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Retrogradely Transported Fluorogold Accumulates in Lysosomes of Neurons and is Detectable Ultrastructurally using Post-embedding Immuno-gold Methods

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Abstract

For ultrastructural studies, it is of great interest to be able to combine anatomical tracer techniques with sensitive immunohistochemical methods. Fluorogold (FG) is a fluorescent and retrogradely transported anatomical tracer, which is commonly used to label neurons in the brain and spinal cord for light microscopic studies. We here describe a method for detecting FG-labeled somata in the electron microscope using a high resolution post-embedding immuno-gold method. For this purpose, spinal motoneurons were retrogradely labeled by an intraperitoneal injection of FG in the adult rat. The rats were intravascularly perfused with a fixative solution containing 2% paraformaldehyde and 1–2% glutaraldehyde. Vibratome sections of spinal cord tissues were cryo-protected in glycerol, freeze-substituted in methanol containing uranyl acetate, and embedded in the Lowicryl HM20 resin at low temperatures. Electron microscopic analysis demonstrated atypical lysosome-like structures in the cytoplasm of FG-labeled motoneurons. Subsequent post-embedding immuno-gold labeling demonstrated prominent accumulation of FG in numerous lysosomes but not in other organelles or cytoplasmic compartments of the labeled neurons. The protocol is versatile and allows for combining anatomical tracing of neurons with e.g. neuro-transmitter studies in the electron microscope. We suggest that the described method for sensitive detection of FG in the spinal cord may also have broad applicability to other areas of the central nervous system.

Keywords

Electron microscopy; ultrastructural; immuno-gold; fluorogold; spinal cord; lysosome; rat

Introduction

Anatomical tracers and retrograde labeling techniques are used to identify select neuronal populations in the nervous system. A variety of tracers, including fluorogold (FG), fast blue, dextran conjugates, horseradish peroxidase (HRP), and the B subunit of the cholera toxin (CTb), may be injected into the brain, individual muscles and peripheral autonomic ganglia,

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or applied to cut ventral roots or peripheral nerves for light microscopic fluorescent and light stable identification of brain and spinal cord neurons (Nadelhaft et al., 1986; Schmued and Fallon, 1986; Hosoya et al., 1994; Novikova et al., 1997; VanderHorst and Holstege, 2000). Preganglionic autonomic and motor neurons in the brain stem and spinal cord may also be identified after a systemic administration of FG, fast blue, or CTb (Ambalavanar and Morris, 1989; Leong and Ling, 1990; Alisky et al., 2002; Akhavan et al., 2006). The latter anatomical tracers do not cross the blood-brain barrier and may therefore label only central neurons with peripheral axons. Therefore, no interneurons are labeled in the brain or spinal cord.

Retrograde transport methods are also commonly used to identify spinal cord neurons in the electron microscope. For instance, both HRP and HRP conjugated to CTb (B-HRP) may be used as retrogradely transported tracers, and a histochemical protocol using, for instance, tetramethyl benzidine (TMB) as a chromogen allows for the ultrastructural detection of an electron dense reaction product (Mesulam, 1978; Olucha et al., 1985; Ichiyama et al., 2006). Spinal cord autonomic and motor neurons may also be retrogradely labeled and similarly detected ultrastructurally after systemic administration of B-HRP (Havton and Broman, 2005; Persson and Havton, 2008). However, these protocols for the detection of the TMB reaction product involve multiple steps and strict adherence to sensitive experimental conditions, thereby limiting opportunities for combining ultrastructural identification of retrogradely labeled neurons with post-embedding immuno-gold studies.

Here, electron microscopic studies were performed to investigate whether FG may be detected in spinal motoneurons after systemic delivery of the tracer. A protocol for post-embedding immuno-gold detection was developed and used to validate the presence of FG in retrogradely labeled neurons. We show that FG preferentially accumulates in lysosomal-like organelles in the cytoplasm of retrogradely labeled motoneurons. Our protocol may be particularly helpful for detailed electron microscopic studies of synaptic inputs to retrogradely labeled cells, as the freeze substitution technique used here for slow dehydration and plastic-embedding of tissues at low temperatures allows for immuno-gold detection of both FG and a variety of markers associated with synaptic function.

