

The efficacy of the fluorescent conjugates of cholera toxin subunit B for multiple retrograde tract tracing in the central nervous system

William L. Conte · Hiroaki Kamishina · Roger L. Reep

Received: 14 May 2009 / Accepted: 7 July 2009
© Springer-Verlag 2009

Abstract Cholera toxin subunit B (CTB) is a sensitive neuroanatomical tracer that generally transports retrogradely in the nervous system, and has been used extensively in brightfield microscopy. Recently, Alexa Fluor (AF) conjugates of CTB have been made available, which now allows multiple tracing with CTB. In this study, we examined the efficacy of these new AF-CTB conjugates when injected into the brain, and compared the results to our previous experiences using fluorescent 3k dextran amines. To test this, we injected AF 488 and AF 594 CTB into the anterior cingulate cortex and the medial agranular cortex in the rat, and examined the retrograde transport to the lateral posterior nucleus of the thalamus. We found that CTB was very viscous but yet very sensitive: small injection sites revealed very intense and detailed retrograde labeling. Anterograde transport was seen only when tissue at the injection site was damaged. These findings suggest that AF-CTB is a very reliable and sensitive retrograde tracer, and should be the first choice retrograde tracer for

experiments examining multiple pathways within the same brain.

Keywords Cholera toxin · Retrograde tracer · Alexa Fluor · Tract tracing · Fluorescence · Neuroanatomy

Introduction

Cholera toxin subunit B (CTB) is a neuroanatomical tracer that selectively transports retrogradely (Krout et al. 2002; Llewellyn-Smith et al. 2000; Zin-Ka-Ieu et al. 1997). Historically, CTB has been utilized as an unconjugated form, with subsequent immunohistochemical detection using the avidin–biotin complex detection method for brightfield microscopic visualization (Luppi et al. 1990). Although very useful for single-labeling experiments, this methodology is not useful for those wanting to compare multiple connections of brain regions in a single animal. The introduction of TRITC and FITC conjugates of CTB allowed researchers to conduct double-labeling experiments using CTB, but the rapid fading and less intense signal of these conjugates proved difficult.

Recently, Molecular Probes (part of Invitrogen, Inc.) has successfully attached their Alexa Fluor (AF) line of fluorescent probes to CTB, which now allows experiments to be undertaken that compare the connections of two or more regions in a single brain using a robust fluorescent dye. The AF family of dyes, which has proven to be more photostable, bright, and pH-insensitive than conventional fluorescent dyes, has never been successfully attached to a retrograde tracer (Panchuk-Voloshina et al. 1999).

The mechanism of action is based on the binding of CTB to the GM1 ganglioside of the nerve cell membrane (Masco et al. 1991; Wang et al. 1998). More specifically,

W. L. Conte (✉) · R. L. Reep
Department of Physiological Sciences, University of Florida,
Box 100144, Gainesville, FL 32610, USA
e-mail: billy.conte@mbi.ufl.edu

W. L. Conte · R. L. Reep
McKnight Brain Institute, University of Florida,
Gainesville, FL, USA

W. L. Conte
Stritch School of Medicine, Loyola University Chicago,
Chicago, IL, USA

H. Kamishina
Department of Veterinary Medicine, Iwate University,
Morioka, Japan

the larger B subunit contains cholera toxin which binds to the GM1 receptor of the cell membrane and facilitates entry into the cell (van Heyningen 1974). The A subunit does not bind to GM1, but is responsible for changing activity in the cell by activating adenylate cyclase (Fishman 1982; van Heyningen 1974). Nervous tissue is a large source of gangliosides, and they are concentrated on synaptic membranes (van Heyningen 1974). These findings support the assertion that injected CTB is taken up by the cell membranes, but does not produce any morphological changes in the cell.

