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Biochemical analysis of axon-specific phosphorylation events using isolated squid axoplasms

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Abstract

Appropriate functionality of nodes of Ranvier, presynaptic terminals, and other axonal subdomains depends on efficient and timely delivery of proteins synthesized and packaged into membrane-bound organelles (MBOs) within the neuronal cell body. MBOs are transported and delivered to their final sites of utilization within axons by a cellular process known as fast axonal transport (FAT). Conventional kinesin, the most abundant multisubunit motor protein expressed in mature neurons, is responsible for FAT of a large variety of MBOs and plays a major role in the maintenance of appropriate axonal connectivity. Consistent with the variety and large number of discrete subdomains within axons, experimental evidence revealed the identity of several protein kinases that modulate specific functional activities of conventional kinesin. Thus, methods for the analysis of kinase activity and conventional kinesin phosphorylation facilitate the study of FAT regulation in health and disease conditions.

Axonal degeneration, abnormal patterns of protein phosphorylation, and deficits in FAT represent early pathological features characteristic of neurological diseases caused by unrelated neuropathogenic proteins. Interestingly, some of these proteins were shown to produce deficits in FAT by modulating the activity of specific protein kinases involved in conventional kinesin phosphorylation. However, experimental systems that facilitate an evaluation of molecular events within axons remain scarce. Using the isolated squid axoplasm preparation, we describe methods for evaluating *axon-autonomous* effects of neuropathogenic proteins on the activity of protein kinases. Protocols are also provided to evaluate the effect of such proteins on the phosphorylation of endogenous axonal substrates, including conventional kinesin and neurofilaments.

INTRODUCTION

Axons comprise the bulk of the total cell volume for most neurons, yet the vast majority of components needed for the generation and maintenance of this compartment, including lipids and membrane proteins, are synthesized in the neuronal cell body. Within the neuronal soma, these components are sorted and packaged into specific membrane-bound organelles (MBOs), which are later delivered to their final sites of utilization in axons by a cellular process collectively known as fast axonal transport (FAT) (Morfini, Stenoien, Brady, 2011). Anterograde FAT of MBOs from the cell body to axons and dendrites is largely executed by

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microtubule (MT)-based motor proteins of the kinesin superfamily, whereas MBOs moved in retrograde FAT are transported by cytoplasmic dynein (Hirokawa & Takemura, 2005).

Conventional kinesin represents the best characterized and most abundant kinesin superfamily member in mature neurons (Wagner, Pfister, Brady, & Bloom, 1991) and is responsible for anterograde FAT of a wide variety of MBOs including mitochondria, plasmalemma, and synaptic vesicle precursors (Leopold, McDowall, Pfister, Bloom, & Brady, 1992). This multisubunit motor protein exists as a heterotetramer comprised of two kinesin heavy chain (kinesin-1, KHC) and two kinesin light chain (KLC) homodimer subunits and is responsible for specific functional activities of the holoenzyme (DeBoer et al., 2008). Kinesin-1 activities involve adenosine triphosphate (ATP) hydrolysis and MT-binding, which are essential for processive movement of conventional kinesin along axonal MTs (Wagner et al., 1991). KLCs contain tandem repeat domains that play a major role in the binding of conventional kinesin to MBOs (Stenoien & Brady, 1997). Three kinesin-1 and two KLC genes are expressed in mammalian nerve tissue, and isoform-specific sequences located at the carboxy terminus of each subunit appear to mediate the sorting of biochemically heterogeneous forms of conventional kinesin to specific MBOs (DeBoer et al., 2008). The critical role of conventional kinesin and cytoplasmic dynein on neuronal function and the maintenance of axonal connectivity is illustrated by the identification of mutations in specific subunits of these motor proteins, which cause neurodegenerative diseases featuring axonal degeneration as a major pathological hallmark (Morfini, Burns, et al., 2009; Roy, Zhang, Lee, & Trojanowski, 2005).

The restricted distribution of selected kinesin-1 MBO cargoes at specific axonal subcompartments (i.e., sodium channels at nodes of Ranvier, or synaptic vesicle precursors to presynaptic terminals) long implied the existence of regulatory mechanisms that allow for localized unloading of MBOs (Morfini, Szebenyi, Richards, & Brady, 2001). A hint on such mechanisms first came from studies showing that both kinesin-1 and KLC subunits are phosphorylated in vivo (Hollenbeck, 1990). More recently, a number of specific protein kinases were identified, which regulate selected functional activities of conventional kinesin by phosphorylating specific subunits. For example, both c-jun amino-terminal kinase 3 (JNK3) and p38α MAP kinase phosphorylate kinesin-1 subunits at residues located in close proximity to MT-binding domains. Consistent with the enzymatic activities of kinesin-1, these phosphorylation events were found to inhibit processive movement of this subunit along axonal MTs (Morfini et al., 2013; Morfini, You, et al., 2009). On the other hand, both glycogen synthase kinase 3 (GSK3β) and casein kinase 2 (CK2) were shown to phosphorylate KLCs instead, and these events were associated with detachment of conventional kinesin from MBOs (Morfini, Szebenyi, Elluru, Ratner, & Brady, 2002; Morfini et al., 2001). Other proteins were found to regulate this motor protein indirectly, by modulating the activity of these kinases above (Morfini et al., 2004; Ratner, Bloom, & Brady, 1998).

Relevant to human diseases, several neuropathogenic proteins were shown to alter FAT by modulating the activity of protein kinases involved in conventional kinesin phosphorylation (Morfini, Burns, et al., 2009). For example, mutant forms of huntingtin protein that cause Huntington's disease inhibited kinesin-1-based FAT through a mechanism involving JNK3

activation (Morfini, You, et al., 2009). Similarly, both mutant and misfolded forms of superoxide dismutase 1 (SOD1) associated with familial and sporadic forms of amyotrophic lateral sclerosis similarly inhibited conventional kinesin-based FAT. However, their inhibitory effect was mediated by p38 MAP kinase (Bosco et al., 2010; Morfini et al., 2013). In addition, inhibitory effects of the Alzheimer's disease-related proteins tau- and beta-amyloid on conventional kinesin function were mediated by GSK3 and CK2, respectively (Kanaan et al., 2011; Pigino et al., 2009). Because many neurodegenerative diseases feature an early loss of axonal connectivity associated with deficits in FAT, the identification of specific kinases modulating kinesin-1 phosphorylation in the context of these diseases has important implications for delineating pathogenic mechanisms (Morfini, Burns, et al., 2009).

