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Suitability of the retrograde tracer DiI for electrophysiological studies of brainstem neurons: Adverse ramifications for G-protein coupled receptor agonists

Liang Zhang [a,1], Amy C. Jongeling [c,2], Donna L. Hammond [a,b,c,*]

[a] *Department of Anesthesia, The University of Iowa, Iowa City, IA 52242, USA*
[b] *Department of Pharmacology, The University of Iowa, Iowa City, IA 52242, USA*
[c] *Interdisciplinary Graduate Program in Neuroscience, The University of Iowa, Iowa City, IA 52242, USA*

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Abstract

Despite the acknowledged advantages of studying identified populations of neurons, few studies have convincingly established that fluorescent retrograde tracers do not alter the passive membrane properties, action potential characteristics, or effects of drugs on the labeled neurons. Whole-cell patch clamp recordings were made from spinally-projecting serotonergic neurons in the rostral ventromedial medulla (RVM) and spinally-projecting noradrenergic neurons in the locus coeruleus (LC) that were retrogradely labeled with 1,1′-dioactadecyl-3,3,3′,3′-tetramethylindocarbodyanine perchlorate (DiI). The passive membrane and the action potential properties of DiI-labeled (0.2%) and non-labeled serotonergic neurons in the RVM did not differ. Similarly, the passive membrane and action potential properties of non-labeled noradrenergic LC neurons did not differ from neurons labeled with 0.2% or 5% DiI. Although the *mu* opioid receptor agonist [D-Ala^2-NMePhe^4-Gly-ol^5]enkephalin (DAMGO) produced equivalent outward currents in non-labeled noradrenergic LC neurons and those labeled with 0.2% DiI, significantly smaller currents were recorded in LC neurons labeled with 5% DiI. Baclofen, a γ-aminobutryic acid$_B$ receptor agonist, also produced smaller currents in RVM neurons labeled with 5% DiI compared to 0.2% DiI. These results indicate that 0.2% DiI is suitable for retrograde labeling of brainstem neurons in vivo for subsequent in vitro electrophysiological study. However, 5% DiI is likely to confound studies of the postsynaptic actions of G-protein coupled receptor ligands.

Keywords: DiI; Retrograde tracer; Rostral ventromedial medulla; Locus coeruleus; Opioid; GABA$_B$; Patch clamp

1. Introduction

Whole-cell patch clamp recording methods are increasingly used in conjunction with in vivo retrograde labeling to study identified populations of neurons in the slice preparation (Kangrga and Loewy, 1994; Johnson et al., 2001; Marinelli et al., 2002). This approach facilitates identification of the neurons in terms of their morphology, axonal projections and neurotransmitter content, and it enables drug effects to be related to specific populations of neurons. An implicit assumption of such studies is that the methods used for identification of these neurons do not alter their electrophysiological characteristics. However, it is not entirely clear that these assumptions are routinely met, despite assertions that the retrograde tracers and intracellular labels are without effect. For example, biocytin, a commonly used intracellular label, can nonspecifically block G-protein coupled inwardly-rectifying K^+ channels and occlude the ability of *mu* opioid receptor agonists to produce outward currents in substantia gelatinosa neurons at concentrations greater than 1% (Eckert et al., 2001). Lucifer Yellow, another intracellular label, can affect inactivation of Na^+ channels (Higure et al., 2003).

Many reports in the literature contain undocumented statements that the passive membrane properties, action potential characteristics, or drug effects on the neurons under study were unaffected by the presence of a retrograde tracer. Yet, high concentrations (12.5%) of the retrograde

* Corresponding author at: Department of Anesthesia, The University of Iowa, 200 Hawkins Drive 6 JCP, Iowa City, IA 52242, USA. Tel.: +1 319 384 7127; fax: +1 319 356 2940.
E-mail address: donna-hammond@uiowa.edu (D.L. Hammond).
[1] Present address: Department of Anesthesia, The University of Iowa, 200 Hawkins Drive 3000 Med Labs, Iowa City, IA 52242, USA.
[2] Present address: Interdisciplinary Graduate Program in Neuroscience, The University of Iowa, 200 Hawkins Drive 3000 Med Labs, Iowa City, IA 52242, USA.

