Alexa Fluor 488-conjugated cholera toxin subunit B optimally labels neurons 3–7 days after injection into the rat gastrocnemius muscle

Abstract

Neural tract tracing is used to study neural pathways and evaluate neuronal regeneration following nerve injuries. However, it is not always clear which tracer should be used to yield optimal results. In this study, we examined the use of Alexa Fluor 488-conjugated cholera toxin subunit B (AF488-CTB). This was injected into the gastrocnemius muscle of rats, and it was found that motor, sensory, and sympathetic neurons were labeled in the spinal ventral horn, dorsal root ganglia, and sympathetic chain, respectively. Similar results were obtained when we injected AF594-CTB into the tibialis anterior muscle. The morphology and number of neurons were evaluated at different time points following the AF488-CTB injection. It was found that labeled motor and sensory neurons could be observed 12 hours post-injection. The intensity was found to increase over time, and the morphology appeared clear and complete 3–7 days post-injection, with clearly distinguishable motor neuron axons and dendrites. However, 14 days after the injection, the quality of the images decreased and the neurons appeared blurred and incomplete. Nissl and immunohistochemical staining showed that the AF488-CTB-labeled neurons retained normal neurochemical and morphological features, and the surrounding microglia were also found to be unaltered. Overall, these results imply that the cholera toxin subunit B, whether unconjugated or conjugated with Alexa Fluor, is effective for retrograde tracing in muscular tissues and that it would also be suitable for evaluating the regeneration or degeneration of injured nerves.

Key Words: Alexa Fluor-conjugated cholera toxin subunit B; calcitonin gene-related peptide; microglia; motor neurons; neural tract tracing; optimal time window; sensory neurons; somatotopic organization; sympathetic neurons; tibialis anterior muscle
In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., Waltham, MA, USA) for 2 hours at room temperature. The spinal cord and 4′,6-diamidino-2-phenylindole (1:50,000; Cat# D3570; Thermo Fisher, 647 (1:500; Cat# B3540; Life Technologies Corporation, Waltham, MA, USA) were cryoprotected overnight in 25% sucrose. The gastrocnemius muscle sections were stained with α-bungarotoxin AF (250 mg/kg, Sigma-Aldrich, Darmstadt, Germany) and transcardially perfused after 0.5 (12 hours), 1, 2, 3, 5, 7, and 14 days following the AF488-CTB injection. The labeled neuronal components were found to be similar. However, the DRG sections from days 3 and 14 were examined for Nissl, CGRP, ChAT, CI, and CD11b, and carried out as follows: (1) CGRP + Nissl to label the motor and sensory neurons; (2) ChAT + Nissl to label the motor neurons; (3) CI Casp-3 + Nissl to show neuronal degeneration; and (4) CD11b + Nissl to show the microglia around the labeled neurons. For (1), the DRG sections and spinal sections were both stained; for the other sections, the spinal sections were stained with 0.1 M PB containing 0.05% Triton X-100 and 0.5% BSA. The DRG sections were then transferred to mouse anti-CGRP monoclonal antibody (1:1000; Cat# AB14133; Thermofisher) and incubated overnight in a dilution of 1% normal donkey serum and 0.5% Triton X-100, and left overnight at 4°C. After washing three times with 0.1 M PB, the sections were exposed to the secondary antibodies of donkey anti-mouse AF 547 (1:1000; Cat# A21203; RRID: AB_2079751; Millipore, Temecula, CA, USA), rabbit anti-CI Casp-3 (1:500; Cat# 96615; RRID: AB_2341188; Cell Signaling Technology, Danvers, MA, USA), and CD11b (1:1000; Cat# MCA275R; RRID: AB_2341188; Bio-Rad, Hercules, CA, USA) in a dilution of 1% normal donkey serum and 0.5% Triton X-100. Sample observation The samples were viewed using the Virtual Slide System (V5120, Olympus, Tokyo, Japan), and representative regions were selected to view in more detail using a confocal imaging system (FV1200, Olympus). The images were collected in successive frames of 5 μm for the whole-mount DRG, sympathetic sensory chain, and in the 0.1 mm thick transverse sections of the DRG for the spinal sections and the longitudinal DRG sections; these were then integrated into a single in-focus image. The data were analyzed using the Olympus Image Processing Software and processed using Adobe Photoshop/Illustration CS5 (Adobe Systems, San Jose, CA, USA). The quality of the neuronal labeling was mainly judged according to the morphology of the labeled motor neurons, especially their dendritic branches.

