

Learning Something ORIGINAL at the *Drosophila* Neuromuscular Junction

Genetic screens seek to identify molecules based on phenotype, without regard to the expectations of the experimenter. A classic example is the screen by Nusslein-Volhard and Weischaus (1980) that identified most of the genes required for embryonic pattern formation. Such an approach is being applied to a question of special interest to neurobiologists—what are the molecules and mechanisms required for learning and memory? *Drosophila* can be taught to associate an odor with a footshock, and single gene mutants have been identified that are defective in either learning or remembering this task. Most such mutants show defects in two areas, brain development and synaptic plasticity (Dubnau and Tully, 1998). Previously identified plasticity mutants conform to expectations, encoding proteins involved in signal transduction. Now, however, genetics presents us with a surprise. The gene for the learning mutant *latheo* (*lat*) encodes a component of the origin replication complex (ORC), which is important for DNA replication, but the protein is also present at the synapse and regulates synaptic function and plasticity.

latheo (Greek for “to cause a person not to know”) was identified in a genetic screen for *Drosophila* mutants with defects in associative learning and memory (Boynnton and Tully, 1992). In this issue of *Neuron*, a collaboration between the Tully and Dutta groups presents the cloning of *lat* and the characterization of its biochemical function (Pinto et al., 1999). Tully and colleagues identify the transcript disrupted in *lat* and find that it encodes a novel gene homologous to a human expressed sequence tag (EST). In independent experiments, Dutta and colleagues have isolated new constituents of the human ORC, a group of proteins essential for DNA replication (Rowles and Blow, 1997). Pinto, Quintana, and colleagues (1999) find that an ORC protein is encoded by the human *lat* homolog, and show that human and *Drosophila* *latheo* (LAT) proteins biochemically associate with the ORC and are homologous to the yeast protein ORC3.

Why was a DNA replication protein identified in a screen for learning and memory mutants? A weak allele of *lat* shows the learning and memory defect, while stronger alleles are pupal lethal. Pinto et al. (1999) demonstrate that null mutations of *lat* have severe defects in cell proliferation; the mutants lack imaginal discs, proliferating cell clusters that form much of the adult body. In addition, neuroblast proliferation is disrupted, so these mutants have small brains. The mushroom bodies, structures crucial for olfactory learning (Heisenberg et al., 1985), are reduced in both weak and strong *lat* mutants, providing a satisfying explanation for *lat*'s learning and memory defect.

This, however, is not the entire story. Many *Drosophila*

learning and memory mutants have morphological and functional synaptic defects that can be assayed at the neuromuscular junction (NMJ) (Dubnau and Tully, 1998). In this issue of *Neuron*, work from Broadie and colleagues (Rohrbough et al., 1999) analyzes the synaptic phenotype of *lat* mutants. The most unexpected result is that the LAT protein is localized to synaptic boutons of the fly NMJ. While the staining intensity varies at different synaptic sites, the protein appears to be present at all neuromuscular synapses. Double staining with a variety of synaptic markers suggests that the protein is present in the presynaptic terminal. Having demonstrated that LAT is present at the NMJ, the authors characterize synaptic structure and function in *lat* mutants. They find only subtle alterations of synaptic morphology but a profound change in the function of the synapse.

latheo mutants show a large increase in the amplitude of evoked synaptic events, with no change in the postsynaptic responsiveness to transmitter. Hence, in the mutant many more vesicles are released when the nerve is stimulated. In addition to this alteration of basal synaptic transmission, *lat* has defects in synaptic plasticity. The *Drosophila* NMJ exhibits many forms of activity-dependent synaptic plasticity including paired-pulse facilitation, augmentation, and posttetanic potentiation (Zhong and Wu, 1991). All of these plasticity mechanisms are disrupted in *lat*. These defects are not merely a result of the increase in basal transmission in the mutant. When basal transmission is decreased in the mutant to wild-type levels (by decreasing the extracellular calcium concentration), there is still an impairment in all forms of activity-dependent plasticity.

The involvement of a DNA replication protein in functional synaptic plasticity is, to put it mildly, unexpected, and leaves us with three interpretations of the synaptic phenotype of *latheo* mutants. First, the synaptic defects may be downstream of LAT's role in the nucleus, and the presence of LAT at the synapse may be a fluke. Second, the ORC has two functions, one in the nucleus and one at the synapse. Third, LAT has two functions, one as a part of the ORC and a second at the synapse. Fortunately, the reagents exist to test these three models. LAT biochemically interacts with another subunit of the *Drosophila* ORC, DmORC2. Mutants for *DmORC2* show an identical proliferation phenotype to *lat*, with late larval lethality, missing imaginal discs, and defective cell proliferation (Gatti and Baker, 1989; Landis et al., 1997). In addition, antibodies have been generated to DmORC2 (Gossen et al., 1995). Future experiments can address whether *DmORC2* mutants have a synaptic phenotype and if the protein is synaptically localized. If *DmORC2* mutants do show the *latheo* synaptic phenotype but DmORC2 is not present at the synapse, this would suggest that the synaptic defects are downstream of the ORC's role in replication. If DmORC2 is at the NMJ, this would lead to the exciting hypothesis that the ORC has a second function at the synapse. Finally, if *DmORC2* mutants have no synaptic phenotype, this would argue that LAT has a second function

at the synapse. Regardless of the final explanation for the unexpected results with LAT, genetics has served its purpose in calling attention to a molecule that is not a usual suspect at the synapse.