Materials and Methods

Four adult female Sprague-Dawley rats (180-220 g, corresponding to 7-10 weeks of age, Charles River Laboratories, Raleigh, NC) were included in the studies. All rats were housed in a room with a 12:12 hour light: dark cycle and had access to food and water ad libitum. All animal procedures followed the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1996) and were approved by the Chancellor's Animal Research Committee at UCLA.

Retrograde labeling of motoneurons and tissue processing

For retrograde labeling of motoneurons, 5 mg of FG (Fluorochrome, Denver, CO), was dissolved in 400 µl sterile water and injected i.p. in each rat according to established protocols (Akhavan et al., 2006). Five days after the tracer injection, the rats were perfused intravascularly with 50 ml of phosphate buffered saline (PBS, pH 7.4), followed by 250 ml of a tissue fixative solution containing 2% paraformaldehyde + 1% glutaraldehyde or 2% paraformaldehyde + 2% glutaraldehyde in phosphate buffer at room temperature. The spine was next removed, and the thoracolumbar portion of the spinal cord was dissected out. Transverse spinal cord sections were cut using an oscillating tissue slicer (Electron Microscopy Sciences, Fort Washington, PA) and collected for both light and electron microscopic studies. For light microscopic detection of FG-labeled spinal cord neurons, lumbar sections (100 µm thickness) were placed on glass slides, cover-slipped, and analyzed using a Nikon E600 light microscope equipped for epifluorescence detection.

For ultrastructural studies, thoraco-lumbar sections (250 μm thickness) were collected, cryo-protected in a graded series of glycerol in PBS and kept in a 30% glycerol solution overnight. Next, the tissue sections were snap frozen in liquid propane at -180°C using the Leica EM CPC cryo workstation (Leica Microsystems, Wetzlar, Germany). The frozen tissue sections were transferred to a Leica EM AFS2 automatic freeze-substitution system (Leica Microsystems) and dehydrated in methanol containing 1-2 % uranyl acetate at -90°C for 35 h. The temperature was subsequently raised by $5^{\circ}\text{C}/\text{h}$ to -45°C , rinsed in methanol, and infiltrated in graded solutions of Lowicryl HM20 (Electron Microscopy Sciences) in methanol (2 h each in 1:1, 2:1, 1:0, and 1:0 overnight). The infiltrated spinal cord sections were polymerized using ultraviolet light for 30 h at -45°C , and, following a rise in the temperature of $5^{\circ}\text{C}/\text{h}$, for 40 h at 0°C .

Semi-thin spinal cord sections (about 1 μm thickness) were cut using a PowerTome X ultramicrotome (RMC Products, Boeckeler Instruments, Tucson, AZ) and stained with toluidine blue for light microscopic examination. For electron microscopic studies, ultrathin sections (60-80 nm thickness) were cut using a PowerTome X ultramicrotome (RMC Products), collected on formvar-coated copper grids, and stained with uranyl acetate and lead citrate. For post-embedding ultrastructural immuno-gold studies, the ultrathin sections were collected on formvar-coated nickel grids.

Post-embedding immuno-gold labeling and analysis

All grids were placed in drops containing the reagents and kept in humid chambers for the immuno-gold incubation steps. For immuno-gold detection of FG, formvar-coated nickel grids with ultrathin sections of the spinal cord ventral horn were first immersed in 0.1% sodium borohydride + 50 mM glycine in Tris-buffered saline (pH 7.4) containing 0.1% Triton X-100 (TBST) for 10 minutes, in order to block free aldehyde residues in the tissue. This was followed by three rinses of the grids in TBST. Next, the sections were incubated in TBST containing normal goat serum (NGS, 1:20) for 10 minutes. The sections were subsequently incubated in a primary antibody solution containing a polyclonal antibody to FG raised in rabbit (1:500, Fluorochrome) in Tris buffer with 0.1% Triton X-100 and NGS (1:20) for 4 h, followed by three rinses of the grids with TBST, and incubation of the grids in TBST with NGS (1:20) for 10 min. The sections were incubated in a secondary antibody solution containing a goat anti-rabbit antibody conjugated to 15 nm colloidal gold (Amersham, Arlington Heights, IL), diluted 1:20 in TBST for 2 h. The grids were rinsed in ddH_2O , air dried, and contrasted with uranyl acetate and lead citrate. As a control experiment, a few grids from each animal were processed using a protocol where the primary antibody was replaced with normal rabbit serum. In all other regards, the experimental and control protocols were identical. The sections were finally examined in a JEOL 100CX transmission electron microscope (JEOL, Japan), and images were captured on photographic film.