Even though axons represent the largest neuronal compartment, addressing axon-specific effects of neuropathogenic proteins remains a major challenge. Indeed, most cellular and animal models to date rely on the expression of exogenous DNA constructs that are translated within the neuronal soma. Therefore, it is difficult to determine whether effects triggered from proteins derived from those constructs take place within axons, or represent an epiphenomenon of alterations initiated in the neuronal cell body. Within this context, isolated squid axoplasms obtained from the Atlantic squid *L. pealeii* provide a unique experimental system for the study of *axon-specific* molecular events. This experimental model was instrumental in the original discovery of conventional kinesin (Brady, 1985; Lasek & Brady, 1985), novel pathways for FAT regulation (Morfini, Burns, et al., 2009), and axonal-specific phosphorylation events (Grant, Diggins, & Pant, 1999). Unlike experiments based on cDNA transfection, the lack of permeability barriers (i.e., plasma membrane) in the axoplasm preparation allows for perfusion of various effectors at precisely controlled concentrations (Brady, Richards, & Leopold, 1993). Isolated axoplasm has been used to evaluate the impact of various neuropathogenic effectors on FAT, including filamentous and soluble forms of tau (Kanaan et al., 2011; Lapointe et al., 2009; Morfini, Pigino, Mizuno, Kikkawa, & Brady, 2007), mutant versions of huntingtin (Morfini, You, et al., 2009), androgen receptor (Morfini et al., 2006), spastin (Solowska et al., 2008), and SOD1 (Bosco et al., 2010; Morfini et al., 2013), as well as the parkinsonian drug MPP+ (Morfini, Pigino, Opalach, et al., 2007). In many cases, changes in conventional kinesin-dependent FAT were confirmed in mammalian animal models of associated diseases, thus establishing the axoplasm as a valuable model for the identification of pathways triggered by pathogenic proteins (Morfini, Burns, et al., 2009).

1. METHODS

1.1 THE ISOLATED SQUID AXOPLASM PREPARATION

Procedures for the preparation of isolated axoplasm from the Atlantic squid *Loligo pealeii* have been published (Brady et al., 1993; Song & Brady, 2013), and an update on those procedures can be found in Chapter Fast Axonal Transport in Isolated Axoplasm from the Squid Giant Axon of this issue. Squid are seasonally available from April through October at the Marine Biological Laboratory in Woods Hole, MA, USA. Suitable squid are approximately 30 cm in length and their giant axons are approximately 400–500 μm in diameter. Owing to intrinsic differences in baseline kinase activity among individual squid

(see Figures 2(B) and 3(A) and (B)), comparative studies require the dissection of two giant axons per squid, which are referred to as “sister” axons. Such differences are minimal between “sister” axons, thus allowing for reproducible comparison between two experimental conditions. After extrusion (see Chapter Fast Axonal Transport in Isolated Axoplasm from the Squid Giant Axon), axoplasms are typically placed on glass slides (Corning, Cat. #2948-75X25) and available for biochemical studies (Figure 1). The quality of extrusion is critical for these experiments, as activation of endogenous axoplasmic kinases by exogenous effectors often depends on the structural integrity of the axoplasm preparation.

Below, we provide protocols to analyze the effects of mutant neuropathogenic proteins on the activity of axonal kinases (Figure 2), and the phosphorylation of axonal substrates, including kinesin-1 and neurofilaments (NFs) (Figure 3). An overview of these methods is shown in Figure 1.

1.2 EFFECTS OF SPECIFIC PROTEINS ON AXONAL KINASE ACTIVITY

Buffer X was developed to mimic the unique nonprotein composition of the squid axoplasm. Potassium aspartate serves as the primary organic anion, taurine as the major reducing agent, betaine as an organic osmolyte, and glycine as an amino acids component (Song & Brady, 2013). To permit direct comparisons with vesicle trafficking studies (see Chapter Fast Axonal Transport in Isolated Axoplasm from the Squid Giant Axon), biochemical experiments described below involve perfusion of specific effectors diluted in half strength *Buffer X* (*Buffer X/2*). In some experimental settings, an isolated axoplasm is typically perfused with *Buffer X/2* alone (control condition), whereas its “sister” axoplasm counterpart is perfused with a given effector (i.e., a neurotoxin or any other compound) diluted in *Buffer X/2* (experimental condition) (LaPointe et al., 2013; Morfini, Pigino, Opalach, et al., 2007). In other cases, one axoplasm is perfused with a nonpathogenic protein (i.e., wild-type SOD1), whereas its “sister” is perfused with a pathogenic version of that protein (i.e., oxidized or mutant forms of SOD1), both proteins being diluted in *Buffer X/2* (see Table 1) (Bosco et al., 2010; Morfini et al., 2013).

1.2.1 Materials

1. Timer.
2. Water bath with thermometer.
3. Tabletop microcentrifuge.
4. 1.5 mL microcentrifuge tubes.
5. Super Pap Pen (Invitrogen, Cat. #00-8899) or equivalent liquid blocker pen.
6. Humidity chambers. Prepared by placing a piece of wet paper towel in a plastic box with a lid large enough for two glass slides. Empty pipet tip containers are typically used.
7. 50 mM HEPES pH 7.2.
8. Buffer X/0.8: 437.5 mM potassium aspartate, 162.5 mM taurine, 87.5 mM betaine, 62.5 mM glycine, 25 mM HEPES, 16.25 mM $MgCl_2$, 12.5 mM EGTA,

3.75 mM $CaCl_2$, 1.25 mM glucose, pH 7.2. Buffer X/0.8 is a concentrated (1.2X) version of full strength *Buffer X* that facilitates preparation of perfusion mixes containing recombinant proteins and pharmacological agents.

