N- and L-Type Voltage-Gated Calcium Channels Mediate Fast Calcium Transients in Axonal Shafts of Mouse Peripheral Nerve

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In the peripheral nervous system (PNS) a vast number of axons are accommodated within fiber bundles that constitute peripheral nerves. A major function of peripheral axons is to propagate action potentials along their length, and hence they are equipped with Na+ and K+ channels, which ensure successful generation, conduction and termination of each action potential. However little is known about Ca2+ ion channels expressed along peripheral axons and their possible functional significance.

The goal of the present study was to test whether voltage-gated Ca2+ channels (VGCCs) are present along peripheral nerve axons in situ and mediate rapid activity-dependent Ca2+ elevations under physiological circumstances. To address this question we used mouse sciatic nerve slices, Ca2+ indicator Oregon Green BAPTA-1, and 2-photon Ca2+ imaging in fast line scan mode (500 Hz). We report that transient increases in intra-axonal Ca2+ concentration take place along peripheral nerve axons in situ when axons are stimulated electrically with single pulses. Furthermore, we show for the first time that Ca2+ transients in peripheral nerves are fast, i.e., occur in a millisecond time-domain. Combining Ca2+ imaging and pharmacology with specific blockers of different VGCCs subtypes we demonstrate that Ca2+ transients in peripheral nerves are mediated mainly by N-type and L-type VGCCs. Discovery of fast Ca2+ entry into the axonal shafts through VGCCs in peripheral nerves suggests that Ca2+ may be involved in regulation of action potential propagation and/or properties in this system, or mediate neurotransmitter release along peripheral axons as it occurs in the optic nerve and white matter of the central nervous system (CNS).

Keywords: Ca2+ channels, peripheral nervous system, axonal shafts

Abbreviations: ACSF, artificial cerebrospinal fluid; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N, N′, N′, N′-tetraacetic acid; Ca2+, calcium; Cd2+, cadmium; ChAT, choline acetyltransferase; DAPI, 4′,6-diamidino-2-phenylindole; K+, potassium; Kd, dissociation constant; MBP, myelin basic protein; Na+, sodium; NF200, neurofilament 200 kDa; OGB-1, Oregon Green BAPTA-1; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RRX, rhodamine red X; TBS, tris-buffered saline; TTA-P2, 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide; TTX, tetrodotoxin; VGCCs, voltage-gated calcium channels.
INTRODUCTION

In the peripheral nervous system (PNS) a vast number of axons are accommodated within fiber bundles that constitute peripheral nerves. The major function of peripheral axons is to propagate action potentials along their length, therefore axons are equipped with voltage-gated Na\(^+\) and K\(^+\) channels which ensure successful generation, conduction and termination of each action potential. In addition to Na\(^+\) and K\(^+\) channels voltage-gated Ca\(^{2+}\) channels (VGCCs) are expressed on peripheral axons. However only few groups have so far directly studied these channels and very little is known about their sub-types, developmental regulation, and function. Ca\(^{2+}\)-conductance probably mediated by VGCCs was detected in rat preganglionic cervical sympathetic nerves (Elliott et al., 1989) and in unmyelinated fibers of biopsied human sural nerve (Quasthoff et al., 1995, 1996). Increase in intra-axonal Ca\(^{2+}\) level along peripheral axons was reported during prolonged (0.3–10 s) repetitive electrical stimulation of rat vagus nerve and of biopsied human sural nerves (Wächtler et al., 1998; Mayer et al., 1999), yet the exact channel subtypes mediating Ca\(^{2+}\) influx remain unknown. In mouse postganglionic sympathetic axonal bundles Ca\(^{2+}\) transients could be detected not only during train stimulation but also in response to a single stimulus (Jackson et al., 2001). VGCCs located along axonal shafts in the PNS could be of great significance for modulation of action potential conduction velocity and/or frequency (François et al., 2015), fast axonal transport (Chan et al., 1980), or release of neuropeptides (Eberhardt et al., 2008; Spitzer et al., 2008). To play a modulatory role during these fast cellular processes, Ca\(^{2+}\) transients in the peripheral axons should occur in a millisecond time domain. Yet, it remains unclear whether rapid activity-dependent Ca\(^{2+}\) elevations take place along peripheral axons under physiological conditions, because in the previous studies either image acquisition has been done using relatively slow frame scanning mode and low sampling rate (Wächtler et al., 1998; Mayer et al., 1999; Jackson et al., 2001), or Ca\(^{2+}\) conductance has been measured with blockers of K\(^+\) channels in the bath or strongly elevated extracellular K\(^+\) concentration (Elliott et al., 1989; Quasthoff et al., 1995, 1996). It is also un-clear which types of VGCCs mediate rapid Ca\(^{2+}\) elevations in the peripheral axons. Remarkably, in the central nervous system (CNS) VGCCs are present along the axons in several structures including retina (Sargoy et al., 2014), cerebellum (Callewaert et al., 1996; Forti et al., 2000), corpus callosum (Kukley et al., 2007), optic nerve (Lev-Ram and Grinvald, 1987; Fern et al., 1995; Sun and Chiu, 1999; Brown et al., 2001; Zhang et al., 2006; Alix et al., 2008) and spinal dorsal column (Ouardouz et al., 2014). They open in a millisecond time domain upon action potential arrival and mediate fast Ca\(^{2+}\) transients which are similar to those observed in presynaptic nerve terminals at conventional neuronal synapses (Lev-Ram and Grinvald, 1987; Sun and Chiu, 1999; Kukley et al., 2007). Axonal Ca\(^{2+}\) transients in the CNS are involved in synaptic signaling between axons and oligodendrocyte progenitor cells (Kukley et al., 2007; Ziskin et al., 2007), modulation of axonal excitability, and regulation of intracellular Ca\(^{2+}\) level during axonal growth (Sun and Chiu, 1999; Bucher and Goaillard, 2011).

