Journal of Neuroscience Methods 220 (2013) 92–98

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Activated caspase detection in living tissue combined with subsequent retrograde labeling, immunohistochemistry or *in situ* hybridization in whole-mounted lamprey brains

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HIGHLIGHTS

- Often there is a need to detect early apoptosis before tissue is fixed and processed for molecular correlates.
- Activation of caspases was detected in living lamprey brains with fluorescently labeled inhibitors of caspase (FLICA).
- The same tissue was then fixed and processed for immunohistochemistry or *in situ* hybridization.
- "Bad-regenerator" neurons undergo slow apoptosis post-axotomy and selectively express the mRNA for the CSPG receptor PTPσ.

ARTICLE INFO

Article history:
Received 2 April 2013
Received in revised form 14 August 2013
Accepted 16 August 2013

Keywords:
Caspase
Apoptosis
Wholemount *in situ* hybridization
Wholemount immunohistochemistry
Lamprey
Brain

ABSTRACT

In the lamprey brain, there are 18 pairs of identified spinal-projecting neurons whose regenerative abilities have been characterized. The "bad-regenerating" neurons show a very delayed form of apoptosis after axotomy (Shifman et al., 2008). Theoretically, this should provide a long window of opportunity to intervene therapeutically, so it would be helpful if we could identify the early stages of this process *in vivo*. Until now, there has been no method to link mRNA or protein expression directly to early-stages neuronal apoptosis *in vivo*. Here we describe a double-labeling protocol in whole-mounted lamprey brain for simultaneous detection of early stage apoptosis, using Fluorochrome-Labeled Inhibitors of Caspases (FLICA), and either mRNA, using *in situ* hybridization, or protein expression, using immunohistochemistry. To improve brain preservation, the working temperature during the FLICA stage was lowered from 37 °C to 4 °C (Barreiro-Iglesias and Shifman, 2012). Using this method, neurofilament protein was demonstrated by immunohistochemistry in neurons previously reacted by FLICA. The method also revealed that mRNA for the receptor protein tyrosine phosphatase PTPσ is expressed selectively in FLICA-positive neurons. In addition, our study showed that a retrograde labeling technique can be used in the context of FLICA labeling. FLICA label colocalized with TUNEL staining, confirming that FLICA labeling is a reliable marker of apoptosis in lamprey brain. Our results suggested that we can combine caspase detection with other techniques *in vivo* to investigate the roles and mechanisms of activated caspases and other molecules in retrograde cell deaths and regenerative abilities of neurons.

1. Introduction

Apoptosis is regulated by genetically encoded mechanisms and can be recognized by many biologically and morphologically distinct characteristics (Arama and Steller, 2006). Cells undergoing apoptosis may have changes in mitochondrial membrane potential, caspase activation, nuclear DNA fragmentation, and membrane blebbing. Many different assays have been designed to detect apoptotic cells, including Annexin-V binding, caspase enzyme activity, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL). For a long time, however, only TUNEL has been recommended and used on tissue specimens to detect late-stage apoptosis. Excitingly, Fluorochrome-Labeled Inhibitors of Caspases (FLICA) have been shown to detect activated caspases in live lamprey brain (Barreiro-Iglesias and Shifman, 2012), which may provide new insights into apoptosis *in vivo*. The lamprey has several advantages for neurobiological investigation, including an absence of myelin (Bullock et al., 1984). This makes the whole-mounted brain less opaque and facilitates immunohistochemistry, *in situ* hybridization and other imaging procedures. A schematic of large larval lamprey brain is shown in Fig. 1. There are

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http://dx.doi.org/10.1016/j.jneumeth.2013.08.016