**Materials and Methods**

**Ethics statement**

This study was approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (approval No. 2021-04-15-1) on April 15, 2021, and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington DC, USA). All of the experiments were designed and reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020).

**Animals**

The study focused on male rats because the cyclic hormonal changes in 2-month-old female rats may affect their physiological state (Sarkar and Mitsugi, 1990). Twenty-seven adult male specific-pathogen-free Sprague-Dawley rats (weight 220–250 g, aged 7–8 weeks) were obtained from the National Institutes of Food and Drug Control (license No. SCXK (Jing) 2017-0005). The rats were provided with free access to food and water, and kept in a 12-hour light-dark cycle with controlled temperature and humidity.

**Surgical procedure for tracer injection**

The rats were anesthetized by inhalation of 2% isoflurane (YiPin, Shijiazhuang, China) at 0.5 L/min. The body temperature was maintained at 37°C by using a heating pad (Zhike, Zhengzhou, China) during surgery. Twenty-four randomly-selected rats were used for the single-tracer experiment with AF488-CTB. After a small skin incision, 4 μL 0.1% AF488-CTB (Cat# C22841, Invitrogen-Molecular Probes, Eugene, OR, USA) was slowly injected (within 1 minute) into the medial gastrocnemius muscle of the left hind limb. As the DRG and the sympathetic chain are both small and transparent, the AF488-CTB and α-bungarotoxin labeled the axon terminals and motor endplates, using laser scanning confocal microscopy. It was found that the AF488-CTB labeling revealed the neuromuscular junctions, which were observed in a 6–7 mm in diameter area.

**Tissue processing**

For the single-tracer experiment, randomly-selected rats were euthanized after 0.5 (12 hours), 1, 2, 3, 5, 7, and 14 days following the AF488-CTB injection (n= 6 after 3 days; n=3 for the other time points). For the dual-tracer experiment using AF488-594-CTB, the three rats were euthanized after 3 days. The rats were anesthetized intraperitoneal with 2,2,2-tribromoethanol (250 mg/kg, Sigma-Aldrich, Darmstadt, Germany) and transcardially perfused with 0.1 M phosphate buffer (PB; pH 7.4) containing 0.1% paraformaldehyde and 0.1% phosphate buffer (PB; pH 7.4). The labeled motor neurons, spinal cord, dorsal root ganglion (DRG), and lumbar sympathetic (paravertebral) chain were dissected and post-fixed for 2 hours using the same fixative, and then cryoprotected overnight in 25% sucrose.

Using a microtome (REI-710, Yamato Koki Industrial, Asaka, Japan), the gastrocnemius muscle was sliced into 80-μm-thick horizontal sections, and the spinal cord was sliced into 40-μm-thick coronal sections. However, for three of the single-tracer rats euthanized on day 3, the spinal cords were sliced into 100-μm-thick horizontal sections. All of the sections were collected in order in a six-hole Petri dish with 0.1 M PB (pH 7.4). As the DRG and the sympathetic sensory chain are both small and transparent, the AF488-CTB- and AF594-CTB-labeled neurons were directly observed in whole-mounts using a fluorescence microscope or confocal imaging system. The DRG from days 3 and 14 were then sliced into 40-μm-thick longitudinal sections for further examination, but the sympathetic chains were not sliced.

Free-floating sections from every sixth spinal coronal section and all of the spinal horizontal sections were mounted on silane-coated glass slides; these were then coveredslipped with 50% glycerin to better visualize the labeled neurons.

**Fluorescence histochemical and immunohistochemical staining**

Fluorescence histochemical or immunohistochemical staining was carried out on the sections of gastrocnemius muscle, spinal cord, and DRG. The gastrocnemius muscle sections were stained with α-bungarotoxin AF 647 (1:500; Cat# B35450; Life Technologies Corporation, Waltham, MA, USA) and 4′,6-diamidino-2-phenylindole (1:50,000; Cat# D3570; Thermo Fisher, Waltham, MA, USA) for 2 hours at room temperature. The spinal cord and DRG sections from days 3 and 14 were examined for Nissl, CGRP, ChAT, CI Casp-3, and CD11b, and carried out as follows: (1) CGRP + Nissl to label the motor and sensory neurons; (2) ChAT + Nissl to label the motor neurons; (3) CI Casp-3 + Nissl to show neuronal degeneration; and (4) CD11b + Nissl to show the microglia around the labeled neurons. The distribution of AF488-CTB labeling around the injection site was examined in the gastrocnemius muscle; this showed the distribution to be limited to an area 6–7 mm in diameter (Figure 2). Histochemical analyses were performed using GraphPad Prism 8 software (GraphPad Prism Software, La Jolla, CA, USA). The data were expressed as the mean ± standard error of the mean (SEM). The number of labeled motor and sensory neurons were determined for the 21 rats injected with AF488-CTB. For this, the motor neurons were counted in every sixth slice from the spinal cord, and the sensory neurons were counted in the whole-mount DRG.