A major goal of the present study was to test whether VGCCs mediate rapid (in a millisecond time domain) activity-dependent Ca\(^{2+}\) elevations along mammalian peripheral nerve axons in situ under physiological conditions. Answering this question is of great importance for the follow-up research on the functional role of VGCCs in peripheral nerves in situ and in vivo, and is also of clinical and pharmaceutical relevance. Using 2-photon Ca\(^{2+}\) imaging in line scan mode we found that action potentials trigger fast Ca\(^{2+}\) transients along peripheral nerve axons in situ; these Ca\(^{2+}\) transients involve activation of N- and L-type VGCCs.

MATERIALS AND METHODS

Animals

C57BL/6N mice were originally obtained from Charles River and bred in house. All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee at the University of Tübingen.

Preparation of Sciatic Nerve Live Slices

Newborn mouse pups (P0–2) were sacrificed by decapitation without anesthesia and both sciatic nerves were isolated. The nerves were transferred to a Petri dish and maintained for ~15 min in ice-cold high-Mg\(^{2+}\) ACSF containing in mM: 124 NaCl, 1.25 NaH\(_2\)PO\(_4\), 10 MgSO\(_4\), 2.7 KCl, 26 NaHCO\(_3\), 2 CaCl\(_2\), 2 ascorbic acid, 18.6 glucose. Subsequently nerves were embedded into 2.5% low-melting agarose dissolved in 1.3 MgSO\(_4\), 0.3 NaHCO\(_3\), 10 NaCl, 1.3 MgSO\(_4\), 2.7 KCl, 26 NaHCO\(_3\), 2 CaCl\(_2\), 2 ascorbic acid, 18.6 glucose and cooled down to 37°C. One hundred micrometres-thin longitudinal nerve slices were prepared on a vibratome (VTI200S, Leica Biosystems), using ice-cold high-Mg\(^{2+}\) ACSF. The slices were placed into a Haas-type interface chamber and maintained at room temperature up to 8 h perfused with normal ACSF gassed with 95% O\(_2\) and 5% CO\(_2\).

Ca\(^{2+}\) Indicator Injection

Individual sciatic nerve slices were loaded with a high-affinity Ca\(^{2+}\) indicator Oregon-green-BAPTA-1 (OGB-1 AM) or low-affinity indicator Magnesium Green, as described previously (Regehr, 2000). Briefly, 50 µg of Ca\(^{2+}\) indicator was dissolved in 20 µl of pluronic acid in dimethyl sulfoxide (20% w/v). Four hundred microliter normal ACSF was added to this solution. The final Ca\(^{2+}\) indicator concentration was ~100 µM. A volume of 5 µl of the indicator solution was loaded into a glass micropipette (diameter ~3–6 µm) which was lowered into a nerve slice; a small positive pressure was applied for 10–15 min. Subsequently the slice was washed with ACSF for ~15 min.

Ca\(^{2+}\) Imaging with Two-Photon Excitation Microscopy

Individual nerve slices filled with Ca\(^{2+}\) indicator were transferred to a recording chamber mounted on the stage of a 2-photon
**TABLE 1 | List of antibodies used for immunohistochemistry.**

| Antibody name                             | Antibody number and the company name | Dilution |
|-------------------------------------------|--------------------------------------|----------|
| **Primary antibodies**                    |                                      |          |
| Rabbit anti-Ca_{1.2} (L-type VGCCs)       | A85156, Millipore                    | 1:100    |
| Rabbit anti-CACNA 1B                      | ab66426, Abcam                      | 1:100    |
| (N-type VGCCs)                            |                                      |          |
| Goat anti-choline acetyltransferase (ChAT)| AB144P, Millipore                   | 1:100    |
| Rat anti-myelin basic protein (MBP)       | ab7349, Abcam                       | 1:125    |
| Chicken anti-neurofilament 200 kDa       | ab4680, Abcam                       | 1:1000   |
| **Secondary antibodies**                  |                                      |          |
| Goat anti-chicken Alexa-Fluor-488         | A-11039, Invitrogen                 | 1:500    |
| Goat anti-rabbit Rhodamine-Red-X          | 111-295-003, Dianova                | 1:500    |
| Goat anti-rat Alexa-Fluor-633             | A-21094, Invitrogen                 | 1:500    |
| Donkey anti-rabbit Alexa-Fluor-488        | A-21206, Invitrogen                 | 1:500    |
| Donkey anti-goat-Cy3                      | 705-165-003, Dianova                | 1:500    |
| Donkey anti-chicken-Cy5                   | 703-176-155, Dianova                | 1:500    |

Analysis of Ca^{2+} Imaging Data

The amplitude of the Ca^{2+} fluorescence signal was measured in parts of axonal bundles positioned in the focal plane, as the ratio of the difference between the peak fluorescence and the resting fluorescence $\Delta F = F - F_0$ and the resting fluorescence $F_0$, after background subtraction. Background region was chosen as the less bright area in the field of view (FOV; not more than 10 µm away from the recorded axon). The analysis was performed using custom-written macros for IgorPro (WaveMetrics, Lake Oswego, OR, USA). 10–90% rise-time of Ca^{2+} transients was measured manually using hairline cursors in IgorPro. To determine the decay time constant, a mono-exponential function was fitted to the decaying part of the transient, from the peak until $\sim$500 ms after the peak. The graphs show mean ± standard error (SEM).