Transported CTB produces sensitive labeling of neuronal processes (Dederen et al. 1994). CTB can be injected either through pressure injections or iontophoresis, and can be fixed with paraformaldehyde (Luppi et al. 1990). CTB has been found to be taken up by fibers of passage, which is a common problem for most neuronal tracers (Chen and Aston-Jones 1995). However, it is unknown whether this uptake is due to damaged fibers or undamaged fibers, because some studies have found that gangliosides are also present on the axolemma (Chen and Aston-Jones 1995; DeVries et al. 1981). On the other hand, it has been suggested that CTB is only taken up by damaged fibers of passage (Luppi et al. 1990). CTB is able to be mixed and simultaneously co-injected with other tracers, such as dextran amine (Coolen et al. 1999).

Due to the novelty of these new fluorescent conjugates of CTB, few studies have examined the efficacy of these conjugates in relation to pathway tracing within the central nervous system. These conjugates have been successfully utilized in studies examining retinal connections (Muscat et al. 2003), and the PNS (Christianson et al. 2007; Kreier et al. 2006; McDavid et al. 2006; Niedringhaus et al. 2008). In light of these new developments, we successfully used CTB to examine the thalamocortical connections related to directed attention and hemispatial neglect in rats (Conte et al. 2008). Here, we present a formalized methodology for use of AF-CTB conjugates in experiments examining the topography of retrograde connections between different areas of the brain [for a step-by-step protocol, see (Conte et al. 2009)]. Our laboratory has extensively utilized the fluorescent conjugates of 3k dextran amine (3kDA), and thus below we informally use our previous experience with 3kDA as a comparison with CTB (Kamishina et al. 2008, 2009).

Materials and methods

All experiments were performed on adult male Long Evans Hooded rats ($n = 49$; 300–350 g; obtained from Harlan Sprague Dawley, Inc.). All procedures were conducted in accordance to the US Public Health Service Guide for the

Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1996) and were approved by the University of Florida IACUC. All efforts were made to minimize the amount of pain experienced and number of animals used. Animals were placed in an induction chamber with a small inhalation dose of Isoflurane (Webster Labs) before being given an injection of ketamine/xylazine cocktail (87 mg/kg ketamine + 13 mg/kg xylazine; 0.1 ml/100 g body weight; Webster Labs). Supplemental doses were given throughout the surgery as needed. Once deep anesthesia was confirmed, the animals were placed in a stereotaxic surgical device with blunt earbars. After bregma was noted, the two sites of injection were marked, and a craniotomy was performed using a Dremel drill. Glass pipettes (30–40 μm diameter) were filled with the respective tracer and injected into the region of interest. Injections were made into various parts of the medial agranular cortex (AGm) and cingulate cortex (CG1). Coordinates were defined using the Paxinos and Watson (2005) atlas. After injections were finished, the surgical site was rinsed with saline, packed with gelfoam, skin sutured, and the rat given an injection of ringers lactate.

Tracers

For CTB injections, AF 488 CTB and AF 594 CTB were used (Molecular Probes, Invitrogen Inc.). The tracers were dissolved in neutral phosphate buffer at either 0.5% (500 μg dissolved in 100 μl buffer) or 1% (500 μg dissolved in 50 μl) concentration. Any extra solutions were separated into aliquots and stored in a freezer. The tracer was loaded into a glass pipette using a Hamilton syringe. Injections were made using a picospritzer (6–10 pulses, 10–15 ms duration, approximately 20 psi).

Post-operative protocols

After a survival period of 7 days, the animals were perfused transcardially with PBS and 4% paraformaldehyde and the brain was removed and placed into a postfix sucrose solution for cryoprotection. Brains were cut coronally on a sliding microtome at thickness of 40 μm and placed serially into PBS. The sections were mounted on slides, dehydrated through a series of alcohol submersions, cleared with xylene, and coverslipped with Entellan mounting median. For each brain we mounted at least two spaced series of fluorescent sections to account for photobleaching and one cresyl violet series. Fluorescent sections were viewed using a Zeiss Axioplan II microscope with the following filter sets: FITC (model 31001, Chroma Technology Corp.) and Texas Red (model 31004, Chroma Technology Corp.). Confocal microscopy was performed on an Olympus IX81 spinning disk confocal microscope.

