiple C9 (C5b-9).

Complement activation as well as the recruitment and activation of blood derived phagocytes are responsible for rapid myelin clearance (Bendszus and Stoll, 2003; Bruck et al., 1996; Hirata and Kawabuchi, 2002). Previous studies showed that complement C6 deficient rats ($C6^{-/-}$), which are unable to form MAC (C5b-9), have a much slower WD after a sciatic nerve crush (Ramaglia et al., 2007) compared to wild type (WT) animals. Surprisingly, the slower WD resulted in an earlier functional recovery. Reconstitution of functional complement C6 diminished the protective effect of complement inhibition on functional recovery (Ramaglia et al., 2009).

It is still unclear how complement inhibition results in a more rapid functional recovery in C6 deficient animals. In this study, we tested whether the observed effect of complement C6 deficiency on nerve regeneration is the result of the inability to form MAC,

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or if there is an underlying intrinsic difference in regrowth properties of the nerves in the C6 deficient animals that results in for example faster neurite outgrowth or axon elongation. To this end, we used a model with only modest activation of the complement system. In this way we could study nerve de- and regeneration in an experimental setting that excluded confounding effects of high levels of complement activation, MAC deposition, in WT animals, while still sufficient damage was delivered to induce WD. To this end we developed a crush injury model with only modest complement activation. We ensured reduced complement activation by using a standardized crush injury model but with a small pair of tweezers (1.5 mm wide *vs* 2.0 mm wide, which was used in previous experiments see Ramaglia et al., 2007, 2009). The smaller crush size reduces the amount of complement activation, possibly by exposing less complement activating molecules. This model can be used to determine the contribution of C6 status and genetic background on axonal regrowth.

We studied the degeneration by morphological analysis of axons and myelin as well as complement deposition at 5 mm distal of the crush area. The morphology of the regenerated nerves was also studied in the distal tibial nerves over time. Sensory recovery was measured *in vivo* with a foot flick test. Additionally, both the degeneration and regeneration phase were studied by *ex vivo* electrophysiology (Sta et al., 2013). The *ex vivo* electrophysiology allowed us to visualize functional degeneration and regeneration over time, by measuring nerve conduction over the entire length of the nerve.

2. Materials and methods

2.1. Animals

Twelve-week old male wild type (WT) Piebold Virol Glaxo (PVG) rats (Harlan, UK) (www.harlan.com, 2013) and C6$^{-/-}$ PVG rats (Animal Research Institute Amsterdam, Academic Medical Center (AMC), University of Amsterdam, The Netherlands) of approximately 250 g were used in this study. Care and use of the animals in this study were approved by the Animal Care and Use Committee of the AMC and accorded with national guidelines. Animals were housed in standard cages with 12 h light:12 h dark cycle, and received food and water *ad libitum*.

2.2. Genotyping of PVG C6$^{-/-}$ rats

C6$^{-/-}$ animals carry a 31 base pair deletion in exon 10 of the C6 gene (Bhole and Stahl, 2004). Breeding couples were genotyped according to Ramaglia et al. (2007) to confirm the homozygous deletion in the C6 deficient group of rats.

2.3. Crush experiment

For the crush injury, animals were anesthetized (2.5 vol% isoflurane, 2 L/min O_2). The left sciatic nerve was exposed and crushed with a "1.5 mm" wide pair of tweezers above the intersection of the *musculus semimembranosus* and the *musculus vastus lateralis*, about 9.5 mm above the trifurcation of the sciatic nerve.

Calibrated force of 5.3 N was exerted three times for a period of 10 s each; in between the crushes the nerve was released. The right sciatic nerve served as a control and was exposed for 30 s. This crush procedure is similar to the procedure previously described by Ramaglia et al. (2007, 2008, 2009) with a few exceptions: (1) the force is calibrated and (2) the crush and control sites were marked with black dye (East India Ink, Talens, The Netherlands) in contrast to previous experiments where, sutures trough the epineurium were used to mark crush and control sites. After surgery, the wounds were clipped closed, and buprenorfin hydrochloride (Temgesic; Reckitt Benckiser, RB pharmaceuticals, Slough Berkhire, UK), a synthetic opioid, was administered by subcutaneous injections of 0.05 mg/kg for three consecutive days after surgery. Previous experiments did administer Temgesic after injury, when needed. This synthetic opioid does not affect the inflammatory processes following peripheral nerve damage. Animals were tested at 11 time points after the crush injury (early degeneration: 1, 3, 6 h post injury (h PI); rapid degeneration: 1, 2, and 3 days PI (d PI); regeneration: 7, 14, 21, 28 and 35 d PI.

2.4. In vivo functional test

Sensory recovery was assessed *in vivo* with a foot flick test twice a week from 3 d PI until 35 d PI. An electrical stimulus was given on a fixed position on the rat's foot sole (de Koning et al., 1986). The stimuli ranged from 20 to 50 μA, in 12 fixed steps of 2.5 μA each. The minimal current needed to elicit a retraction of the paw was recorded. The same test applied to the uninjured leg was used for normalization. To control for learned responses, trials included application of electrodes without current as well as desensitizing the animal to the sound of the switch (van der Hoop et al., 1988). These tests were also conducted prior to the experimental measurement to prevent confounding responses due to hyperalgesia and allodynia. Animals were not tested if hyper responsiveness or hypo responsiveness occurred.

2.5. Ex vivo electrophysiology

After crush, the animals were allowed to recover for 1 h to 28 days before they were killed with 300 μL 20% pentobarbital sodium (Produlab pharma, Raamsdonkveer, The Netherlands) cardially injected under deep isoflurane anesthesia. Both left and right hind leg sciatic and tibial nerves still connected at the trifurcation, were dissected. Excess fat and connective tissue were removed and the nerves were transferred within 12 min after death into a 0.9% saline solution at room temperature. Nerves were kept in saline solution until the start of the recording session on the electrode array.

Compound action potentials (CAPs) were recorded with a custom-made 32-channel silver-wire electrode-array (inter electrode distance 1.8 mm) in an airtight, moisturized holder (Cappaert et al., 2013; Sta et al., 2013). Electrical stimulation and simultaneous CAP recording was done by custom-made software written in MATLAB (The MathWorks, Inc., Natick, MA) that controlled a data acquisition system (NI-6259, National Instruments, Austin, TX). Charged-balanced biphasic rectangular voltage pulses (500 kHz sampling rate) with phase durations of 100 μs and separated by an interphase gap of 100 μs were used for stimulation (Cappaert et al., 2013). Stimulus intensities varied between 0 and V_{max}: the intensity required to evoke a CAP of maximal amplitude. V_{max} varied slightly between nerves and ranged between 0.4 and 0.9 V in the uninjured sciatic nerves and between 0.3 and 0.8 V in the injured sciatic nerves. The CAP was simultaneously recorded (25 kHz sampling rate) from the remaining 30 channels against a chosen common reference (one of the electrodes) as it propagated over the nerve. It took about 30 min to realize a complete isolated nerve measurement protocol and no decline in response was seen during the measurements period (Cappaert et al., 2013).

2.6. CAP data processing

The analysis of CAP propagation along the nerve was performed with custom-made software, written in MATLAB (Cappaert et al., 2013). The scaled response to a sub-threshold stimulus was subtracted from each recording to suppress the stimulus artifact. CAP