Measurement of Axon Diameter

To estimate the diameter of single axons within small axonal bundles from which Ca^{2+} transients were recorded, 3D line-scan pictures (z-stack) of the axons loaded with OGB-1 were recorded. The brightness profile of the line-scans was plotted, and the diameter of single axons was measured in the plane where the axons were in focus. ImSpector Pro Software (LaVision Biotech, Germany) was employed for these measurements.

Immunohistochemistry and Confocal Laser Scanning Microscopy

Newborn mouse pups (P0–2) were sacrificed by decapitation without anesthesia and both sciatic nerves were isolated. The nerves were transferred to a Petri dish and maintained for $\sim$15 min in ice-cold high-Mg^{2+} ACSF containing in mM: 124 NaCl, 1.25 NaH2PO4, 10 MgSO4, 2.7 KCl, 26 NaHCO3, 2 CaCl2, 2 ascorbic acid, 18.6 glucose. The nerves were fixed for 1 h in 4% paraformaldehyde (PFA) prepared in phosphate-buffered saline (PBS). PBS contained, in mM: 4.3 Na2HPO4, 1.6 NaH2PO4, 150 NaCl. Afterwards the nerves were washed with PBS (3 times × 15 min) and transferred to 30% sucrose solution in PBS, where they were kept overnight at 4°C. The nerves were then embedded into Tissue-Tek (Sakura Finetek Europe, Netherlands) and frozen at $\sim$80°C. Ten micrometre thick slices were prepared with a Leica CM3050S Cryotome and Leica 819 Microtome blades, and transferred onto the glass slides. The slices were washed (3 times × 15 min) with tris-buffered saline (TBS), and incubated in blocking solution for 2 h at room temperature. TBS contained, in mM: 100 Sigma 7–9, 154 NaCl. Blocking solution contained: 3% bovine serum albumin and 0.2% Triton-X in TBS. The slices were incubated with primary antibody overnight at 4°C, washed in TBS (3 times × 15 min), and incubated with secondary antibody coupled to a fluorescent dye for 3–4 h at room temperature. For double and triple immune-labeling the antibodies were applied sequentially, i.e., first primary followed by first secondary, followed by second primary, followed by second secondary, etc. All antibodies were applied in the blocking solution. Washing of slices with TBS (3 times × 15 min) was performed after incubation with each antibody. At the end of the immuno-labeling procedure counterstain 4’,6-diamidino-2-phenylindole (DAPI, 5 mg/ml) was applied for 5 min at room temperature. The slices were washed with water, dried, covered with Vectashield (Vector Laboratories, Inc, Burlingame, CA, USA), and sealed with nail-polish. The list of antibodies used in this study is given in Table 1. Confocal images were acquired with confocal laser scanning microscope LSM-710 (Zeiss, Germany) equipped with 40× objective (Plan-Apochromat 40×/1.3 Oil DIC M27, Zeiss, Germany). The dyes were excited with the following laser-lines: 405 nm for DAPI, 488 nm for Alexa-Fluor-488, 568 nm for rhodamine-red-X (RRX) or Cy3, and 633 nm for Alexa-Fluor-633 or Cy5. The pinhole was set to 34–38 µm depending on the wave-length, and was adjusted so that the optical section for each channel was 0.9 µm. Images for multiple channels were acquired sequentially, and care was taken that parts of the emission spectra from which the light was collected for different dyes do not overlap.
FIGURE 1 | Electrical stimulation triggers Ca\(^{2+}\) transients along axonal shafts in neonatal mouse sciatic nerve. (A) Left: 2-photon image (single plane) of an axonal bundle filled with Ca\(^{2+}\) indicator Oregon Green BAPTA-1 (OGB-1) in a mouse sciatic nerve slice. The white dotted line delineates the contour of the axonal bundle. The white arrow shows orientation of the axons in the bundle. The yellow line indicates the ROI located \(\sim 300 \mu m\) away from the injection site. The white star indicates the position of stimulation electrode. Scale bar 20 \(\mu m\). (A) Right, top: plot of fluorescence intensity in the ROI, vs. time. The plot was obtained during the line scan along the ROI (500 lines scanned at 500 Hz). (A) Right, bottom: corresponding example trace showing increase in fluorescence intensity of OGB-1 upon electrical stimulation of axonal bundle with a single pulse. The trace is an average of three successive sweeps. The red bar indicates the time point of electrical stimulation. (B) Examples of imaged axons included in the study. White arrows show the orientation of axons in each neuronal bundle. The yellow line indicates the region of interest ROI located \(\sim 300 \mu m\) away from the injection site. Scale bars 12 \(\mu m\). (C) Bar-graphs showing average 10–90% rise time of 7.73 ± 0.56 ms (mean ± SEM; \(n = 6\)) and average decay time constant of 323 ± 30 ms (mean ± SEM; \(n = 7\)) for Ca\(^{2+}\) transients recorded with OGB-1. (D) Example traces showing increase in fluorescence intensity of Magnesium Green (left) or OGB-1 (right) upon electrical stimulation of sciatic nerve axons with a single pulse. Each trace is an average of three successive sweeps. Red lines show mono-exponential fit to the decaying phase of each transient. (E) Histogram showing the diameter size distribution of the thin axons within bundles. Fifty-two axons within 18 bundles were analyzed.