9. 200 mM ATP stocks, pH 7.2. Stocks are prepared in 50 mM HEPES pH 7.2 and stored in 20–50 μL aliquots at −80 °C until use. Avoid multiple freeze-thaw cycles.

10. *Buffer X+10 mM ATP*: Prepare the necessary volume (see Table 1 for an example) from the stocks 7, 8, and 9 above before use. Keep on ice. See **Note 1**.

    Note 1: ATP is added to facilitate the maintenance of ATP-dependent processes ex vivo.

11. Lysis buffer (*LB*): 1% w/v sodium dodecyl sulfate (SDS), diluted in 10 mM HEPES pH 7.2.

12. 6X gel loading buffer (*6X GLB*: 375 mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, 9% 2-mercaptoethanol, 0.03% bromophenol blue). Aliquot and store at −20 °C for up to 6 months. Thawed aliquots are stored at room temperature up to 2 weeks. Before use, make sure SDS is properly solubilized by warming at 37 °C for 1–5 min.

13. Effector(s) of interest: These may include recombinant polypeptides, neurotoxins, and pharmacological inhibitors. Stocks of recombinant-purified proteins are typically prepared in 10 mM HEPES pH 7.2. Small aliquots are flash-frozen in liquid nitrogen and stored at −80 °C until use. Avoid multiple freeze-thaw cycles. Unless the exact physiological concentration of an effector is known (Morfini, Pigino, Mizuno, et al., 2007), recombinant proteins are initially perfused at 0.1–2 μM. See **Note 2**.

    Note 2: Many commonly used buffers such as Tris, PIPES and MOPS, chaotropic agents (Cl and Na), detergents (Triton X-100), and organic reagents (glycerol) have a toxic inhibitory effect on FAT and should be eliminated by dialyzing samples in 10 mM HEPES pH 7.2.

1.2.2 Procedure

1. Set water bath at 90 °C.

2. Prepare perfusion mixes. Depending on the number of “sister” axoplasm pairs to be extruded, prepare the necessary volumes of control and experimental perfusion mixes and keep on ice. Effectors (i.e., recombinant proteins) in perfusion mixes should be diluted in Buffer X/2 plus 5 mM ATP. An example on how to prepare perfusion mixes is shown in Table 1, where enough “control” and “experimental” perfusion mixes were prepared to perfuse seven “sister” axoplasm pairs. In this specific example, the “control” perfusion mix contains properly folded, recombinant superoxide dismutase protein (SOD1-WT, 5 μM), whereas the “experimental” perfusion mix contains a misfolded, oxidized (SOD1-ox, 5 μM) version of SOD1 (Bosco et al., 2010). If effector availability is

not a concern, prepare enough perfusion mix for one additional axoplasm to account for small pipetting errors.

3. After extrusion on glass slides (see Chapter Fast Axonal Transport in Isolated Axoplasm from the Squid Giant Axon), draw along the contour of each axoplasm using the Pap Pen (Figure 2(A)). Avoid touching the axoplasms. As a reference, the liquid blocker should be placed at approximately 3-mm distance from the axoplasm border. Allow 20–30 s for the liquid blocker to dry.

4. Place coverslips with "sister" axoplasms in a humidity chamber and keep in a fridge (at 4 °C) for 5 min. See **Note 3**.

   Note 3: In our experience, this preincubation step increases the consistency of results, likely by suppressing an initial stress response induced by the extrusion procedure.

5. Remove the humidity chamber from the fridge.

6. Add 30 μL of *control perfusion mix* to one axoplasm, and 30 μL of *experimental perfusion mix* to the "sister" axoplasm, perfusing them 1–2 min apart (Figure 2(B)). If needed, slightly incline the coverslips to ensure the entire axoplasm is perfused.

7. Incubate perfused "sister" axoplasm pairs in the humidity chamber at room temperature for 50 min. See **Note 4**.

   Note 4: This incubation time has been chosen to allow correlations between results from biochemical experiments and vesicle motility assays (see Chapter Fast Axonal Transport in Isolated Axoplasm from the Squid Giant Axon). Some experiments might require different incubation times and those should be determined empirically.

8. To stop the incubation, use a P100 or P200 pipettor to slowly add 30 μL of *LB* to each axoplasm. The surface tension of the solution should prevent spilling of *LB* beyond the liquid blocker line.

9. Using a P100 or P200 pipettor, carefully collect the axoplasm and all of the perfusion mix to transfer to a 1.5 mL microcentrifuge tube. If needed, carefully incline the slide to collect all the perfusion mix. See **Note 5**.

   Note 5: Practice caution when pipetting up the axoplasm, which after addition of 1% SDS in *LB* becomes "gooey." Instead of pipetting up the entire axoplasm, "grab" the axoplasm with the negative pressure inside the pipette tip and carefully transfer to a microcentrifuge tube. Collect any remaining *LB* on the glass slide as well.

10. Briefly spin down axoplasm lysates in a tabletop centrifuge and incubate for 5 min at 90 °C in water bath. See **Note 6**.

    Note 6: This step, along with the addition of SDS in *LB*, minimizes potential postlysis modification (i.e., dephosphorylation) of endogenous squid proteins.

11. Add 30 μL of *6X GLB* to each microcentrifuge tube.

12. Store collected samples at −20 °C until needed for immunoblot analysis. See **Note 7**.

    Note 7: Antibodies used in immunoblot analysis of squid axoplasms need to be validated, as they may or may not be able to detect endogenous proteins depending on sequence homology between target species (see Figure 2(C)). In our experience, antibodies recognizing evolutionarily conserved activation domains within protein kinases are among the most likely to cross-react with squid variants. A list of selected phosphorylation-dependent and independent antibodies that effectively recognize their target protein in squid lysates is provided in Table 2.

An example of results from these type of experiments is shown in Figure 2(D), and others can be found in our published work (Bosco et al., 2010; Morfini et al., 2013; Morfini, You, et al., 2009).