Photos were normalized in Adobe Photoshop CS3. We limited our focus to labeling patterns within the lateral posterior nucleus of the thalamus (LP).

Results

Notably, as compared to 3kDA, the injection sites for CTB showed a considerably more focused dispersion of tracer at the injection site, and displayed almost no halo (Fig. 1). Compared to the 3kDA injections, the CTB injections were smaller and more specific for the coordinates. For example, we were able to inject a red conjugate of 3kDA into CG1 and a green conjugate in AGm at the same A-P location, and found that the width of the 3kDA injection was approximately 262 μm at its maximum width while the CTB injection width was only 140 μm wide (Fig. 1f). In case 431, which had double injections of CTB into CG1 and AGm, the injection sites both had widths of less than 100 μm , yet produced fully adequate labeling (Fig. 1c–e). There were also markedly fewer labeled cell bodies within the CTB injection sites, as demonstrated by the co-injection of 3kDA and CTB on case 420 (Fig. 1f). However, we often noted that some of the CG1 injection tracer leaked into the superior sagittal sinus, and was absorbed by layer I cells, both contralaterally and ipsilaterally (Fig. 1a). This was most likely due to our selection of injection sites, since CG1 is located so closely to midline.

Both conjugates of CTB produced almost entirely retrograde labeling in LP (Fig. 1). No obvious axonal labeling was noted in cases with intact injection sites. On some cases that had tissue damage within the injection site, we noted some anterograde axonal labeling (Fig. 2). In addition to thalamic labeling, corticocortical labeling was noted in areas that were difficult to see with 3kDA (not illustrated). Similarly, injections of the two conjugates of CTB made in nearby areas showed comparable labeling, but there was no axonal labeling present in either tracer conjugate (Fig. 1). The labeling was focused inside vesicles within the cell bodies, and the vesicles were concentrated on the periphery of the plasma membrane (Fig. 3). Furthermore, most of the labeled cells also showed proximal dendritic labeling (Fig. 3).

Discussion

The present study has independently tested the use of fluorescent conjugates of CTB and has found reliable results. Not only was CTB comparable to other fluorescent tracers such as 3kDA, but it surpassed expectations by producing very fine and detailed labeling even after very focal injections.