FIGURE 1 | Continued

Electrical Stimulation of Nerve Bundles Triggers Ca\(^{2+}\) Transients Along Sciatic Nerve Axons

The first goal was to test whether activity-dependent Ca\(^{2+}\) transients occur along mouse sciatic nerve axons in a millisecond time domain, and to assess whether high- or low-affinity indicator works best to measure these transients. We performed 2-photon Ca\(^{2+}\) imaging in nerve slices filled with a high-affinity Ca\(^{2+}\) indicator OGB-1 AM (K\(d = 170\) nM) or a low-affinity Ca\(^{2+}\) indicator Magnesium Green (K\(d = 6 \mu M\)), while stimulating axons electrically (Figures 1A,D). We aimed to image small axonal bundles which had constant diameter (in the range of 3–12 \(\mu m\)) over the length of tens of micrometers (Figure 1B). We estimated that the diameter of thin axons comprising these bundles was in the range of 0.6–2.4 \(\mu m\) (Figure 1E). Each region of interest (ROI) was selected as a line placed perpendicular to the orientation of the axons (Figure 1A). We avoided to image cellular structures appearing as varicosities and potentially being growth cones or cut-and-reseated axons. To ensure that we record Ca\(^{2+}\) transients selectively in axons, but not in the developing Schwann cells, we acquired all scans far from the indicator injection site (>300 \(\mu m\)). This was important, as we observed that at the injected site both Schwann cells and axons took up the dye, while far from the injection site only axons were stained with the indicator and no glial cells were labeled (Figure 1A left,B). Based on the previous studies (Thaxton et al., 2011) and our own unpublished observations, the end-to-end overlap. Images were further analyzed with ZEN Software (Zeiss, Germany).

Chemicals and Drugs

All chemicals were obtained from Sigma (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany). OGB-1, Magnesium Green, and pluronic acid were obtained from Invitrogen (LifeTechnologies GmbH, Darmstadt, Germany). Tetrodotoxin (TTX), TTA-P2, ω-conotoxin GVIA, and ω-agatoxin IVa were obtained from Alomone Labs (Jerusalem, Israel). Stock solutions were prepared according to manufacturer’s instructions and stored at \(-20^\circ C\).

Statistics

Statistical analysis was performed using SPSS statistics Software (Version 23.0, IBM Corp. Armonk, NY, USA). Statistical significance of the drug effect was determined with paired-samples T-test. All values are shown as the mean ± SEM. Differences were considered significant at \(p < 0.05\) (*\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\)).
length of a Schwann cell in the sciatic nerve slice prepared from a neonatal mouse is no longer than 300 µm. In addition, Schwann cells in neonatal sciatic nerve are not coupled via gap-junctions (own unpublished observation). Hence, at the distance of >300 µm from the injection site, which exceeds the length of a Schwann cell in our preparation, we could selectively image the axons.

While stimulating sciatic nerve axons electrically with single pulses every 30 s, we repeatedly executed fast line-scans (500 Hz) perpendicular to the orientation of the axons, and tracked changes in the fluorescence of OGB-1 (Figure 1A right) or Magnesium Green (not shown). Stimulation of axons led to a fast increase of Ca\(^{2+}\)-dye fluorescence which then decayed back to baseline, indicating that changes in Ca\(^{2+}\) level occur in sciatic nerve axons upon electrical activity (Figure 1A right). The peak amplitude of Ca\(^{2+}\) transients in axons loaded with OGB-1 and stimulated with single pulse was typically several times larger than in axons loaded with Magnesium Green, and the signal-to-noise ratio was much better with OGB-1 compared to Magnesium Green (Figure 1D). Further, with Magnesium Green we usually had to stimulate the axons with trains (e.g., 3–50 pulses at 25–100 Hz) rather than with single pulses in order to detect Ca\(^{2+}\) transients. The 10–90% rise-time and the decay time constant of Ca\(^{2+}\) transients recorded with OGB-1 were 7.73 ± 0.56 ms (n = 6) and 323 ± 30 ms (n = 7), respectively (Figure 1C). Ca\(^{2+}\) transients recorded with Magnesium Green were very small upon single pulse stimulation, therefore it was difficult to estimate rise and decay time reliably even when several sweeps were averaged. We could do it only in one experiment where the 10–90% rise-time was 4.48 ms and the decay time constant was 166 ms (Figure 1D). Based on these findings we decided to use a high-affinity Ca\(^{2+}\) indicator OGB-1 for our experiments, aiming for higher signal sensitivity but keeping in mind that OGB-1 likely reports an overestimate of rise- and decay time of Ca\(^{2+}\) transients along the axons (Regehr, 2000).

**Ca\(^{2+}\) Transients Along Sciatic Nerve Axons Depend on TTX-Sensitive Action Potentials**

In brain slices, electrical stimulation of gray and white matter axons results in activation of VGCCs located in presynaptic boutons or along axonal shafts (Koester and Sakmann, 2000; Kukley et al., 2007). This activation depends on action potentials mediated by TTX-sensitive Na\(^{+}\) channels. As peripheral nerves contain both TTX-sensitive and TTX-resistant Na\(^{+}\) channels (Kostyuk et al., 1981), we tested whether Ca\(^{2+}\) transients in sciatic nerve axons are inhibited by TTX. We stimulated the axons electrically with single pulses at 0.033 Hz and acquired line-scans as described above. After verifying that the amplitude of evoked Ca\(^{2+}\) transients remains stable for at least 10 min, we applied TTX (1 µM) via the bath. TTX reduced the peak amplitude of Ca\(^{2+}\) transients by 97 ± 6% (Figures 2A,B) indicating that Ca\(^{2+}\) transients along the axons depend on action potentials mediated by TTX-sensitive Na\(^{+}\) channels. However, in one experiment we found that the amplitude of Ca\(^{2+}\) transients was decreased only by 68% upon TTX application (not shown), suggesting that TTX-resistant Na\(^{+}\) channels and/or Na\(^{+}\)-action-potential independent mechanisms may partially mediate evoked Ca\(^{2+}\) increase along sciatic nerve axons.