1.3 METABOLIC LABELING OF AXONAL PROTEINS

Phosphorylation represents a major regulatory mechanism for conventional kinesin-mediated FAT. A metabolic labeling protocol using radiolabeled ATP γ–^{32}P is described below, which can be used to (1) identify alterations in the phosphorylation of endogenous squid proteins including NFs, kinesin-1, and any other proteins for which antibodies with immunoprecipitation activity and squid cross-reactivity are suitable or (2) to determine phosphorylation of exogenously added proteins by endogenous axoplasmic kinases. Institutional radiation safety guidelines must be followed at all times.

1.3.1 Materials

1. Timer.
2. Water bath with thermometer.
3. Tabletop microcentrifuge.
4. 1.5 mL microcentrifuge tubes.
5. Super Pap Pen (Invitrogen, Cat. #00-8899) or equivalent liquid blocker pen.
6. Humidity chambers (see Section 1.2.1).
7. SDS-PAGE electrophoresis equipment.
8. Orbital shaker.
9. Gel drier (i.e., Hoefer GD2000).
10. Plexiglass screen.
11. Phosphorimager system (i.e., Typhoon from Molecular Dynamics or equivalent) or autoradiography cassettes.
12. Rotator with attachments to hold 1.5 mL microcentrifuge tubes.

13. 30-gauge needles.

14. Vacuum trap.

15. Radiolabeled ATP γ-^{32}P (10mCi, from PerkinElmer, Cat. #NEG035C010MC). Store at −80 °C until use. See **Note 8**.

    Note 8: The half-life of ^{32}P is 14.3 days. Obtain the ATP γ-^{32}P near the date of metabolic labeling in order to assure highest specific activity.

16. Chrompure Mouse IgG agarose resin (Jackson Immunoresearch, Cat. #015-000-052). See **Note 9**.

17. Protein G agarose resin (Thermoscientific, Cat. #20399). See **Note 9**.

    Note 9: Depending on the volume needed, transfer an aliquot of each resin to a microcentrifuge or a 15 mL conical tube. Wash with 10 mM HEPES pH 7.2, and allow beads to settle. Repeat this step for additional three washes. Agarose resins are prepared as 50% slurries (in 10 mM HEPES pH 7.2) and stored at 4 °C. Add sodium azide to 0.01% w/v from a 1% w/v stock and store at 4 °C.

18. 10% bovine serum albumin (BSA) stock. Prepare a 10% w/v solution of IgG-and protease-free BSA (Jackson Immunoresearch, Cat. #001-000-162) in 10 mM HEPES pH 7.2. If stored for over a day, add sodium azide to 0.01% w/v from a 1% w/v stock and keep at 4 °C.

19. Antibody stocks: Anti-kinesin-1 monoclonal antibody (H2 clone, available from Millipore Cat. MAB1614) and anti-KLC mouse monoclonal antibodies (clones 63–90 and KLC-All, both available from the laboratory of Dr Scott Brady upon request). See **Note 10**.

    Note 10: The choice of antibody is based on the target protein. In principle, any endogenous axoplasmic protein could be immunoprecipitated, if antibodies be available that recognize homolog squid variants. The specificities of anti-kinesin-1 antibody H2 (Brady, Pfister, & Bloom, 1990) and anti-KLCs antibodies 63–90 and KLC-All (Stenoien & Brady, 1997) have been previously reported (DeBoer et al., 2008).

20. 1% SDS

21. 50 mM HEPES pH 7.2.

22. *Homogenization Buffer* (*HB*: 50 mM HEPES pH 7.2, 100 mM NaCl, 0.5% Triton X-100, 0.1% sodium deoxycholate, 50 mM NaF, 1 mM ethylenediaminetetraacetic acid (EDTA), 80 mM β-glycerophosphate).

23. Protease inhibitor cocktail, mammalian (Sigma-Aldrich, Cat. #P8340). Prepare 50 μL aliquots and store at −20 °C.

24. Phosphatase inhibitor cocktail II (Calbiochem, Cat. #524625). Prepare 50 μL aliquots and store at −20 °C.

25. Okadaic acid (Calbiochem, Cat. #459620). Prepare a 10 μM stock in methanol and store at −20 °C.

26. Microcystin (Calbiochem, Cat. #475816). Prepare a 10 μM stock in methanol and store at −20 °C.

27. 200 mM Na_3VO_4 (Sigma-Aldrich, Cat. #S6508). Prepare appropriately as described by the manufacturer. Store 50 μL aliquots at −20 °C.

28. *Homogenization Buffer plus inhibitors* (*HB+I*: 50 mM HEPES pH 7.2, 100 mM NaCl, 0.5% Triton X-100, 0.1% sodium deoxycholate, 50 mM NaF, 1 mM EDTA, 80 mM β-glycerophosphate, 100 nM okadaic acid, 100 nM microcystin, 2 mM Na_3VO_4, 1/200 dilution of phosphatase inhibitor cocktail II, 1/200 dilution of protease inhibitor cocktail, mammalian. Prepare 1.1 mL of *HB+I* per axoplasm to account for pipetting errors. See **Note 11**.

Note 11: Conventional kinesin is subject to phosphorylation by multiple kinases, some of which become activated during the homogenization step (Hollenbeck, 1990; Morfini et al., 2001; Tsai, Morfini, Szebenyi, & Brady, 2000). To help minimize potential postlysis phosphorylation artifacts, *HB + I* includes a mixture of general kinase and phosphatase inhibitors. Inhibitors are added to *HB* right before each experiment.

29. *Immunoprecipitation Wash Buffer* (*IPP wash buffer*: 10 mM HEPES pH 7.2, 200 mM NaCl, 0.5% Triton X-100). Prepare 100 mL and store at 4 °C for up to a month.

30. *3X GLB*. Prepared by diluting *6X GLB* in water (see Section 1.2.1).

31. *Preclearing mix*. Prepare in a 15 mL conical tube. For each pair of "sister" axoplasms, combine:

- **a.** 1 mL of *HB + I*.
- **b.** 10 μL of 10% BSA in LB. Final BSA concentration will be 0.1%.
- **c.** 20 μL of Protein G resin. See **Note 12**.
- **d.** 20 μL of Chrompure Mouse IgG agarose resin. See **Note 12**.