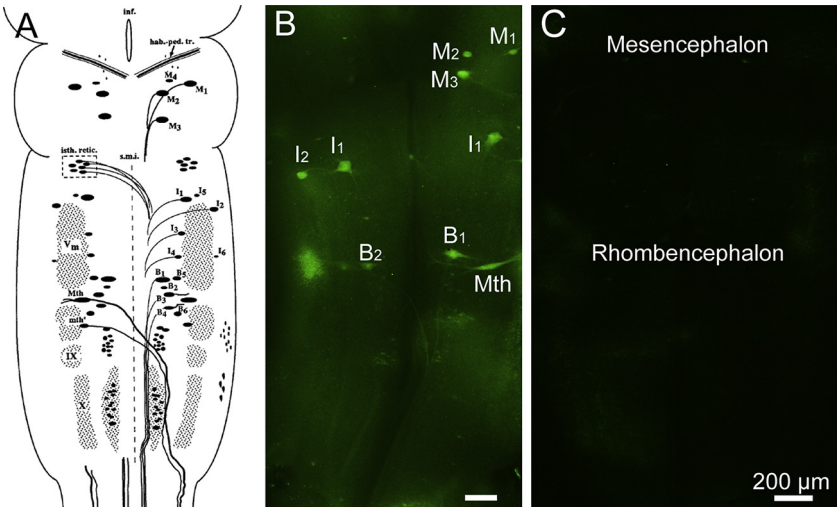

Fig. 1. Cytoarchitecture of the identified reticulospinal neurons in lamprey brain. (A) The spinal projecting neurons in the brain of a large larval sea lamprey were labeled and divided into cytoarchitectonic groups according to the nomenclature in (Swain et al., 1993). In addition to the nuclear groups of small neurons, several giant reticulospinal neurons are seen. These are the Mv̈ller and Mauthner neurons and also will be described in subsequent figures. M, Mesencephalic Mv̈ller cells; I, isthmic Mv̈ller cells; B, bulbar Mv̈ller cells; Mth, Mauthner cell; mth, auxiliary Mauthner cell; hab.-ped. tr., habenulopeduncular tract; inf., infundibulum; isth. retic., isthmic (anterior rhombencephalic) reticulospinal nucleus; s.m.i., sulcus medianus inferior; Vm, trigeminal motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus. The unlabeled cell mass lateral to mth and lying between V and IX is the facial motor nucleus. Modified from (Jacobs et al., 1997). M2, M3, I1, B1, B2, B4, Mth are considered bad regenerators, while M1, M4, I2, I3, I4, I5, I6, B2, B5, B6 and mth as good regenerator (Davis and McClellan, 1994; Jacobs et al., 1997). (B) FLICA labeling in the whole-mounted brain from a larval lamprey after 2 weeks post spinal cord transection. The identified neurons which are FLICA positive have been marked. (C) FLICA labeling in the whole-mounted brain from a control larval lamprey without spinal cord transection. Images showed the mesencephalon and rhombencephalon as marked. Scale bar: 200 μm.

18 pairs of large reticulospinal neurons in the lamprey brain. Some are bad regenerators and others good regenerators (Jacobs et al., 1997); the probability that each identified neuron will regenerate after spinal cord transection has been defined previously (Davis and McClellan, 1994; Jacobs et al., 1997). Recent evidence shows that spinal cord transection induces delayed cell death in lamprey spinal-projecting neurons (Shifman et al., 2008). The neuronal death, shown by TUNEL staining, began 4 weeks after spinal cord transection and reached its peak at 12–16 weeks (Shifman et al., 2008). Interestingly, at earlier times, the neurons were swollen and lacked Nissl-staining, which indicated that changes in these neurons began long before death. However, the mechanisms by which neurons in the brain detect and respond to spinal cord transection are unknown. It would be very useful to develop techniques to detect the early stages of apoptosis and to define its relationship to the expression of other important peptides or mRNAs.

Caspases are a family of cysteine proteases that mediate apoptosis, which plays a critical role in development. Accumulating evidence suggests that in the mature nervous system, caspases are not only involved in apoptosis, but also have other important roles in physiological and pathological processes such as dendritic pruning (Kuo et al., 2006; Williams et al., 2006), synaptic plasticity (Li et al., 2010; Lu et al., 2006) and Alzheimer's disease (D'Amelio et al., 2011; de Calignon et al., 2009; Jo et al., 2011). The multiple roles of caspases make it even more important to develop methods for combining *in vivo* caspase detection with other imaging techniques.

In the present study, FLICA labeling was subsequently combined with either immunohistochemistry for NF-180, the most prominent of the neurofilament (NF) subunits in lamprey, or *in situ* hybridization for protein tyrosine phosphatase sigma (PTPσ). NFs comprise most of the axonal cytoskeleton, providing mechanical support and regulating the diameter of axons. Thus far, four NF subunits have been identified in lamprey: NF-180, L-NFL, NF132 and NF95 (Zhang et al., 2004; Jin, Zhang, Pennicooke, Laramore, & Selzer, 2011; Zhang, Jin, & Selzer, 2011). NF-180 was the first discovered and is required for the formation of normal NF bundles (Zhang et al., 2011). It is of interest to our laboratory because after axotomy, NF-180 mRNA expression first disappeared, but then returned selectively in identified reticulospinal neurons that are "good regenerators", whereas in bad-regenerating neurons, NF-180 expression was permanently downregulated (Jacobs et al., 1997; Zhang et al., 2011). PTPσ, a member of transmembrane receptor protein tyrosine phosphatase (RPTP) family, is of interest to our laboratory because it acts as a functional receptor for chondroitin sulfate proteoglycans (CSPGs) and appears to mediate a significant part of their inhibitory effects on axon regeneration (Shen et al., 2009).

In summary, using the lamprey nervous system as a model, we have modified the FLICA method to detect activated caspases *in vivo* and combined this with immunohistochemistry, *in situ* hybridization and retrograde labeling. These combinatorial labeling techniques permit exploration of caspase-involved activities *in vivo* (Hyman and Yuan, 2012).

2. Materials and methods

2.1. Spinal cord transection and retrograde labeling of reticulospinal neurons

Wild-type larval and adult lampreys, *Petromyzon marinus*, 10–14 cm in length, obtained from the Connecticut River (Massachusetts) or from streams feeding Lake Champlain (Vermont), were maintained in fresh water tanks at room temperature (RT) until brains were removed for the experiments described below. Animals were anesthetized by immersion in 0.1% tricaine methanesulfonate, and the spinal cord was exposed from the dorsal midline at the level of the fifth gill. Transection of the lamprey spinal cord was performed with Castroviejo scissors. Completeness of full transections was confirmed by retraction and visual inspection of the cut ends. For retrograde labeling, a pledget of Gelfoam soaked in 5% dextran tetramethylrhodamine (DTMR, 10 kDa, Molecular Probes) was placed at the site of a complete spinal cord transection. Transected lampreys recovered on ice for 2 h and then returned to fresh water at RT for 2 weeks until brainstems were removed for the following experiments.