**Results**

The distribution of AF488-CTB labeling around the injection site was examined in the gastrocnemius muscle; this showed the distribution to be limited to an area 6–7 mm in diameter (Figure 2). Histochemical analyses were performed using GraphPad Prism 8 software (GraphPad Prism Software, La Jolla, CA, USA). The data were expressed as the mean ± standard error of the mean (SEM). The number of labeled motor and sensory neurons were determined for the 21 rats injected with AF488-CTB. For this, the motor neurons were counted in every sixth slice from the spinal cord, and the sensory neurons were counted in the whole-mount DRG.

**Distribution of labeled neurons**

In the single-tracer experiment, with the injection of AF488-CTB into the gastrocnemius muscle, the labeled neurons were found to include motor, sensory, and sympathetic neurons (Figure 2). All of the labeled neurons were located ipsilateral to the injection, and they were distributed in a segmental or regional pattern. They included motor neurons located in the spinal ventral horn (VH), lumbar (L) segments, and sympathetic neurons in the L3–L6 DRG, and sympathetic neurons in the lumbar sympathetic chain.

In the dual-tracer experiment, with injections of AF488-CTB and AF594-CTB into the gastrocnemius muscle and the tibialis anterior muscle, respectively, the labeled neuronal components were found to be similar. However, the motor, sensory, and sympathetic neurons that were labeled from the gastrocnemius muscle were scattered more exterioirly within the spinal ventral horn than those associated with the tibialis anterior muscle. The labeled neurons associated with the tibialis anterior muscle and the gastrocnemius muscle were concentrated in the L4 and L5 DRG, respectively; the sympathetic neurons associated with each muscle were scattered separately within the lumbar sympathetic chain.
Neuronal morphology

Neuronal labeling with AF488-CTB was evaluated for the motor and sensory neurons at different time points following the AF488-CTB injection (Figures 4 and 5). After 12 hours (0.5 days), weak AF488-CTB labeling was observed in the cell bodies of both types of neurons, as well as in the primary dendritic arbors of the motor neurons (Figures 4 and 5). The intensity of the labeling was seen to increase on days 1 and 2 (Figures 4 and 5). On day 3, the spinal motor neurons’ secondary and tertiary dendritic arbors could be clearly seen, similar to the images typically obtained following Golgi staining (Figure 4). This detailed morphology was also apparent on days 5 and 7 (Figure 4). On day 14, however, most of the fluorescent neuronal labeling had faded to give a punctate appearance, especially on the dendritic arbors (Figures 4 and 5).

Neurochemical substances in the labeled neurons

Certain neurochemical substances were examined in the labeled neurons and compared between days 3 and 14 following the AF488-CTB injection. It was found that there were no marked changes in CGRP expression in the labeled motor and sensory neurons (Figure 6), nor in ChAT expression in the labeled motor neurons (Figure 7), even though the AF488-CTB labeling faded with time. Nissl-labeling of the AF488-CTB neurons was also found to be similar to that seen in the unlabeled neurons (Figures 6 and 7).

Potential pathological changes around the labeled neurons

We found that Cl Casp-3 was distributed around the AF488-CTB-labeled neurons forming a dotted pattern (Figure 8). There were no obvious differences between days 3 and 14. The microglia were labeled with CD11b and were shown to have small cell bodies and thin cellular processes. They were evenly distributed around the labeled motor neurons, and there were no marked morphological differences between days 3 and 14, it was also found that they were similar to the microglia around the unlabeled motor neurons in the corresponding contralateral area (Figure 8).
In this study, we examined the use of AF488-CTB for the retrograde tracing of motor, sensory, and sympathetic neurons associated with the gastrocnemius muscle in the rat. As a sensitive retrograde tracer, AF488-CTB can be used for tracing the innervation patterns of a particular muscle, or, when used together with AF594-CTB, for comparing the innervation patterns of different muscles. Our study showed that there is an optimal time-window for visualizing AF488-CTB-labeled neurons in morphological detail. In addition, we found that certain neurochemical substances were unaffected by the labeling and that there were no signs of pathology.