  Note 12: Volumes refer to 50% slurries. Thoroughly resuspend resins before pipetting as they quickly settle.

32. Buffer X/0.8 (see Section 1.2.1).

33. "*Hot*" *Buffer X* (*Buffer X* supplemented with 2 mM ATP ATP γ–^{32}P [2 μCi/μL]). See Table 3 for an example preparation. Store 35 μL aliquots at −80 °C.

34. See **Note 13**.

Note 13: The concentration of ATP in "Hot" *Buffer X* is lower than that used in Section 1.2.1. This is to increase the incorporation of radiolabeled ATP γ–^{32}P into endogenous proteins. Because "*Hot*" *Buffer X* is diluted twofold in perfusion mixes, a 15 μL volume of "*Hot*" *Buffer X* should be prepared per

single axoplasm (30 μL per axoplasm pair). For instance, if 10 pairs of "sister" axoplasms are used for metabolic labeling experiments, the total volume of "*Hot*" *Buffer X* needed is 330 μL, which includes 30 μL extra volume to account for pipetting errors.

1.3.2 Metabolic labeling and immunoprecipitation

1. Prepare perfusion mixes. Split an aliquot of "Hot" Buffer X + 2 mM ATP in half. One half is used for the perfusion of the control buffer and the other half is for the perfusion of the desired effector. Prepare half-strength "Hot" Buffer X/2 by diluting the buffer in half in 10 mM HEPES along with desired effectors. Refer to Table 4 for an example. The final concentration of perfusion mix per axoplasm should contain "Hot" Buffer X/2, 1 mM ATP, 30 μCi ATP γ–^{32}P, and desired effector(s).
2. Complete *Steps 3–5*, as described in Section 1.2.2.
3. Add 30 μL of "control" hot perfusion mix to one axoplasm, and 30 μL of "experimental" hot perfusion mix to its "sister" axoplasm. Perfuse "sister" axoplasms 1–2 min apart. If needed, slightly incline the coverslips to ensure the entire axoplasm is perfused. Use Plexiglass screens to minimize exposure to radiation.
4. Incubate axoplasms in the humidity chamber for 50 min at room temperature.
5. Stop the incubation by adding 20 μL of 1% SDS on each axoplasm.
6. Transfer each axoplasm and the surrounding solution into properly labeled microcentrifuge tubes (see Section 1.2.2, *Step 9*). Total volume should be approximately 50 μL.
7. Carefully wash each glass slide by adding 50 μL of *HB* + *I* within the boundary of the liquid blocker pen, and transfer the solution to the same microcentrifuge tube.
8. Briefly spin down samples and incubate the samples in a water bath at 60 °C for 2 min to eliminate any residual phosphatase activity and to solubilize NFs.
9. Place samples on an orbital shaker and rotate for 30 min. See **Note 14**.

   Note 14: This step helps solubilize the highly stable NF cytoskeleton, which is referred to as the axoplasm "ghost" (Song & Brady, 2013).
10. Add 1 mL of *preclearing mix* to each tube and incubate samples at 4 °C with rotation for 1 h. See **Note 15**.

    Note 15: 0.1% BSA in *preclearing mix* helps prevent nonspecific binding of radiolabeled proteins to the tube wall and lids. This step also reduces background signal in immunoprecipitates, as it eliminates proteins that nonspecifically bind to Protein G or to IgGs.
11. While waiting for incubation, label the necessary number of 1.5 mL microcentrifuge tubes, each containing 20 μL of Protein G resin (50% slurries)

and a fixed amount of antibody(ies), which should be determined empirically. For immunoprecipitation of conventional kinesin in squid axoplasm, we typically use 4 μg of anti-KHC antibody (H2) and 4 μg of anti-KLC antibody (63–90) per axoplasm.

12. Centrifuge tubes from *Step 10* at 14,000 rpm for 2 min on a tabletop centrifuge.

13. Transfer 30 mL aliquot of clarified supernatant to a tube containing 15 mL of GLB and store at −20 °C. See **Note 16**.

    Note 16: This aliquot is referred to as "input," and it serves as a control for similar levels of protein radiolabeling among saxoplasms. NFs are the most highly phosphorylated proteins in metabolically labeled axoplasm lysates (Pant, Shecket, Gainer, & Lasek, 1978). SDS-PAGE analysis of "input" samples can reveal alterations in the activity of NF kinases triggered by a given effector (see Figure 3(A)–(B)).

14. Carefully transfer 900 μL of the remaining clarified supernatant to microcentrifuge tubes containing Protein G and antibody (from *Step 11*). Avoid pipetting any pellet.

15. Incubate radiolabeled axoplasm lysates containing Protein G agarose beads and antibodies for 4 h to overnight at 4 °C with rotation.

16. Centrifuge samples at 1000 rpm for 1 min on a tabletop centrifuge to settle Protein G beads. See **Note 17**.

    Note 17 (**optional**): The supernatant resulting from this centrifugation can be used to immunoprecipitate additional axoplasmic proteins. If needed, transfer to a new set of labeled microcentrifuge tubes and store at −80 °C. Otherwise, discard following institutional radiation safety guidelines.

17. Wash Protein G agarose resin pellets with 1 mL of *IPP wash buffer*.

18. Centrifuge samples at 1000 rpm for 1 min on a tabletop centrifuge.

19. Using a vacuum trap connected to a pipette tip, carefully aspirate *IPP wash buffer*, leaving approximately 50 μL volume untouched behind. Avoid disturbing the pellet!

20. Repeat *Steps 17–19* three times for a total of four washes with *IPP wash buffer*, and a final wash in 10 mM HEPES pH 7.2. See **Note 18**.

    Note 18: This last wash removes most Triton X-100 and NaCl from *IPP wash buffer*, which interfere with SDS-PAGE analysis.

21. Using a 30-gauge needle tip attached to a vacuum source, carefully aspirate as much HEPES as possible. The small diameter of this gauge prevents beads from being aspirated. Wash the needle tip between each tube by briefly immersing it on a 50 mL conical tube containing 10 mM HEPES pH 7.2.