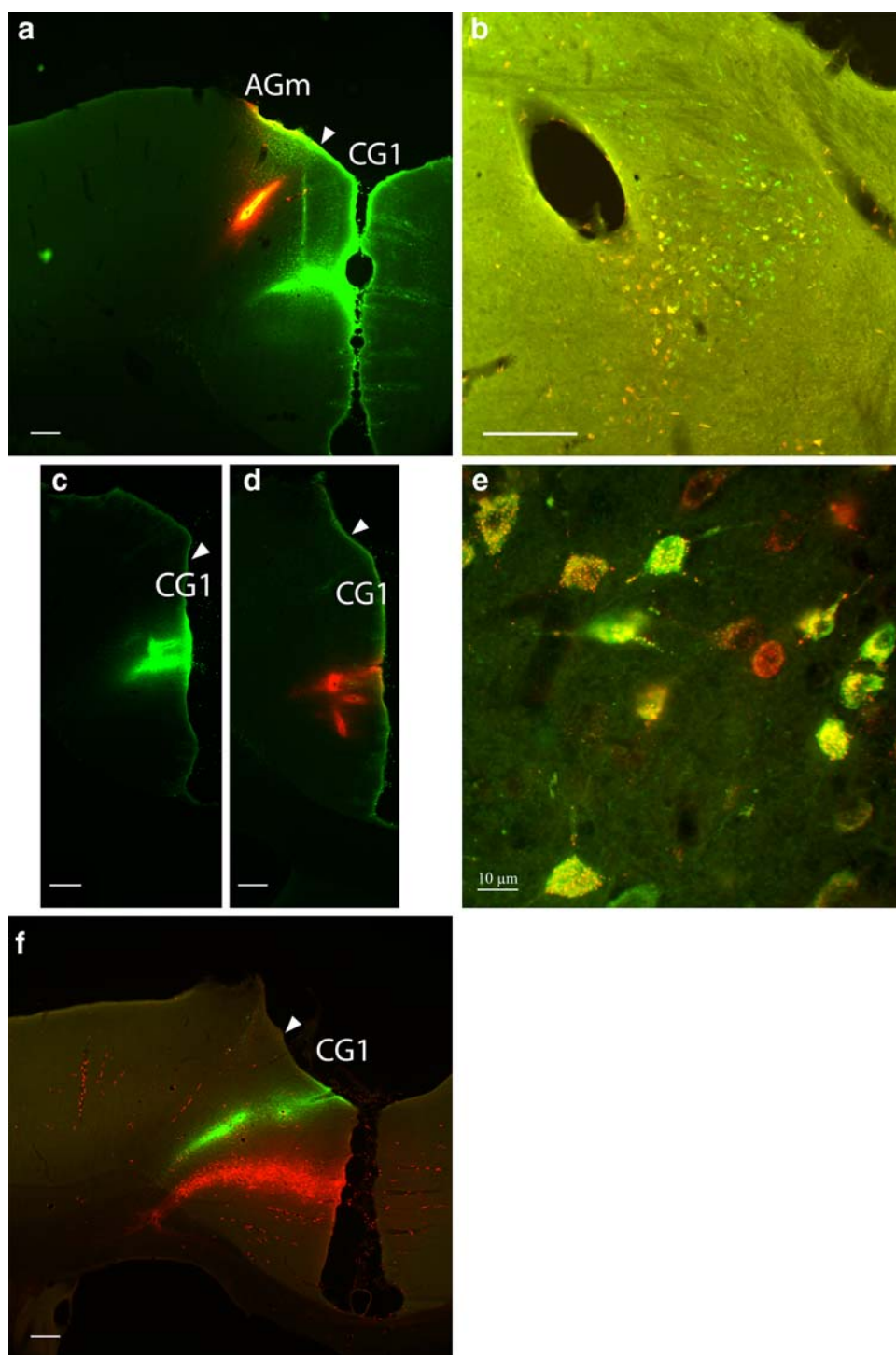
After an initial testing phase of CTB, we found that the fluorescent CTB conjugates were not only very sensitive, but were able to be used at smaller volumes and lower concentrations than 3kDA. To further test the capabilities of CTB, we attempted an injection into cingulate cortex area CG1 with an additional injection into the laterally adjacent area AGm, at the same AP level. This provided a good testing environment, since the injection site centers were only about 100 μm away from each other. When injected this close, the CTB conjugates showed no overlap and were focused within their intended injection site. We were unable to perform similar injections using 3kDA in previous experiments because the tracers would diffuse too far from their intended injection sites and mix. For most cases, we found that CTB was able to be injected into a very small area but produced detailed labeling. Furthermore, even though the injection site centers were nearby, the two CTB injections did not mix. Indeed, CTB was notably more thick and viscous than 3kDA even in solution. These properties of CTB allowed us to discover a previously unknown topography of the connections within our area of interest (Conte et al. 2008).

Cholera toxin subunit B was very effective and sensitive at 0.5% concentration. At 1% concentration, there was slightly more background labeling, although it is unclear whether or not this had to do with the higher concentration of CTB. The AF conjugates appeared very photostable and resisted photobleaching, in line with previous reports (Berlier et al. 2003; Panchuk-Voloshina et al. 1999). Indeed, we have been able to view labeling several months after coverslipping with no significant loss of signal.

In addition to the 488 and 594 conjugates of AF-CTB, a 555 and 647 version is also available (Conte et al. 2009). These could, in turn, be combined for use in triple labeling experiments, by using a Cy3 filter set for AF 555 and a Cy5 filter set for AF 647. For investigators wanting to use AF-CTB for single labeling experiments, we suggest use of the AF 594 conjugate, since this conjugate appears to have a much higher contrast to background tissue than AF 488 CTB.