**Ca\(^{2+}\) Transients Along Sciatic Nerve Axons Involve Ca\(^{2+}\) Influx from the Extracellular Space**

To investigate the origin of Ca\(^{2+}\) transients in peripheral nerve axons, we perfused the slices with ACSF containing reduced Ca\(^{2+}\) concentration (1.8, 1.2 or 0.5 mM Ca\(^{2+}\) instead of 2.5 mM). The total divalent concentration was maintained constant by adjusting the levels of Mg\(^{2+}\) ions in the bath. Under these conditions, the peak amplitude of Ca\(^{2+}\) transients was reversibly reduced by 11 ± 2% in 1.8 mM Ca\(^{2+}\) (Figures 3A,D; n = 3), 29 ± 3% in 1.2 mM Ca\(^{2+}\) (Figures 3B,D; n = 3), and 55 ± 3% in...
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Ca2+ indicate time-period when solution with reduced Ca2+ concentration: 1.8 mM (after bath application of extracellular solution containing reduced Ca2+). Amplitude of Ca2+ transients evoked by single pulse stimulation, before, during and after bath application of extracellular solution with reduced Ca2+. Figures 3C,D; n = 3. These findings suggest that evoked Ca2+ transients along sciatic nerve axons involve Ca2+ influx from the extracellular space.

Notably, in the experiments with various Ca2+ concentration in the bath we observed that relationship between the peak amplitude of Ca2+ transients (ΔF/F) and extracellular Ca2+ concentration is not linear, but Ca2+ influx tends to saturate with increasing Ca2+ level in the bath (Figure 3D). This non-linearity may be explained by the fact that Ca2+-binding site(s) at the membrane surface or within the channel pore, to which Ca2+ ions have to bind in order to pass through the channel, get saturated at higher extracellular Ca2+ concentration (Augustine and Charlton, 1986; Mintz et al., 1995). Alternatively, the observed non-linearity may be explained by slight saturation of OGB-1 when Ca2+ transients are recorded with 2.5 mM Ca2+ in the bath.

Ca2+ Transients Along Sciatic Nerve Axons are Mediated By VGCCs

In physiological or pathological conditions Ca2+ transients along the axons can be mediated by Ca2+ entry through different routes, e.g., VGCCs (Lev-Ram and Grinvald, 1987; Fern et al., 1995; Sun and Chiu, 1999; Brown et al., 2001; Zhang et al., 2006; Alix et al., 2008; Sargoy et al., 2014), reversal of Na+/Ca2+ exchanger (Lehning et al., 1996), release from intracellular stores (Matute, 2010; Villegas et al., 2014), or ionotropic Ca2+-permeable receptor channels (Kinkelin et al., 2000; Matute, 2010). Our goal was to test whether VGCCs contribute to Ca2+ entry along peripheral nerve axons. To address this question, we stimulated sciatic nerve axons electrically, acquired fast line-scans and perfused the slices with ACSF containing Cd2+ (50 μM), a broad-spectrum VGCCs blocker. Cd2+ strongly decreased the peak amplitude of Ca2+ transients, i.e., by 86 ± 2% (Figure 4A; n = 6). We also tested whether application of higher concentration of Cd2+, i.e., 100 μM, results in a stronger inhibition of evoked Ca2+ transients. This was indeed the case, as Ca2+ transients were inhibited by 94 ± 3% p < 0.01 (not shown; n = 3). However, application of 100 μM Cd2+ also caused a steady increase of both basal and peak fluorescence of OGB-1. Increase of Ca2+ indicator fluorescence in the presence of Cd2+ has also been reported in other preparations, e.g., in cultured hippocampal neurons (Ermolyuk et al., 2013) or in rat vagus nerve fibers (Wächtler et al., 1998). Possible explanations of this phenomenon are: rise of intracellular Ca2+ levels triggered by Cd2+ (Ermolyuk et al., 2013), binding of Cd2+ ions to the Ca2+ indicator (Regehr and Atluri, 1995) or block of Ca2+-ATPase by Cd2+ with subsequent accumulation of intracellular.
FIGURE 4 | Ca$^{2+}$ transients in mouse sciatic nerve are mediated by voltage-gated Ca$^{2+}$ channels (VGCCs). (A) Left: time course of the amplitudes of axonal Ca$^{2+}$ transients evoked by single pulse stimulation before and during application of Cd$^{2+}$. “a” and “b” indicate the time-period from which 10 successive sweeps were averaged (corresponding average is shown on the right of the time course). Horizontal bar indicates time-period of Cd$^{2+}$ application. (A) Middle: example traces recorded before (black) and during (green) application of 50 µM Cd$^{2+}$. Each example trace represents an average of 10 successive sweeps. Black vertical bar indicates time-point of electrical stimulation. (A) Right: summary bar graphs showing the effect of Cd$^{2+}$ on the amplitude of Ca$^{2+}$ transients vs. control. Fifty micromolar Cd$^{2+}$ reduces the fluorescence by 86 ± 2% (mean ± SEM) of control values (n = 6; ***p < 0.001). (B–E) Left: time course of the amplitudes of axonal Ca$^{2+}$ transients evoked by single pulse stimulation, before and after application of a specific blocker of voltage-gated Ca$^{2+}$ channels, VGCCs: N-type VGCCs (Continued)
Ca\textsuperscript{2+} (Yuan et al., 2013). Taken together, our findings suggest that VGCCs contribute to Ca\textsuperscript{2+} transients along sciatic nerve axons.