22. Add 30 μL of *3X GLB* to each microcentrifuge tube containing immunoprecipitate.

23. Place samples in a water bath (at 90 °C) for 2 min to reduce IgGs and facilitate separation of immunoprecipitated proteins by SDS-PAGE.

24. Separate immunoprecipitates by SDS-PAGE electrophoresis. See **Note 19**.

Note 19: Optimization of SDS-PAGE conditions may be needed depending on the target protein. For studying conventional kinesin, good separation of kinesin-1s from KLCs can be obtained with 7.5–16% Tris-glycine acrylamide gels or 4–12% Bis-Tris acrylamide gels with MOPS-based running buffer (Life Technologies, Cat. #NP0001).

25. Dry gels and expose in a Typhoon phosphorimager cassette (Molecular Dynamics) or to X-ray film in an autoradiography cassette.

An example of these type of experiments is shown in Figure 3. Figure 3 depicts the effects of wild type and oxidized forms of SOD1 on the phosphorylation of NFs (Figure 3(A) and (B)) and conventional kinesin (Figure 3(C)).

CONCLUSIONS

Using extruded axoplasms obtained from the Atlantic squid *L. pealeii*, we presented biochemical approaches to evaluate phosphorylation events triggered by neuropathogenic effectors in axons without the influence of neuronal cell bodies. These procedures should help researchers determine the effects of protein and nonprotein effectors on the phosphorylation status of specific axonal proteins, including protein kinases, conventional kinesin, and NFs.

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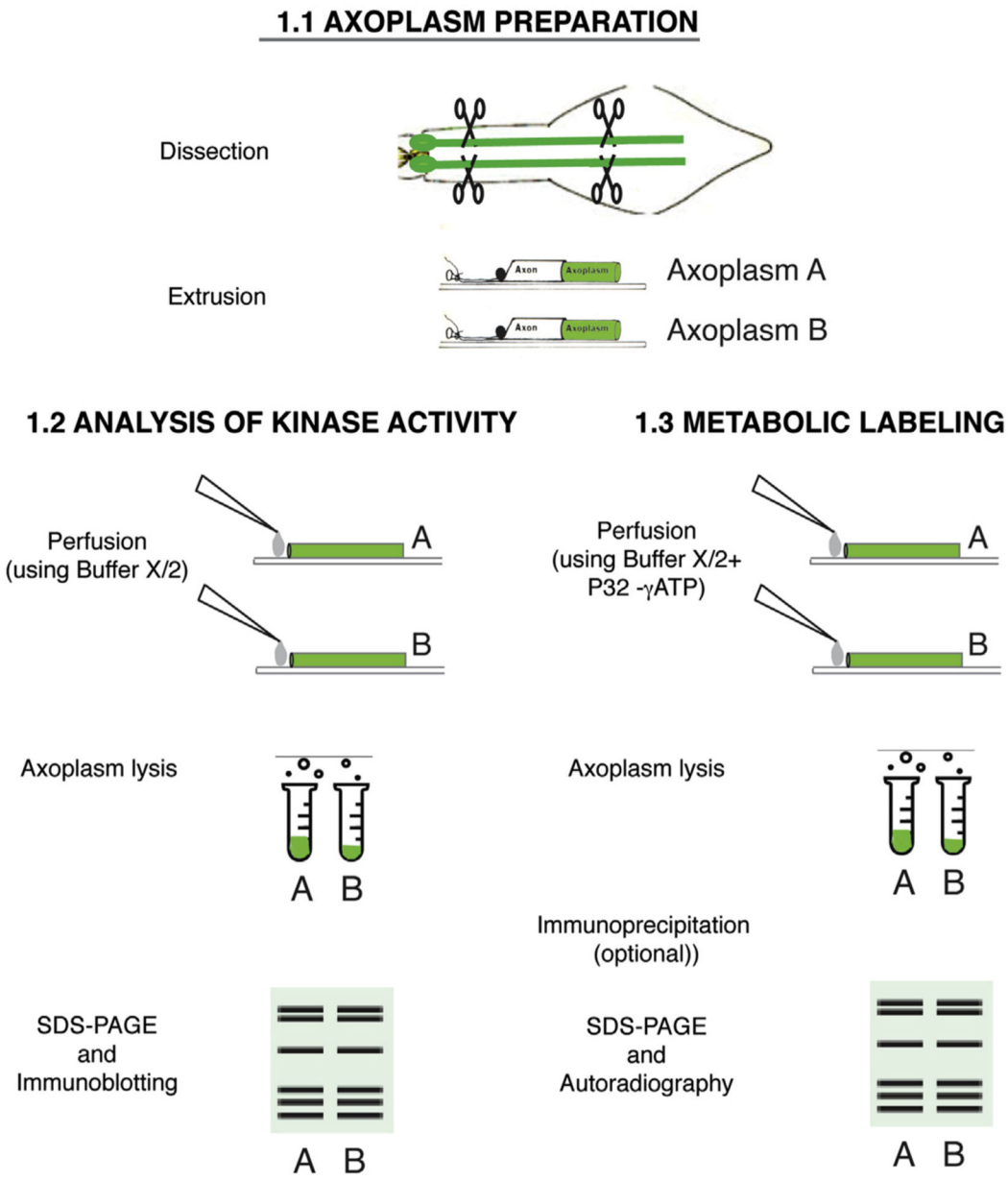

FIGURE 1.

Outline of procedures in this chapter. Owing to intrinsic differences between different squid, two “sister” axons need to be dissected from the same squid, extruded, and placed on glass coverslips (see Chapter Fast Axonal Transport in Isolated Axoplasm from the Squid Giant Axon). One axoplasm is perfused with *control perfusion mix* and its “sister” axon with *experimental perfusion mix* (see Table 1 for an example). Metabolic labeling experiments require perfusion mixes containing P^{32}-radiolabeled adenosine triphosphate. After 50-min incubation, axoplasms are lysed and processed for immunoblotting (see Section 1.2). For metabolic labeling procedures, radiolabeled axoplasmic proteins are immunoprecipitated and analyzed by autoradiography (see Section 1.3).