How, in the end, should we think about the learning phenotype of *latheo* mutants? Is it a result of defective mushroom body development, impaired synaptic plasticity, or both? A satisfying answer must await the identification of mutants that separate the roles of *lat* at the chromosome and the synapse, or the creation of conditional mutants that rescue only the developmental defects. In the meantime, these two papers in *Neuron* may inspire neurobiologists to head to the library for a refresher course on DNA replication.

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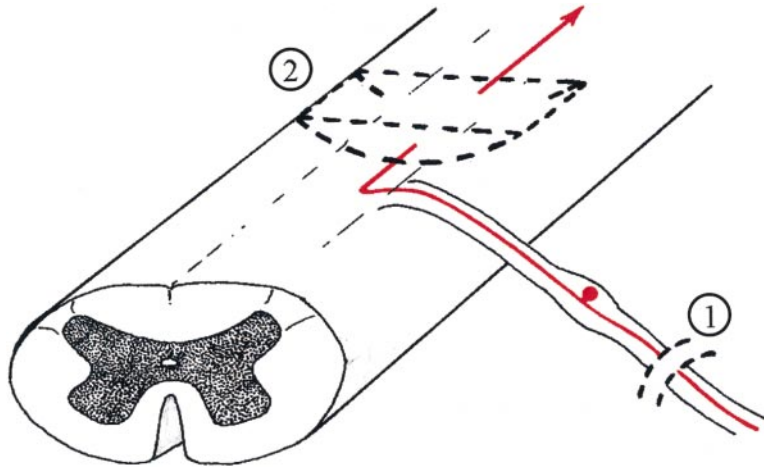
The Benefits of Adding Insult to Injury

A persistent, central question in neurobiology is, why do the axons of the PNS regenerate after injury while CNS axons do not? Several factors are known to prevent CNS regeneration: glial scarring (which presents both a physical barrier and inhibitors to regeneration), myelin-specific inhibitors, and possibly the loss of general growth capacity by adult CNS axons. In contrast, the PNS regenerates largely because of the environmental changes following injury. Myelin debris is cleared and Schwann cells dedifferentiate, downregulating expression of myelin proteins and thus becoming permissive for regeneration. Not surprisingly, a number of studies, going back over a decade, demonstrate that CNS axons

readily grow in vivo into an implanted graft of PNS tissue (Richardson et al., 1980; David and Aguayo, 1981).

An environment favorable to regeneration, however, is not all that is needed. Further work showed that a primary lesion in the peripheral branch of the dorsal root ganglion (DRG), followed by a second lesion in the spinal branch of the same nerve at the same time or weeks later, results in more extensive growth into peripheral nerve grafts situated in the dorsal columns. The conditioning peripheral nerve lesion somehow encourages growth for the majority of lesioned spinal axons to the most distal edge of the graft but still not into the host CNS tissue beyond. In spite of the conditioning lesion effect, it appears that a glial scar at the graft–host border, along with myelin-specific inhibitors, ultimately halts axonal growth (Richardson and Issa, 1984; Oudega et al., 1994). What is notable about these animal experiments is that the CNS axons grow better if their peripheral branch has been previously cut. Even a conditioning lesion in the peripheral branch of a DRG, followed by a second peripheral branch lesion of the same nerve rather than a central lesion, leads to more rapid regeneration (McQuarrie and Grafstein, 1973). Together, these experiments suggest a strong environmental influence on regeneration, but they also point to some intrinsic properties of the neuron that must affect the extent of regeneration. A conditioning lesion in the peripheral branch of the sciatic nerve induces an intrinsic change in the neuron that allows either the peripheral or central branch to subsequently grow better after injury.