Results

Spinal motoneurons were retrogradely labeled with FG after i.p. administration of the tracer in neurologically intact female rats and studied in the epifluorescence, light, and electron microscope. For this purpose, sections of the thoracolumbar spinal cord were prepared for morphological studies. For light and electron microscopic studies, the sections underwent dehydration and were embedded in Lowicryl HM20 in the absence of osmium at low temperatures using an automatic freeze substitution protocol.

Epifluorescent and light microscopic observations

When studied in an epifluorescence microscope, FG was detected in ventral horn motoneurons in the thoracolumbar spinal cord. The tracer presented as bright granulae, which typically

surrounded the nucleus and, to some extent, extended into proximal dendritic branches (Figure 1).

Following embedding in Lowicryl HM20, semi-thin sections of the lumbosacral spinal cord were stained with toluidine blue and examined in the light microscope. The ventral horn neuropil appeared normal and demonstrated large neuronal cell bodies, which were presumed to represent motoneurons. These somata typically showed a prominent nucleus and nucleolus. The motoneuron cytoplasm exhibited patchy areas of fine granular staining, suggestive of Nissl substance. One or more dendritic branches commonly extended from the somata. However, no tracer was detected in the cytoplasm of the presumed motoneurons by light microscopy. The latter finding is in agreement with previous observations by Schmued et al. (1989), who observed FG granules in osmicated sections and when using epifluorescent detection methods, but not when examining unosmicated sections in the light microscope.

Electron microscopic observations in the lumbosacral ventral horn

Large neuronal somata were identified in the ventral horn gray matter ultrastructurally and demonstrated a large nucleus and nucleolus. The cytoplasm of these presumed motoneurons exhibited prominent endoplasmic reticulum and associated ribosomes, several mitochondria, and a mixed population of atypical lysosomes. Many lysosomal-like structures were irregularly shaped, contained irregularly shaped electron-dense structures, as well as one or more electron-lucent vacuoles (Figure 2A). Other lysosomes showed an electron-dense and coarse grained appearance (Figure 2A). The morphological appearance of these organelles is similar to the ultrastructure of lysosomes in retrogradely labeled neurons in the cerebral cortex and brain stem after select spinal cord and brain injections of FG in rats (Schmued et al., 1989).

Electron microscopic examination of FG immuno-gold labeling

A total of 15 neurons in the motor nuclei of lumbar spinal cord sections from three rats were examined ultrastructurally using immuno-gold methods for the detection of FG. A selective pattern of immuno-gold labeling was detected with a high density of gold particles over a subset of organelles in the cytoplasm of all motoneurons. Specifically, marked immuno-gold labeling was detected over lysosomal-like structures, which typically demonstrated electron-dense content and vacuoles (Figure 2B and C). In contrast, there was only sparse background immuno-labeling associated with e.g. mitochondria, endoplasmic reticulum or the nucleus. The vast majority of immuno-gold labeled lysosomes were detected in the somata of motoneurons, although dendritic structures of the adjacent ventral horn neuropil on occasion demonstrated lysosomes exhibiting similar immuno-labeling (Figure 3).

Replacing the primary antibody with normal rabbit serum in the immuno-labeling protocol abolished virtually all labeling, with the exception of a very faint background staining.

Discussion

Here, we demonstrate a method for ultrastructural detection of retrogradely transported FG in motoneurons. The procedure includes tissue fixation using mixed aldehydes, freeze substitution, embedding in Lowicryl HM20 at low temperatures, and post-embedding immuno-gold studies. In the electron microscope, putative FG-labeled motoneurons exhibited lysosomal structures with an atypical appearance within the cell body cytoplasm. The presence of FG within lysosomes of retrogradely labeled motoneurons was confirmed ultrastructurally by immuno-gold detection using a primary antibody to FG.