We found that CTB consistently transported exclusively in a retrograde manner, with no obvious axonal labeling. Labeled somata appeared granular in appearance, and the nucleus was unstained; staining was focused on the plasma membrane (Fig. 3). Retrogradely transported CTB appears granular in a cell soma because CTB remains in vesicles (Kobbert et al. 2000). One interesting note is that we did notice anterogradely labeled axons in a small number of cases (Fig. 2). These cases all had noticeably damaged injection sites, which suggests that anterograde labeling by CTB is facilitated through uptake by damaged cells in the injection site. A future study should be conducted to examine this hypothesis.

Fig. 1 Injection sites of AF-CTB in the cortex (CG1 and AGm) with corresponding labeling patterns in thalamic nucleus LP. **a, b** Injection sites of AF 488 and 594 CTB with corresponding labeling patterns. The injection sites are very small and focused, but show clear labeling patterns in the thalamus. The thalamic labeling is almost exclusively retrograde since only the cell bodies are labeled. **c, d** Injection sites of AF 488 and 594 CTB in the same brain. The labeling patterns (**e**) for this case showed retrogradely labeled cells and double-labeled cells (yellow in appearance). **f** Case 420 had an injection of 3kDA (red) in ventral CG1 and AF-CTB (green) in dorsal CG1. The CTB injection has a smaller width than 3kDA and is more focused in the intended injection site. Compared to the 3kDA injection, the CTB injection also has fewer cell body labeling within in the injection site. Scale bars = 200 μ m, unless otherwise noted. Figure adapted with permission from Conte et al. (2008)