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label 1,1′-dioactadecyl-3,3,3′,3′-tetramethylindocarbodyanine perchlorate (Dil) injected intraspinally are apparently toxic to rostral ventromedial medulla (RVM) neurons (Kangrga and Loewy, 1994), while neurons in the subfornical organ retrogradely labeled with 5% Dil exhibit smaller responses to acetylcholine (Johnson et al., 2001). To the best of our knowledge, a systematic analysis of the potential effects of retrograde tracers on the passive membrane or action potential properties of neurons in the slice preparation has not been conducted. The present study compared the passive membrane properties and action potential properties of serotonergic neurons in the rostral ventromedial medulla retrogradely labeled with a very low concentration of Dil (0.2%, w/v) to those of serotonergic RVM neurons recorded in rats that did not undergo retrograde labeling (i.e. non-labeled neurons). The analysis was then extended to noradrenergic neurons in the locus coeruleus (LC). Finally, this study determined whether retrograde labeling with 0.2% Dil or with 5% Dil, which is often used in the literature, affected the responses of LC neurons to the *mu* opioid receptor agonist [D-Ala^2]-NMePhe^4-Gly^5-ol]enkephalin (DAMGO) or of RVM neurons to the γ-aminobutyric acid $(GABA)_B$ receptor agonist, baclofen.

2. Materials and methods

2.1. Retrograde labeling of spinally projecting neurons in RVM and LC

These experiments were approved by the University of Iowa Animal Care and Use Committee, and were conducted in accordance with the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Nine- to 18-day old male Sprague-Dawley rats (Charles River; Portage, IN) were used. After induction of anesthesia with halothane, a small laminectomy was performed and a 0.5 mm^3 pledget of Gelfoam®, which had been dipped in 0.2% or 5% Dil (w/v; Molecular Probes, Eugene, OR) was tucked beside the dorsal root and juxtapositioned to the dorsolateral aspect of the spinal cord bilaterally (Zhang et al., 2006). The muscle and skin were closed as separate layers. Pups that exhibited hindlimb paralysis or an abnormal response to pinch of the hindpaws (<10% of total number undergoing surgery) were not used.

2.2. Slice preparation

Three to five days after surgery, the pups were decapitated and the brain was rapidly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 dextrose, and equilibrated with 95% O_2/5% CO_2. Coronal slices of 160-μm thickness were cut through the medulla as previously described (Zhang et al., 2006).

2.3. Electrophysiological recording

After equilibration in gassed ACSF for at least 1 h at 34 °C, slices were transferred to the recording chamber and continuously perfused with oxygenated ACSF at 32–34 °C (4 ml min^{-1}); a consistent temperature was maintained throughout each recording. Retrogradely labeled neurons were identified by the presence of fluorescent granules in the cytoplasm under rhodamine illumination. Whole-cell patch clamp recordings were made using glass recording pipettes of 3–6 MΩ resistance that contained (in mM): 140 K^+ methane sulphonate, 10 HEPES, 2 $MgCl_2$, 0.6 EGTA, 2 MgATP, 0.25 Na_2GTP; pH 7.3, 280–290 mOsm. Biocytin (0.01%) was included in the pipette for subsequent identification of the neurons. Recordings were made with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Series resistance was compensated by 70–80%. A seal resistance of ≥3 GΩ and access resistance of <30 MΩ (LC mean 15.0 ± 0.8 MΩ, $N = 67$; RVM mean 18.5 ± 0.6 MΩ, $N = 104$) was required. Liquid junction potentials of −14 mV were uncorrected. A Digidata 1322A board and pCLAMP 9.0 software (Axon Instruments) were used for data acquisition and analysis.

Passive membrane properties were measured in voltage clamp mode using the pClamp membrane test before whole-cell capacitance and series resistance compensation were made. The holding potential was maintained at −60 mV. For measurement of membrane resistance and capacitance values, a test pulse of −10 mV was applied at a frequency that allowed the slow membrane capacitance-induced current transient to decline to a steady-state level. Ten such determinations were made and the average reported. Resting membrane potentials and action potentials were recorded in current clamp mode, sampled at 10 kHz, and filtered with a 5 kHz low-pass filter. Action potential amplitude was measured from the threshold (onset of the rapidly polarizing phase) to its peak. Action potential half-width was determined as the width of the action potential measured at one-half peak amplitude. The amplitude of the slow after-hyperpolarization (AHP) was measured as the difference between resting membrane potential and the negative peak amplitude of the slow AHP. In neurons that did not exhibit spontaneous activity, action potentials were evoked by injection of depolarizing current. It was therefore not possible to measure the amplitude of the slow AHP relative to the resting membrane potential. A similar problem was encountered with a sizable proportion of neurons that repetitively fired action potentials and in which resting membrane potential could not be reliably determined. Therefore, the amplitude from the threshold of the action potential to the maximum negative excursion of the slow AHP was measured in neurons that lacked spontaneous activity and in neurons that fired repetitive action potentials. The absence or presence of a fast AHP was documented for each neuron; amplitude of the fast AHP was not determined.

Outward currents induced by bath application of DAMGO or baclofen were recorded in voltage clamp gap-free mode (holding at −60 mV, sampled at 10 kHz, pClamp low-pass filter 50 Hz). DAMGO or (+) baclofen hydrochloride (Sigma, St. Louis, MO) was added to the ACSF and delivered as previously described (Zhang et al., 2006). Equilibrium concentrations of drug were achieved in the chamber by 90 s.