4.1. FG is localized in lysosomes of retrogradely transported motoneurons

Fluorogold was initially introduced as a retrogradely transported fluorescent dye providing bright fluorescent labeling of neuronal somata and proximal dendrites (Schmued and Fallon, 1986). The tracer was subsequently described in more detail as 2-hydroxy-4, 4'-diamidino stilbene (hydroxystilbamidine), an aromatic diamidine antibiotic (Schmued, 1990; Wessendorf, 1991). When neurons are lightly labeled with FG, fluorescent gold colored granules or vesicles are readily visualized in the cytoplasm of their somata and proximal dendrites, and it was therefore proposed that the tracer may accumulate within lysosomes (Schmued and Fallon, 1986). As hydroxystilbamidine is a weak base, it was later suggested that such accumulation of FG in lysosomes could take place by initial passive diffusion of its uncharged form and later trapping of the protonated form of the tracer due to the acidic milieu within lysosomes (Wessendorf, 1991).

Early attempts of visualizing FG ultrastructurally used a photo-conversion strategy to convert the fluorescence of retrogradely labeled neurons into a stable diaminobenzidine reaction product and demonstrated electron-dense material within lysosomes and scattered in the cytoplasm (Balercia et al., 1992). With the introduction of an antibody to FG, pre-embedding immunohistochemical studies, using the electron dense diaminobenzidine reaction product, demonstrated diffuse cytoplasmic staining of the somata and dendrites in well-labeled neurons (Chang et al., 1990). Additional studies on the ultrastructural localization of immuno-peroxidase-labeled FG or silver-intensified immuno-gold labeling for FG demonstrated the reaction product both in lysosomes and throughout the cytoplasm of labeled neuronal somata and dendrites (Van Bockstaele et al., 1994). The post-embedding immuno-gold labeling pattern for FG in the present study shows that the tracer is principally or exclusively localized in lysosomes, as other organelles or intracellular compartments, such as, for instance, the mitochondria, endoplasmic reticulum, nucleus, nucleolus, or cytoplasm did not show any specific immuno-gold labeling for FG in the present study.

4.2. Use of FG as an anatomical tracer for ultrastructural detection

Previous studies have demonstrated that intracellular labeling of neurons, using FG as a retrogradely transported anatomical tracer, may alter the ultrastructural appearance of lysosomal structures within their somata (Schmued et al., 1989). Specifically, the lysosome-like cytoplasmic organelles showed three distinct morphological appearances in the form of lamellar bodies, heterogeneous or lipofuscin-like lysosomes, and coarse grained lysosomes (Schmued et al., 1989). In the present study, we also encountered the heterogeneous or lipofuscin-like lysosomes, as well as the coarse grained lysosomes, but not the lamellar bodies in the cell body cytoplasm of FG-labeled motoneurons. It is possible that the absence of lamellar bodies in the present investigation may be due to methodological differences between the studies, as there is no osmification step in our freeze substitution/plastic embedding protocol. It is also possible that this is an effect of a sampling bias; Schmued et al. (1989) noted that the lamellar bodies were more prevalent in dendrites, while this study concentrated on finding FG in the cell somata. Although we administered FG by systemic delivery instead of by intraparenchymal injection, as was the case in the study by Schmued et al. (1989), the electron microscopic appearance of lysosomal structures in retrogradely labeled neurons were very similar between the two studies. Specifically, in both investigations, lysosomal structures showed atypical heterogeneity of their electron density and the occasional presence of single or multiple electron lucent vacuoles.

There may be situations wherein the altered morphology of lysosomal structures may be used to identify a neuron as FG-labeled in the electron microscope. However, some caution is suggested, as the lysosomes of FG-labeled neurons share key ultrastructural features with lysosomes containing the age-associated pigment, lipofuscin (Jung et al., 2007). Therefore,

this confounding factor limits the potential use of the above ultrastructural features alone when identifying FG-labeled neurons, especially in tissues obtained from older animals. In contrast, the method for post-embedding immuno-gold identification of FG-labeled neurons presented here provides a sensitive method for reliable detection of FG-labeled neurons.