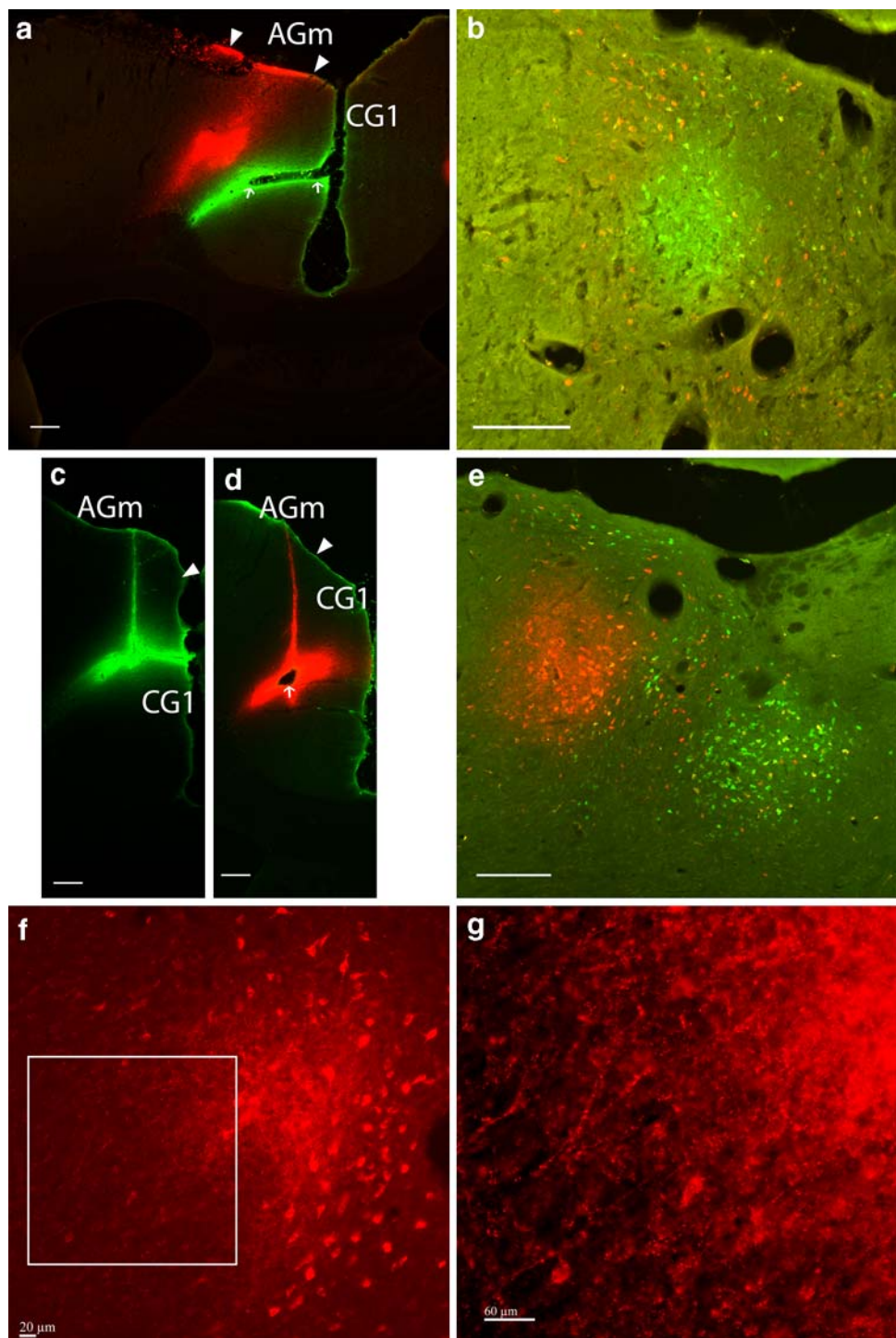


A brief technical note that may be easily overlooked should be mentioned. After seeing several successful cases with CTB, we encountered a series of cases where there was no CTB labeling present. After a discussion with the manufacturer, the problem appeared to be that we were vortexing the solution when mixing the tracer and buffer. Since CTB is a protein complex, vortexing denatures the

proteins and this contributed to our lack of labeling. Once we began to just gently mix the solutions without a vortex, we once again saw good consistent labeling.

Our results using the AF conjugates of CTB confirm previous studies claiming the AF dye line is the brightest and most photostable fluorescent probe available (Berlier et al. 2003; Kumar et al. 1999; Panchuk-Voloshina et al.

Fig. 2 Examples of damaged CTB injection sites in CG1 and AGm that show anterograde labeling in the thalamus. **a** The *green* injection site of CTB shows a central necrotic region (between the *arrows*), while the *red* injection site is intact. **b** Thalamic labeling for the *green* conjugate shows fine, granular axonal labeling indicative of anterograde transport intermingled with retrograde cellular labeling, while the *red* conjugate exhibits only retrograde neuronal labeling. There is almost complete overlap between the anterograde and retrograde labeling in the *green* conjugate. **c** An undamaged *green* AF-CTB injection that is rostral to a damaged *red* injection site **d** in the same brain. **e** The undamaged *green* injection produced entirely retrograde labeling while the *red* injection produced a mixture of retrograde and anterograde labeling. In addition to anterograde labeling that is completely intermingled with retrograde labeling as in *panel B*, the anterograde labeling in *panel E* is also present in isolation as a halo surrounding the retrograde labeling. **f** Another section from the same case showing intermingled labeling patterns in addition to isolated retrograde labeling. Some axonal-like processes are visible in addition to a large amount of fine, punctate labeling suggestive of axon terminals, as seen in a high magnification image of the area enclosed in the *white box* (**g**). Scale bars = 200 μ m, unless otherwise noted



1999). We found that AF is highly photostable, and resists fading even after several minutes of continuous exposure, and is pH insensitive (Panchuk-Voloshina et al. 1999). A previous study noted that AF 555 retained 90% of its fluorescence after 95 s of illumination (Berlier et al. 2003). In addition, we were able to view sections after several months in storage with only minor decreases in intensity.

Compared to conventional dyes such as fluorescein and tetramethylrhodamine, the AF dyes were markedly brighter in intensity, and had a high contrast relative to background tissue.