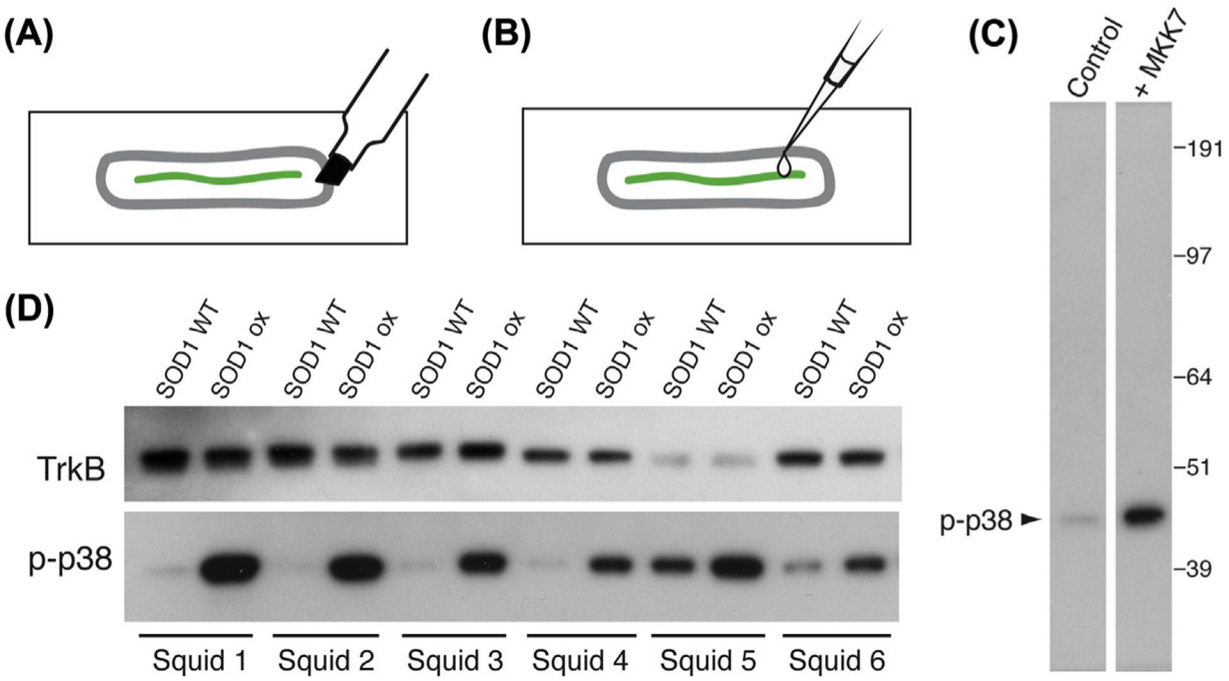

FIGURE 2.

Axon-autonomous effects of properly folded, wild type (SOD1-WT) and oxidized (SOD1-ox) forms of superoxide dismutase 1 on p38 MAPK activity. (A) A schematic of the use of the liquid blocker pen (i.e., Super Pap Pen) described in *Step 3* of Section 1.2.2. A liquid blocking line is drawn along the contour of the axoplasm on the glass slide approximately 3 mm away from the axoplasm. (B) Using a P100 or P200 pipettor, perfusion mixes are placed within the boundary drawn with the Pap Pen. If needed, coverslips are tilted to ensure thorough perfusion of the axoplasm. (C) Validation of rabbit monoclonal antibody clone 3D7 (p-p38 antibody; Cell Signaling #9215) in squid axoplasm. Two axoplasms were homogenized in *Buffer X/2 plus adenosine triphosphate* using a pipettor. The homogenate was briefly centrifuged and similar aliquots of the supernatant were incubated with (+MKK7) or without (Control) recombinant MKK7 (from EMD Millipore), a MAPK that directly phosphorylates and activates p38. After a 30-min incubation, samples were processed for immunoblotting with antibody 3D7. As expected, increased immunoreactivity was observed in the aliquot incubated with MKK7, compared to its control. (D) “Sister” axoplasms were dissected from six squid (Squid# 1–6) and perfused with recombinant forms of superoxide dismutase 1 (SOD1, 5 μM concentration). One axoplasm was perfused with wild-type SOD1 (SOD1-WT) and its “sister” axoplasm with an oxidized, misfolded form of SOD1 (SOD1-ox) associated with sporadic forms of amyotrophic lateral sclerosis (Bosco et al., 2010). After a 50-min incubation, axoplasms were lysed, separated by SDS-PAGE, and processed for immunoblotting using monoclonal antibody 3D7 (as in C). Note the variable levels of active p38 among different squid. Despite this variability, p-p38 immunoreactivity is consistently higher in axoplasms perfused with SOD1-ox than their “sister” counterparts perfused with SOD1-WT. A band recognized by a polyclonal antibody against TrkB (Santa Cruz, Cat. #sc-11, Lot# E0708) provided an internal control for protein loading. A portion of the p-p38 immunoblot (Squid #1–3) was shown in (Bosco et al., 2010).