**Pharmacological Characterization of VGCCs Located Along Sciatic Nerve Axons**

According to their electrophysiological and pharmacological properties VGCCs are classified into L-, N-, P/Q-, R-, and T-type (Dolphin, 2006). L-, N-, P/Q-, and R-type channels (also called high-voltage-activated channels) open to large membrane depolarizations and show a long-lasting current (Bean, 1985). T-type channels (also called low-voltage-activated channels) open by smaller voltage changes and show a transient current (Huguenard, 1996). To investigate which subtypes of VGCCs are involved in Ca\textsuperscript{2+} entry along peripheral nerve axons, we challenged evoked Ca\textsuperscript{2+} transients with specific blockers of different VGCC subtypes. Application of N-type VGCC blockers \(\omega\)-conotoxin GVIA (1 \(\mu\)M) reduced the peak amplitude of Ca\textsuperscript{2+} transients by 40 \(\pm\) 3\% (\textbf{Figures 4B,F}; \(n = 4\)). L-type VGCC blockers nisoldipine (1 \(\mu\)M) or nifedipine (10 \(\mu\)M) inhibited the peak amplitude of Ca\textsuperscript{2+} transients by 15 \(\pm\) 3\% (\textbf{Figures 4C,F}; \(n = 5\)) or by 12 \(\pm\) 7\% \(p = 0.32\) (not shown; \(n = 2\)), respectively. In contrast, application of P/Q-type VGCC blocker \(\omega\)-agatoxin IVA (500 nM) or T-type VGCC blocker TTA-P2 (1 \(\mu\)M) did not significantly change the peak amplitude of Ca\textsuperscript{2+} transients: amplitude in the presence of \(\omega\)-agatoxin IVA was reduced by 4 \(\pm\) 6\% (\textbf{Figures 4D,F}; \(n = 4\)) while in the presence of TTA-P2 it was reduced by 4.2 \(\pm\) 7\% (\textbf{Figures 4E,F}; \(n = 5\)). These results indicate that Ca\textsuperscript{2+} influx along sciatic nerve axons is partially mediated by N- and L-type VGCCs while P/Q and T-type VGCCs are not involved.

**Immunohistological Evidence for VGCCs in the Mouse Sciatic Nerve**

To obtain additional independent evidence for the presence of VGCCs in the developing mouse sciatic nerve, we performed immunohistochemistry. We found that both L- and N-type VGCCs were present in the nerve, but their localization was different. L-type VGCCs appeared on bundles of thin axons which often showed weaker labeling with neurofilament (NF200) than the other axons in the nerve (\(n = 3\) animals, \textbf{Figures 5A–H}). The axons expressing L-type VGCCs showed no co-labeling with myelin basic protein (MBP; \(n = 3\) animals, \textbf{Figures 5I–L}) or choline acetyltransferase (ChAT), a marker of motor axons (\(n = 3\) animals, \textbf{Figures 5M–P}). These findings suggest that L-type VGCCs are expressed by non-myelinated sensory fibers.

N-type VGCCs appeared on myelinated axons (\(n = 3\) animals, \textbf{Figures 6A–D}) while ChAT (\(n = 3\) animals, \textbf{Figures 6I–L}), and other axons expressing N-type VGCCs were negative for ChAT (\(n = 3\) animals, \textbf{Figures 6I–L,P–R}). These data point to the fact that N-type VGCCs are present on myelinated sensory and motor fibers.

**DISCUSSION**

The first important finding of the present study is that transient increases in axoplasmic Ca\textsuperscript{2+} concentration take place in axonal shafts of neonatal mouse peripheral nerve when axons are stimulated electrically with single pulses. Further, we show for the first time that Ca\textsuperscript{2+} transients in peripheral nerves \textit{in situ} are fast, i.e., occur in a millisecond time-domain. Up to now few studies have reported transient activity-dependent Ca\textsuperscript{2+} elevations along peripheral nerve axons \textit{in situ} (Elliott et al., 1989; Quasthoff et al., 1995, 1996; Wächter et al., 1998; Mayer et al., 1999; Jackson et al., 2001). However, no reasonable conclusion regarding kinetic parameters of Ca\textsuperscript{2+} transients can be made from these studies because the time-course of Ca\textsuperscript{2+} transients is rate-limited by slow acquisition, i.e., slow frame scanning mode and low sampling rate (~2.5 Hz; Jackson et al., 2001). At the same time Ca\textsuperscript{2+} transients with fast kinetics have been reported in axons of dorsal root ganglion neurons in culture but the involvement of VGCCs in Ca\textsuperscript{2+} elevations in culture has not been investigated (Lüscher et al., 1996). In the present study we used fast acquisition mode, i.e., line-scanning at 500 Hz, and found that action potentials in mouse sciatic nerve axons \textit{in situ} trigger axoplasmic Ca\textsuperscript{2+} elevations which rise relatively fast (10–90\% rise-time is ~7.7 ms) and decay back to baseline with a slower time constant \(\tau\) of ~320 ms, as estimated with high-affinity Ca\textsuperscript{2+} indicator OGB-1. These values are quite similar to those obtained with a Ca\textsuperscript{2+} indicator of comparable KD in other preparations, including mouse cerebellar mossy fiber boutons (Delvendahl et al., 2015) and presynaptic terminals of rat calyx of Held (Borst et al., 1995). Remarkably, aiming for sufficient sensitivity and good signal-to-noise ratio during imaging of small axons in neonatal mouse nerve, we selected a high-affinity Ca\textsuperscript{2+} indicator OGB-1 (KD = 170 nM) for our experiments. The forthcoming of this experimental design is that OGB-1 may be too slow to precisely follow rapid changes in intra-axonal Ca\textsuperscript{2+} concentration, and most likely also adds some buffer capacity to the axoplasm (Regohr and Atluri, 1995). Hence, the factual activity-dependent Ca\textsuperscript{2+} dynamics in the axoplasm is likely to be even faster than reported by OGB-1. Taken together, our findings indicate that transient activity-dependent Ca\textsuperscript{2+} elevations along
peripheral nerve axons can occur on a rapid time-scale, similar as it happens at synaptic boutons or along axonal shafts in the CNS.