Based on our data, we suggest that AF-CTB is a very suitable and sensitive tracer for fluorescent retrograde tract-tracing in double-labeling experiments. It is very specific

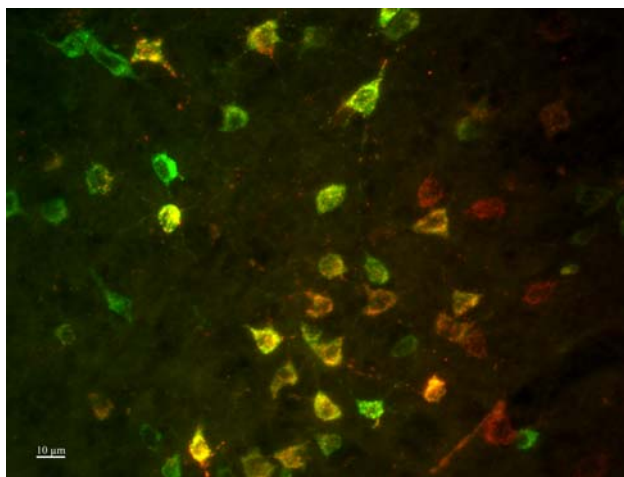


Fig. 3 High magnification confocal image of labeled neurons in thalamic nucleus LP. The neuronal labeling of CTB is focused within the cytoplasm with no nuclear labeling. Some proximal dendrites are also labeled. This field of view exhibits some doubled-labeled cells (yellow in appearance), which represent branching axons of the individual neurons to the two injection sites. However, often times, the two different conjugates are focused in different areas of the cell

for retrograde transport, easy to handle and reliable, available in many of the conjugates of the AF line which allows for exceptional brightness and photostability, and the low concentrations required are cost-effective.

Acknowledgments We greatly appreciate the expert technical support of Maggie Stoll, Harumi Kamishina, Lori Lazar, Elyse Morin, and Rachel Tai. We would also like to thank Doug Smith for microscope consultation. This project was supported by NIMH grant MH60399 and the Maxwell Fund. The authors have no conflicts of interest.