In a study reported in this issue of *Neuron*, Neumann and Woolf (1999) extend these observations by demonstrating that transected dorsal column axons regenerate when a conditioning lesion is first created in the peripheral branch of the sciatic nerve. What makes this study important, and different, is not the substantial distance traversed by the regenerating dorsal column axons, nor the possibly greater number of regenerating axons compared to other studies, but rather that no peripheral nerve graft was used. Regeneration occurred into what is, by all previous criteria, the highly nonpermissive environment of the damaged spinal cord (see figure). After corticospinal tract lesions by Schwab and colleagues, regeneration also occurred over relatively long distances, but these studies employed the monoclonal antibody IN-1, which neutralizes some of the myelin-specific inhibitors of regeneration (Bregman et al., 1995). In the study reported here, regeneration occurred without blocking myelin or glial scar inhibitors. Transecting the dorsal column and simultaneously lesioning the peripheral nerve results in extensive regeneration into the lesion site but not beyond. Significantly, however, Neumann and Woolf (1999) show that when the conditioning lesion in the peripheral nerve is performed 1 week before the dorsal column transection, about 50% of the animals show axon regrowth around the site of injury and into the gray matter surrounding the central canal, with little or no growth into the lesion site itself. Furthermore, these axons continue to grow both caudally and rostrally from the lesion site. In the remaining half of the prelesioned animals, axons did grow into the lesion site, continuing on through the lesion and beyond, to grow mostly



Sites of Preconditioning Peripheral Branch Lesion and Subsequent Central Branch Lesion in Dorsal Root Ganglion Nerves

Preconditioning lesions were created in the sciatic nerve (L4–L6) (1) either simultaneously with, or 1 or 2 weeks prior to, a dorsal column transection at T6–T7 (2). Regeneration occurred through the dorsal column lesion site if the preconditioning lesion preceded the dorsal column lesion by 1 or 2 weeks; regrowth occurred only into the lesion if the preconditioning lesion was inflicted at the same time; no regeneration occurred without a peripheral lesion. The dashed line shows the sites of lesions.

through host gray matter but with considerable regeneration also observed in white matter. A 2-week conditioning lesion resulted in slightly less effective dorsal column regeneration than a 1-week conditioning lesion. Without the peripheral nerve conditioning lesion, dorsal column axons did not regenerate. Finally, in culture, neurite growth from DRG explants was longer following an *in vivo* conditioning lesion than without one.

So, what is responsible for this regrowth? How can a lesion in the peripheral nervous system affect the regeneration capabilities of the CNS branch of the same nerve? The study by Neumann and Woolf (1999), along with previous studies, suggests that the growth capacity of the dorsal column axons is clearly improved by a preconditioning lesion. We do not know, however, the molecular mechanism underlying this improved growth capacity, nor do we know why these axons are not stopped by inhibitory molecules within the glial scar and myelin. One possibility is that growth commences before the glial scar can form. Alternatively, the glial scar might form differently after a conditioning lesion. Since inhibitors in myelin, whether soluble or membrane associated, are exposed/secreted in response to damage regardless of whether there is a lesion to the peripheral nerve, regrowing central axons will confront them in both cases. However, a peripheral nerve lesion might alter the response of regrowing dorsal column axons to myelin inhibitors and inhibitors in general. For example, peripheral lesions may lead to the downregulation of the receptors for these inhibitors on the central regenerating growth cone. Alternatively, inhibitor receptors may still be expressed after a peripheral lesion, but they may no longer signal inhibition. Three recent observations make this latter interpretation compelling. First, like many immature neurons, growth of embryonic and neonatal DRG neurons is not inhibited by myelin (Shewan et al., 1995). For one myelin-specific inhibitor, myelin-associated glycoprotein (MAG), the switch in response from promotion to inhibition occurs sharply at postnatal day 3/4 (DeBellard et al., 1996). Second, inhibition of axonal regeneration by MAG and myelin in culture has been shown to be overcome by elevated neuronal cAMP levels. Conversely, the growth-promoting effect of MAG or myelin on young DRG neurons is blocked by inhibitors of protein kinase A (Cai et al., 1999). Third, the repulsion of

growth cones by a soluble form of MAG, and by a number of other inhibitory molecules, can be switched to attraction by elevating cAMP (Song et al., 1998). Together, these important results suggest that the neuronal response to myelin inhibitors (and, although never tested, possibly also to inhibitors in the glial scar) is dictated by the neuron's endogenous levels of cAMP. Therefore, it is quite likely that transection of the peripheral nerve branch of DRG neurons results in an increase in endogenous levels of cAMP, perhaps reaching levels found in young animals. This may neutralize the effect of myelin inhibitors or effectively switch the response of axons to myelin inhibitors to promotion, thereby allowing them to grow through white matter. Therefore, the combined effect of a conditioning lesion on both increased growth capacity and the ability to grow through inhibitors could account for the pattern of regrowth of these dorsal spinal axons in the damaged spinal cord.