The second important finding of our study is that activity-dependent Ca$^{2+}$ transients along peripheral nerve axons in neonatal mouse depend on Ca$^{2+}$ influx from extracellular...
FIGURE 6 | Immunohistological evidence for N-type VGCCs in the developing mouse sciatic nerve. (A) Confocal image (single plane) showing labeling of sciatic nerve axons with antibody against N-type VGCC (green, Alexa-Fluor-488). (B) Confocal image (single plane) showing labeling of sciatic nerve axons with antibody against neurofilament 200 kDa (blue, Cy5). (C) Overlay of green and blue channels. (D) Negative control, i.e., both primary antibodies are omitted. Scale bar shown in (A) is the same for images (A–D). (E) Confocal image (single plane) showing labeling of sciatic nerve axons with antibody against N-type VGCCs (red, RRX). (F) Confocal image (single plane) showing labeling of sciatic nerve axons with antibody against MBP (blue, Alexa-Fluor-633). (G) Overlay of red and blue channels. (H) Negative control, i.e., both primary antibodies are omitted. Scale bar shown in (E) is the same for images (E–H). (I) Confocal image (single plane) showing labeling of sciatic nerve axons with antibody against N-type VGCCs (red, Alexa-Fluor-488). (J) Confocal image (single plane) showing labeling of sciatic nerve axons with antibody against ChAT (red, Cy3). (K) Overlay of green and red channels. (L) Negative control, i.e., both primary antibodies are omitted. Scale bar shown in (I) is the same for images (I–L). (M) Confocal image (single plane) showing labeling of sciatic nerve axons with antibody against N-type VGCCs (green, Alexa-Fluor-488). (N) Confocal image (single plane) showing labeling of sciatic nerve axons with antibody against ChAT (red, Cy3). (O) Overlay of green and red channels. Arrowheads indicate axons co-labeled with N-type VGCCs and ChAT. Scale bar shown in (M) is the same for images (M–O). (P) Confocal image (Continued)
space and involve activation of N- and L-type VGCCs. We found that a blocker of N-type VGCCs, ω-conotoxin GVIA, reduced the amplitude of Ca$^{2+}$ transients by ∼40%, the blockers of L-type VGCCs nisoldipine or nifedipine caused ∼15% reduction, while the blockers of P/Q- and T-type channels were ineffective. Furthermore, the results of our immunohistological experiments suggest that in the developing mouse sciatic nerve L-type VGCCs are present on non-myelinated sensory fibers, while N-type channels appear on myelinated motor and sensory axons. To the best of our knowledge, this is the first report about subtypes of functional VGCCs present along the peripheral nerve axons in neonatal mice. Interestingly, in neonatal rodent central (optic) nerve likewise L- or N-type VGCCs were suggested to be of functional significance (Sun and Chiu, 1999; Alix et al., 2008), while P/Q-type channels seem to get involved later during development (Alix et al., 2008). L- and/or N-type VGCCs also mediate Ca$^{2+}$ influx in adult optic nerve during pathological conditions (Fern et al., 1995; Brown et al., 2001). When we compared our findings on VGCC subtypes in neonatal mouse sciatic nerve to another preparation of peripheral nerve, i.e., adult mouse postganglionic sympathetic axon bundle, it turned out that also in those axons ∼40% of the total Ca$^{2+}$ influx is carried by N-type VGCCs, however, in contrast to our findings, L-type VGCCs were not involved (Jackson et al., 2001). In adult mouse C-fibers T-type VGCCs have been suggested to play a role in modulating action potential conduction velocity (François et al., 2015), however in the neonatal mouse we could not find T-type VGCC contribution to Ca$^{2+}$ influx along the axons. Remarkably, at the mammalian neuromuscular junction, where some of the peripheral nerve axons terminate, P/Q-type represents the major subtype of VGCCs, although L- and N-type VGCCs also play a role during development, re-innervation or pathological conditions (Katz et al., 1996; Nudler et al., 2003). At the distal nerve endings, in turn, T-type VGCCs have been found in addition to other VGCC subtypes (François et al., 2015). Hence the specific subtypes of VGCCs are likely targeted differently to different functional compartments of the same axon, and may be also differently regulated in developing and adult animals, as well as during pathological conditions. In addition to known VGCC subtypes other yet unidentified subtypes of VGCCs, or alternative routes (e.g., reversed Na$^+$/Ca$^{2+}$ exchanger, release from internal store), may contribute to activity-dependent Ca$^{2+}$ entry into the axoplasm of peripheral nerve axons. In line with this idea are our findings that Ca$^{2+}$ transients in mouse sciatic nerve are reduced only partially by specific blockers of VGCCs. Furthermore, also in the unmyelinated nerve fibers of rat vagus nerve neither L- nor N-type nor P/Q-type VGCCs mediated Ca$^{2+}$ entry along the axons, although Ca$^{2+}$ transients in that preparation were largely inhibited by Cd$^{2+}$ (Wächter et al., 1998). Hence, more experiments in various preparations of central and peripheral nerves/white matter are required to clarify this issue.