4.3 Post-embedding immuno-gold detection of FG allows for the detection of retrogradely labeled neurons to be combined with a variety of other immuno-gold studies in the electron microscope

Historically, a major methodological challenge for ultrastructural studies has been to develop protocols, where anatomical labeling of select neuronal populations may be combined with post-embedding immuno-gold studies. Unfortunately, some of the most commonly used anatomical tracers for ultrastructural studies involve pre-embedding histochemical reactions and treatments that are not amenable to post-embedding immunohistochemical studies. For instance, tissues containing the retrogradely transported tracers HRP and HRP conjugated to the B-fragment of the cholera toxin (B-HRP) or wheat-germ agglutinin (WGA-HRP) typically use tetramethyl benzidine (TMB) or diaminobenzidine (DAB) as a chromogen and require osmium treatment to allow for the ultrastructural detection of an electron dense reaction product (Mesulam, 1978; Olucha et al., 1985; Havton and Broman, 2005; Ichiyama et al., 2006). As a result, antigen detection is much compromised. Interestingly, an osmium-free protocol using freeze-substituted Lowicryl HM20 embedded tissue demonstrated successful detection of neuronally transported CTb using post-embedding immuno-gold techniques (Ragnarson et al., 1998). In the present study, we demonstrated that FG may also be used for neuronal labeling and detectable by a similar protocol. However, FG exhibit some potential methodological advantages, as it remains detectable by immunohistochemistry in labeled neurons over several weeks and is suitable for long-term studies (Akhavan et al., 2006).

The detection of neuro-transmitters and their receptors in the electron microscope has also represented a long-standing technical challenge. Early protocols for post-embedding immuno-gold studies allowed for the detection of amino acid transmitters, including glutamate, GABA, and glycine in the brain and spinal cord (Ottersen, 1987; Örnung, 1996, 1998). Alternatively, freeze-substitution and embedding of fixed brain tissue in Lowicryl HM20 has demonstrated excellent antigen preservation and suitability for immunogold localization of neural antigens in the electron microscope (van Lookeren Campagne et al., 1991). The latter approach, which was used for the immuno-gold detection of FG in the present study, has allowed for the high sensitivity detection of a variety of amino acids, peptides and proteins, including transmitter substances, vesicular transporters, and receptors (Larsson et al., 2001; Mahendrasingam et al., 2003; Persson and Broman, 2004). Thus, FG-labeled neurons and their synaptic inputs may now be studied in the same tissues, allowing for detailed analyses of neuronal circuits.

4.4 Summary

We present a versatile and sensitive method, using freeze substitution techniques and tissue embedding with Lowicryl HM20 in the absence of osmium, for ultrastructural detection of FG in retrogradely labeled motoneurons in the rat spinal cord. Here, sensitive post-embedding immuno-gold detection of FG showed selective accumulation of the tracer in lysosomal structures. Tissues that have been processed using the present protocol may also be used for other post-embedding immuno-gold studies, i.e. experiments to characterize the synaptic inputs to FG-labeled neurons. We suggest that the described methods may have broad applicability to electron microscopic studies on retrogradely labeled neurons in both the brain and spinal cord.

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Figure 1.

Retrogradely labelled neurons in the ventral horn of the lumbar spinal cord following systemic injection of fluorogold, observed using epifluorescence microscopy. A) labeled motoneurons are seen in lamina IX of the spinal cord. B) in higher magnification, the labeling appears as granulae surrounding the nucleus, and extending to some extent into the dendritic processes of the cells. Scale bar: 100 μm when applied to A, 50 μm when applied to B.

Figure 2.

A number of lysosomes in a motoneuron (Mn) next to a glial cell (G). A) the lysosomes seen in a non-immunogold labeled section. The image shows a lipofuscin-like lysosome (thick arrow), surrounded by a number of other lysosomal structures (thin arrows and arrow heads). B) and C) the same structures in an adjacent section, immunogold labeled for fluorogold. The lipofuscin-like lysosome is densely labeled, while labeling in the other lysosomal structures varies, and the small lysosomes indicated by the thin arrows are unlabeled. Scale bar: 1 μ m.

Figure 3.

Lysosome-like structures immuno-labeled for fluorogold, observed in motoneurons. A) two lysosome-like structures (arrows) located in a proximal dendrite of a motoneuron next to a glial cell (G). B) Two distal dendrites (d1 and d2) containing lysosomal structures (arrows) with varying degrees of immuno-labeling. Both dendrites are apposed by synaptic boutons. C) Immuno-labeled lysosomal structures often appear several in the same cell; here near the nucleus in a motoneuron. Note that not all structures with the same general appearance display immuno-labeling (arrow). Scale bars in A, and C (applies to B and C): 2 μ m.