**Technological considerations**

CTB has previously been used as a neural tracer, both in its unconjugated form and when conjugated with horseradish peroxidase, for the anterograde, retrograde, and transganglionic tracing of neural pathways (Hirakawa et al., 1992; Ciriello and Caverson, 2016; Dong et al., 2017; Wang et al., 2018; Cui et al., 2019; Han et al., 2019; Ma et al., 2020). The AF-CTB conjugates were developed more recently and have the advantage of being robust and photostable (Conte et al., 2009); they have now been used extensively in retrograde tracing studies (Conte et al., 2009, Cui et al., 2013, Zhang et al., 2021). AF-CTB labeling can be directly visualized in tissue sections, and for as long as 14 days; however, the optimal time-window was from day 3 and 14 post-injection. It should be noted that, unlike unconjugated CTB and CTB conjugated with horseradish peroxidase (Hirakawa et al., 1992; Panneton et al., 2005; Cui et al., 2019), AF-CTB can be effectively used to label the terminals of sensory neurons in young mice (Li et al., 2011), respectively, and revealed the typical structure of neuromuscular junctions within their corresponding regions and segments, and were distinct from those associated with the tibialis anterior muscle. This was observed in the spinal cord, DRG, and sympathetic chain, and the pattern implies that AF488-CTB and AF594-CTB have similar properties for tracing the peripheral nervous system to the central nervous system. Compared with other types of fluorescent tracers (Byers et al., 2002; Katada et al., 2006; Yu et al., 2015), these markers would appear to be particularly suitable for dual-tracing experiments involving the peripheral nervous system (Cui et al., 2013; Zhang et al., 2021).

**Discussion**

In our study, we were able to visualize the neuromuscular junctions in the gastrocnemius muscle around the injection site. The axon terminals and motor endplates were labeled with AF488-CTB and α-bungarotoxin, respectively, and revealed the typical structure of neuromuscular junctions. The AF488-CTB labeling was seen to fade on day 14 (B, D), but there were no marked changes in the expression of ChAT in either the motor or the sensory neurons (A–D). (A1–B1, A2–D2, and A3–D3) The panels A–D are shown separately for AF488-CTB (A1–D1), CGRP (A2–D2), and Nissl (A3–D3). The images were obtained from an experiment that used three rats per time point. Scale bars: 50 μm. AF: Alexa Fluor; CGRP: calcitonin gene-related peptide; CTB: cholera toxin subunit B.

We examined the labeling efficiency of AF488-CTB when used alone or alongside AF594-CTB. We found that the motor, sensory, and sympathetic neurons associated with the gastrocnemius muscle were only distributed within their corresponding regions and segments, and were distinct from those associated with the tibialis anterior muscle. This was observed in the spinal cord, DRG, and sympathetic chain, and the pattern implies that AF488-CTB and AF594-CTB have similar properties for tracing the peripheral nervous system to the central nervous system. Compared with other types of fluorescent tracers (Byers et al., 2002; Katada et al., 2006; Yu et al., 2015), these markers would appear to be particularly suitable for dual-tracing experiments involving the peripheral nervous system (Cui et al., 2013; Zhang et al., 2021).

It should be noted that, unlike unconjugated CTB and CTB conjugated with horseradish peroxidase (Hirakawa et al., 1992; Panneton et al., 2005; Cui et al., 2019), AF-CTB cannot be transported through the sensory pathway across the blood–brain barrier and into the spinal cord, DRG, and sympathetic chain. This stands in contrast to unconjugated CTB, which needs to be stained using complex immunoperoxidase or immunofluorescence techniques (Hirakawa et al., 1992; Wang et al., 2018; Cui et al., 2019). This therefore enables researchers to rapidly assess whether tracing has been successful or not, and to avoid time-consuming tissue slicing.

In our study, we were able to visualize the neuromuscular junctions in the gastrocnemius muscle around the injection site. The axon terminals and motor endplates were labeled with AF488-CTB and α-bungarotoxin, respectively, and revealed the typical structure of neuromuscular junctions (Chen et al., 2016; Yin et al., 2019). This finding indicates that AF488-CTB is directly taken up by the axon terminals and then transported retrogradely to the neurons.

We examined the labeling efficiency of AF488-CTB when used alone or alongside AF594-CTB. We found that the motor, sensory, and sympathetic neurons associated with the gastrocnemius muscle were only distributed within their corresponding regions and segments, and were distinct from those associated with the tibialis anterior muscle. This was observed in the spinal cord, DRG, and sympathetic chain, and the pattern implies that AF488-CTB and AF594-CTB have similar properties for tracing the peripheral nervous system to the central nervous system. Compared with other types of fluorescent tracers (Byers et al., 2002; Katada et al., 2006; Yu et al., 2015), these markers would appear to be particularly suitable for dual-tracing experiments involving the peripheral nervous system (Cui et al., 2013; Zhang et al., 2021).