References

- Berlier JE, Rothe A, Buller G, Bradford J, Gray DR, Filanoski BJ, Telford WG, Yue S, Liu J, Cheung C-Y, Chang W, Hirsch JD, Beechem JM, Haugland RP, Haugland RP (2003) Quantitative comparison of long-wavelength Alexa Fluor dyes to Cy dyes: fluorescence of the dyes and their bioconjugates. *J Histochem Cytochem* 51:1699–1712
- Chen S, Aston-Jones G (1995) Evidence that cholera toxin b subunit (CTB) can be avidly taken up and transported by fibers of passage. *Brain Res* 674:107–111
- Christianson JA, Liang R, Ustinova EE, Davis BM, Fraser MO, Pezzone MA (2007) Convergence of bladder and colon sensory innervation occurs at the primary afferent level. *Pain* 128:235–243
- Conte WL, Kamishina H, Corwin JV, Reep RL (2008) Topography in the projections of lateral posterior thalamus with cingulate and medial agranular cortex in relation to circuitry for directed attention and neglect. *Brain Res* 1240:87–95
- Conte WL, Kamishina H, Reep RL (2009) Multiple neuroanatomical tract-tracing using fluorescent Alexa Fluor conjugates of cholera toxin subunit b in rats. *Nat Protoc* 4:1157–1166
- Coolen LM, Jansen HT, Goodman RL, Wood RI, Lehman MN (1999) A new method for simultaneous demonstration of anterograde and retrograde connections in the brain: co-injections of biotinylated dextran amine and the beta subunit of cholera toxin. *J Neurosci Methods* 91:1–8
- Dederen P, Gribnau A, Curfs M (1994) Retrograde neuronal tracing with cholera toxin b subunit: comparison of three different visualization methods. *Histochem J* 26:856–862
- DeVries GH, Zetuský WJ, Zmachinski C, Calabrese VP (1981) Lipid composition of axolemma-enriched fractions from human brains. *J Lipid Res* 22:208–216
- Fishman PH (1982) Role of membrane gangliosides in the binding and action of bacterial toxins. *J Membr Biol* 69:85–97
- Kamishina H, Yurcisin GH, Corwin JV, Reep RL (2008) Striatal projections from the rat lateral posterior thalamic nucleus. *Brain Res* 1204:24–39
- Kamishina H, Conte WL, Patel SS, Tai RJ, Corwin JV, Reep RL (2009) Cortical connections of the rat lateral posterior thalamic nucleus. *Brain Res* 1264:39–56
- Kobbert C, Apps R, Bechmann I, Lanciego JL, Mey J, Thanos S (2000) Current concepts in neuroanatomical tracing. *Prog Neurobiol* 62:327–351
- Kreier F, Kap YS, Mettenleiter TC, van Heijningen C, van der Vliet J, Kalsbeek A, Sauerwein HP, Fliers E, Romijn JA, Buijs RM (2006) Tracing from fat tissue, liver, and pancreas: a neuroanatomical framework for the role of the brain in type 2 diabetes. *Endocrinology* 147:1140–1147
- Krout KE, Belzer RE, Loewy AD (2002) Brainstem projections to midline and intralaminar thalamic nuclei of the rat. *J Comp Neurol* 448:53–101
- Kumar RK, Chapple CC, Hunter N (1999) Improved double immunofluorescence for confocal laser scanning microscopy. *J Histochem Cytochem* 47:1213–1218
- Llewellyn-Smith IJ, Martin CL, Arnolda LF, Minson JB (2000) Tracer-toxins: cholera toxin b-saporin as a model. *J Neurosci Methods* 103:83–90
- Luppi P-H, Fort P, Jouvet M (1990) Iontophoretic application of unconjugated cholera toxin b subunit (CTB) combined with immunohistochemistry of neurochemical substances: a method for transmitter identification of retrogradely labeled neurons. *Brain Res* 534:209–224
- Masco D, Van de Walle M, Spiegel S (1991) Interaction of ganglioside GM1 with the B subunit of cholera toxin modulates growth and differentiation of neuroblastoma N18 cells. *J Neurosci* 11:2443–2452
- McDavid S, Lund JP, Auclair F, Kolta A (2006) Morphological and immunohistochemical characterization of interneurons within the rat trigeminal motor nucleus. *Neuroscience* 139:1049–1059
- Muscat L, Huberman AD, Jordan CL, Morin LP (2003) Crossed and uncrossed retinal projections to the hamster circadian system. *J Comp Neurol* 466:513–524
- Niedringhaus M, Jackson PG, Pearson R, Shi M, Dretchen K, Gillis RA, Sahibzada N (2008) Brainstem sites controlling the lower esophageal sphincter and crural diaphragm in the ferret: a neuroanatomical study. *Auton Neurosci* 144:50–60
- Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhalgat MK, Millard PJ, Mao F, Leung W-Y, Haugland RP (1999) Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J Histochem Cytochem* 47:1179–1188
- Paxinos G, Watson C (2005) *The rat brain in stereotaxic coordinates*, 5th edn. Elsevier Academic Press, San Diego
- van Heyningen WE (1974) Gangliosides as membrane receptors for tetanus toxin, cholera toxin and serotonin. *Nature* 249:415–417

Wang HF, Shortland P, Park MJ, Grant G (1998) Retrograde and transganglionic transport of horseradish peroxidase-conjugated cholera toxin b subunit, wheatgerm agglutinin and isolectin b4 from *griffonia simplicifolia* i in primary afferent neurons innervating the rat urinary bladder. *Neuroscience* 87:275–288

Zin-Ka-Ieu S, Roger M, Arnault P (1997) Direct contacts between fibers from the ventrolateral thalamic nucleus and frontal cortical neurons projecting to the striatum: a light-microscopy study in the rat. *Anat Embryol* 197:77–87