Why do peripheral nerve axons express VGCCs along their shafts, and what could be the functional significance of activity-dependent axonal Ca$^{2+}$ transients under physiological circumstances? Ca$^{2+}$ is involved in the majority of cellular functions. Importantly, as cells keep free cytosolic Ca$^{2+}$ level very low (∼100 nM), what determines the specificity and the functional output of each Ca$^{2+}$-dependent process is the amplitude, the time-course and the spatial domain of a transient change in intracellular Ca$^{2+}$ concentration (Berridge et al., 2003). Fast (microseconds to milliseconds) Ca$^{2+}$ transients are usually involved in fast cellular processes, e.g., synaptic transmission, opening of Ca$^{2+}$-dependent channels, muscle contraction, etc (Berridge et al., 2003). At axonal synaptic terminals, for example, highly localized (nano- or microdomains) rapidly rising (<1 ms) and large (∼20 fold) Ca$^{2+}$ elevations mediated by VGCCs trigger rapid release of synaptic vesicles and ensure high precision of synaptic signaling (Kandel et al., 2000). In turn, more global residual Ca$^{2+}$ changes, which are also slower and smaller in amplitude, contribute to modulation of transmitter release, e.g., synaptic potentiation (Swandulla et al., 1991; Wang and Augustine, 2015). We want to emphasize that as Ca$^{2+}$ transients recorded along peripheral nerve axons in our study rise in a range of few milliseconds (10–90% rise time ∼7.7 ms) and this time probably underestimates the true speed of Ca$^{2+}$ influx into the axon upon action potential propagation, these Ca$^{2+}$ transients are well suited to trigger or/and modulate relatively fast axonal processes. For example, transient increase in axoplasmic Ca$^{2+}$ concentration may be involved in regulation of action potential conduction or frequency through e.g., activation of Ca$^{2+}$-dependent K$^+$ and/or Cl$^-$ channels, or inactivation of Ca$^{2+}$ channels (Jirounek et al., 1991; Lüscher et al., 1996; Sun and Chiu, 1999; Alix et al., 2008). Another possible function of VGCCs and fast Ca$^{2+}$ entry in peripheral axons, rarely considered in the literature, could be the contribution to neurotransmitter release (vesicular or non-vesicular) along axonal shafts. Rapid (few milliseconds) increases in Ca$^{2+}$ concentration mediated by VGCCs take place along axonal shafts in white matter of the CNS (Lev-Ram and Grinvald, 1987; Sun and Chiu, 1999; Kukley et al., 2007). They result in build up of axonal Ca$^{2+}$ microdomains which are involved in triggering fast vesicular release of glutamate at synaptic-like junctions between axons and glia cells (Kukley et al., 2007; Ziskin et al., 2007). Intriguingly, peripheral axons also appear capable of releasing neurotransmitters (glutamate and acetylcholine) from their shafts at least in two experimental paradigms: (a) when nerves are dissected from an animal, placed in Ringer solution and stimulated electrically (Lissak, 1939; Vizi et al., 1983); or (b) when dissected nerves are pre-loaded with labeled neurotransmitters, (e.g., $^{14}$C-glutamate, tritiated choline, d-2, 3-(3)H-aspartic acid) and stimulated electrically or magnetically (Wheeler et al., 1966; DeFeudis, 1971; Weinreich and Hammerschlag, 1975; Vizi et al., 1983; Wieraszko and Ahmed, 2009). The mechanisms
of neurotransmitter release from peripheral nerve axons in situ or in vivo remain largely un-investigated. But it is tempting to speculate that peripheral axons utilize similar mechanism of release as callosal and optic nerve axons, i.e., VGCCs located axonal shafts mediate Ca\(^{2+}\) influx followed by fusion and release of neurotransmitter filled vesicles. Subsequently, released neurotransmitter may bind to its receptors on the neighboring Schwann cells. In line with this hypothesis are the recent findings in cell culture demonstrating that vesicular release of glutamate occurs along the axons of dorsal root ganglion neurons and mediates axonal-glial communication important for myelination (Wake et al., 2011, 2015). Notably, L- and N-type VGCCs expressed in peripheral nerve axons are the VGCCs subtypes which are involved in neurotransmitter release at Ribbon synapses and at conventional synapses between neurons, respectively (Catterall, 2011). Few older studies also show that axonal release in peripheral nerves resembles the axon terminal release in many respects, e.g., it depends on extracellular Ca\(^{2+}\) and is stimulated by elevated extracellular K\(^{+}\) (Dettbarn and Rosenberg, 1966; Vizi et al., 1983; Wieraszko and Ahmed, 2009). Yet, other investigators do not support these findings and suggest that the mechanism of axonal release in the peripheral nerves differs from release at synapses (Weinreich and Hammerschlag, 1975).

Finally, evidence is currently accumulating that multiple subtypes of VGCCs may contribute to injury mechanisms of central white matter axons (Fern et al., 1995; Brown et al., 2001; Tsutsui and Stys, 2013). In light of those findings it is likely that in addition to their physiological role, also VGCCs located along peripheral nerve axons may be of significance during pathological conditions, e.g., nerve injury, pain, or peripheral neuropathy.

**AUTHOR CONTRIBUTIONS**

All experiments were conducted in the laboratory of MK at the Centre for Integrative Neuroscience, University of Tübingen. MK and RB designed experiments. RB and FP performed experiments and analyzed data. MK and RB interpreted the findings, prepared the figures, and wrote the manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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