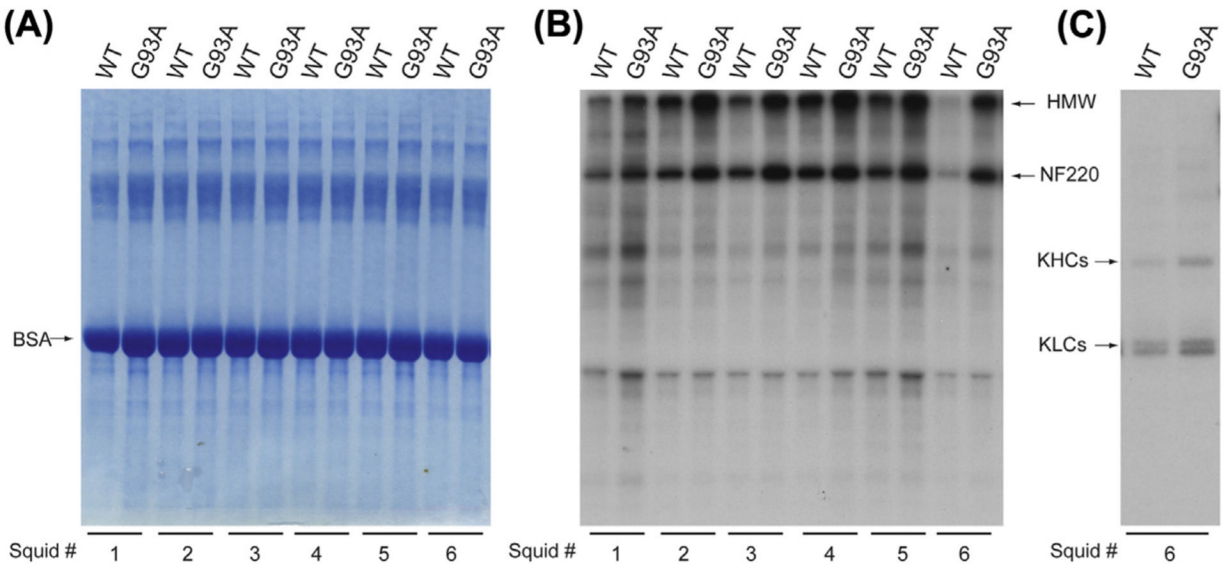

FIGURE 3.

Phosphorylation of squid neurofilaments (A–B) and conventional kinesin (C) was analyzed in "sister" axoplasms perfused with either wild type (WT) or mutant (G93A) forms of recombinant superoxide dismutase 1 (SOD1) using metabolic labeling experiments with ^{32}P-γ-ATP. (A) "Input" aliquots (see Section 1.3.2, *Step 14*) of radiolabeled lysates were separated by SDS-PAGE. Coomassie Blue staining of the gel shows similar levels of axoplasmic proteins. The molecular weight position of bovine serum albumin is indicated. (B) Autoradiogram of the gel in A shows radiolabeled bands corresponding to neurofilament heavy chain subunit (NFH) and NF220 (major NF subunits in squid axoplasm) (Grant et al., 1999). Despite the variability among different squid, "sister" axoplasms perfused with G93A-SOD1 consistently showed increased phosphorylation of NF220 and HMW neurofilament (NF) subunits, compared to their "sister" counterpart (perfused with WT-SOD1). (C) As an example, an autoradiogram shows conventional kinesin immunoprecipitates obtained from "sister" axoplasms prepared from Squid #6. Statistical analysis of additional axoplasms revealed increased phosphorylation of kinesin-1, but not kinesin light chains, in axoplasms perfused with G93A-SOD1, compared to their corresponding "sister" axoplasm perfused with WT-SOD1 (Morfini et al., 2013).

Table 1

An Example of Perfusion Mixes Containing Either SOD1-WT or SOD1-ox

Stock	Control	Experimental	Final Concentration
Buffer X + 10 mM ATP	105 μL	105 μL	Buffer X/2 + 5 mM ATP
10 mM HEPES pH 7.2	70 μL	81.2 μL	
SOD1-WT 30 μM stock	35 μL	0 μL	SOD1-WT 5 μM
SOD1-ox 44 μM stock	0 μL	23.8 μL	SOD1-ox 5 μM
Final volume	210 μL	210 μL	

Table 2

Validated Antibodies that Cross-react with squid Versions of Their Target Proteins

Antigen	Host	Clone	Source
Kinesin-1A, 1B, 1C	Mouse monoclonal	H2	Chemicon
Kinesin light chains	Mouse monoclonal	63–90	In-house *
Kinesin light chains	Mouse monoclonal	KLC-All	In-house *
α-Tubulin	Mouse monoclonal	DM1A	Sigma-Aldrich
Phosphorylated neurofilament	Mouse monoclonal	SMI-31	BioLegend
Phosphorylated neurofilament	Mouse monoclonal	RMO55	EMD Millipore
SNAP-25	Mouse monoclonal	71.1	Synaptic Systems
p-JNK (Thr183/Tyr185)	Mouse monoclonal	G9	Cell Signaling #9255
p-JNK (Thr183/Tyr185)	Rabbit polyclonal		Cell Signaling #9251
p-JNK (Thr183/Tyr185)	Rabbit monoclonal	81E11	Cell Signaling #4668
p-MKK3 (Ser189)/MKK6 (Ser207)	Rabbit monoclonal	22A8	Cell Signaling #9236
p-MKK7 (Ser271/Thr275)	Rabbit polyclonal		Cell Signaling #4171
p-p38 (Thr180/Tyr182)	Rabbit monoclonal	3D7	Cell Signaling #9215
p-GSK3β (Ser9)	Rabbit monoclonal	D85E12	Cell Signaling #5558
p-ERK (Tyr204)	Mouse monoclonal	E-4	Santa Cruz Biotech sc-7383

* Available from the laboratory of Dr Scott Brady upon request.

Table 3

Preparation of “Hot” Buffer X Stock for 10 Pairs of “Sister” Axoplasms

Stock	Volume	Final Concentration
10 mM HEPES pH 7.2	53.9 μL	
Buffer X/0.8	264 μL	Buffer X
200 mM ATP	3.3 μL	2 mM ATP
ATP γ–^{32}P [150 μCi/μL]	4.4 μL	ATP γ –^{32}P [2 μCi/μL]
Final volume	330 μL	

Table 4

An example of “Hot” Perfusion mix Containing Either SOD1-WT or SOD1-ox

Stock	Control	Experimental	Final Concentration
10 mM HEPES pH 7.2	70 μL	81.2 μL	
“*Hot*” *Buffer X* + 2 mM ATP + ATP γ^{-32}P [2 μCi/μL]	105 μL	105 μL	“Hot” Buffer X/2 + 1 mM ATP + ATP γ^{-32}P [1 μCi/μL]
SOD1-WT 30 μM stock	35 μL	0 μL	SOD1-WT 5 μM
SOD1-ox 44 μM stock	0 μL	23.8 μL	SOD1-ox 5 μM
Final volume	210 μL	210 μL	