Changes in neuronal labeling over time

The labeling efficiency of neuronal tracers is affected by many factors, including the dose, concentration, injection procedure, post-injection time, and distance between the injection site and the target area (Vercelli et al., 2000; Lanciego and Wouterlood, 2011, 2020; Ling et al., 2012). We found that AF488-CTB labeling can be observed as early as 12 hours post-injection and for as long as 14 days; however, the optimal time-window was from day...
Neurochemical substances and microglial activation
We investigated whether there were any signs of pathology in the AF488-CTB-labeled neurons. For this, we selected ChAT, AChT, CiAmp, and CD11b to evaluate certain neurochemical substances and the morphological structure of the labeled neurons. These were assessed at both early and later time points post-injection.

As a neuropeptide, CGRP is involved in neuropharmacology and neurotoxicity. There is evidence that it may also be involved in motor and sensory neuron pathology (Chen et al., 2016). Chen et al. (2015) found that there were no differences in Crg expression between the AF488-CTB-labeled neurons and unlabeled neurons. There were also no differences between the early and later stages post-injection. This implies that the labeled neurons were functioning normally. We also examined ChAT in the spinal cord. This enzyme catalyzes acetycholine synthesis, and it is found in motor neurons along with CGRP (Das et al., 2021). We found that ChAT was expressed similarly in the labeled and unlabeled neurons, and also at the different time points. This therefore also implies that the AF488-CTB-labeled motor neurons were functioning normally.

In addition to the neurochemical substances, we also examined other potential signs of pathology in the labeled neurons and their surrounding glial cells using CI-Casp-3 and CD11b (Köbbert and Thanos, 2000; Beggs and Salter, 2003). In this study, we found that there were no marked differences in ChAT expression between the AF488-CTB-labeled neurons and unlabeled neurons; there were also no differences between the early and later stages post-injection. This implies that the labeled neurons were functioning normally. We also examined ChAT in the spinal cord. This enzyme catalyzes acetycholine synthesis, and it is found in motor neurons along with CGRP (Das et al., 2021). We found that ChAT was expressed similarly in the labeled and unlabeled neurons, and also at the different time points. This therefore also implies that the AF488-CTB-labeled motor neurons were functioning normally.

Long-term fluorescence imaging in vivo is currently becoming more widespread (Chen et al., 2019). This was not carried out in the present study because of technical limitations, but this could be examined in future studies. However, this could prove challenging because the labeled motor neurons are located deep in the spinal ventral horn.

Conclusion
Our results provide evidence that AF488-CTB and AF488-CRF are highly effective retrograde tracing from muscular tissue. For optimal results, the labeling should be observed during a particular time window following the injection, so that the morphological detail of the labeled neurons can be clearly seen. This technique could be used to provide neuroanatomical information concerning neuronal connections, as well as to evaluate neuronal degeneration or regeneration following peripheral nerve damage.

Author contributions: Study conception and design: JIC, WZB; neural tracer injection: JIC, JW, DXS, YTL, YHG; histochemical staining and sample observation: JIC, JW, SM, YS, YQW; data analysis and figure preparation: JW, DXS, XH; manuscript drafting: JIC, WZB. All authors approved the final manuscript.

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Availability of data and materials: The data supporting the conclusions of this article will be made available from the corresponding author on reasonable request.

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Additional files:

Additional file 1: Open peer review report 1

Additional file 2: Figure 1: Distribution of labeled motor neurons, sensory neurons and transganglionic axonal terminals on the third day after injection of CTB into the gastrocnemius muscle.

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Additional Figure 1 Distribution of labeled motor neurons, sensory neurons and transganglionic axonal terminals on the third day after injection of CTB into the gastrocnemius muscle.

(A, B) CTB labeled motor neurons at lumbar (L) 4 segment spinal ventral horn (A) and sensory neurons in L5 segment dorsal root ganglion (DRG, B). (C-E) CTB labeled transganglionic axonal terminals in gracile nucleus (C), Clarke's nucleus (D) and the laminae III and V spinal dorsal horn (E). (C1-E1) Higher magnified photo from panels C-E (arrow heads) showing the labeled transganglionic axonal terminals in detail respectively. One independent experiment was performed using three rats. Scale bars: 200 μm in A, C, D and E; 100 μm in B; 50 μm in C1, D1 and E1. AF: Alexa Fluor; CTB: cholera toxin subunit B.