If cAMP does play a major role in this improved growth through damaged spinal tissue, the next obvious questions are (1) what is activated or inactivated to achieve this effect and (2) is the response dependent on protein synthesis? Because growth is better after a 1 week conditioning lesion than when the two lesions are created at the same time, time-dependent changes must occur in the damaged spinal column axon to allow it to regrow. Consistent with this prediction, Smith and Skene (1997) show that after axotomy, adult DRG axons grow differently, becoming more elongated rather than branched, and that this transition requires ongoing transcription for a limited period after injury. An obvious candidate molecule to give improved growth is the growth-associated protein GAP43, which is upregulated in spinal axons after a conditioning lesion. However, preliminary results suggest that there is no improved regeneration in transgenic mice overexpressing GAP43 (Neumann and Woolf, 1999).

At this stage of incomplete understanding, can we suggest strategies for improving spinal cord regeneration in humans? Clearly, for both ethical and practical reasons, a conditioning peripheral nerve injury cannot be inflicted in humans to encourage growth of spinal axons. The peripheral lesion would have to be created before the spinal cord injury itself, an obviously absurd

approach. The solution, instead, is to work out what is happening at the molecular level. How can this effect be induced or mimicked in axons without a conditioning lesion? Can it be induced in neurons that do not have a peripheral nerve branch? Why did the dorsal column axons stop growing before they reached their original target? Perhaps the initial signal induced by the conditioning lesion had subsided. If so, would a second lesion encourage them to start growing again? Answers to these questions could lead to a rational, molecular approach to encouraging CNS axons to regrow after injury. The next problem, of course, is then getting them to their correct destination.

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α Neurotoxins and Their Relatives: Foes and Friends?

In 1963, C. C. Chang and C. Y. Lee initiated a new era in the study of neurotransmitter receptors by showing that a small protein toxin derived from the Taiwanese snake *Bungarus multicinctus* bound tightly and specifically to the nicotinic acetylcholine receptor (nAChR) at the vertebrate neuromuscular junction (Chang and Lee, 1963). At the time of the discovery of α -bungarotoxin (α Btx) and its cobratoxin relatives, the nAChR, although physiologically and pharmacologically well defined, was a molecular enigma. Even the question of whether it was a protein was disputed. The advent of α Btx and related α neurotoxins completely transformed the field. Their extremely high affinity and long off-times (making them essentially irreversible antagonists), their extraordinary

specificity, and the ease with which radioactive or fluorescent labels are incorporated made the α neurotoxins indispensable experimental tools for the study of nAChRs. Their use immediately led to the identification, purification, and localization of these receptors in muscle and in the *Torpedo* electric organ, and made the nAChR the paradigmatic neurotransmitter receptor for the next 20 years.

α Btx is an effective toxin because it inhibits the binding of acetylcholine to the AChR, producing neuromuscular blockade and quick death. This efficient strategy is used by a large number of poisonous snakes in the Elapidae family (cobras, kraits, mambas, and tiger snakes, among others), whose venoms collectively contain over 100 toxins with sequence homology to α Btx. All of these toxins share a common structure consisting of a β sheet core that is tightly cross-linked by four invariant disulfide bonds. Three flexible loops extend from the core to bind the receptor (Love and Stroud, 1986). Most of the toxins in the family bind the nAChR, but some bind other molecules, including muscarinic AChRs and acetylcholinesterase.

The idea that potent toxins have evolved to recognize physiological binding sites for a natural ligand such as acetylcholine is a familiar one. Recent studies by Miwa et al. (1999), however, reported in this issue of *Neuron*, give this concept a new twist. They suggest that the α neurotoxins not only mimic but also may be evolutionarily related to an endogenous ligand for the AChR, one that enhances rather than inhibits the action of acetylcholine. This new finding not only sheds light on the evolutionary origins of this family of toxins, but also poses the question of how the endogenous peptide might function physiologically to modulate the AChR.

The beneficent new relative of the family of elapid neurotoxins is lynx1, a small (11 kDa) protein that was identified in the course of a search for developmentally regulated genes in the cerebellum. A database search using the amino acid sequence encoded by the *lynx1* gene immediately revealed homology with α Btx and with the Ly-6 family, a related group of proteins that are found on the surface of mouse lymphocytes (Gumley et al., 1995). The Ly-6 proteins have tertiary structures that are similar to α Btx and are apparently attached to the surface membrane through a glycolipid anchor, where they participate in cell–cell and cell–substrate interactions. lynx1 shares with both α Btx and Ly-6 the highly conserved motif of eight cysteines, and models show that its predicted three-dimensional structure closely resembles the experimentally determined structures of α Btx and CD59, a member of the Ly-6 family. Moreover, the exon–intron boundaries of all three proteins are the same—a strong indication of a common evolutionary origin.

What distinguishes lynx1, and makes it of unusual interest, is evidence suggesting that it has a functional relationship to AChRs in the nervous system. lynx1 is highly expressed in the brain, where it is associated with neurons in the cortex, in the hippocampus, and in the cerebellum. Interestingly, each of the sites of lynx1 localization in the brain is also a site at which the $\alpha 7$ neuronal AChR is expressed. The $\alpha 7$ AChR, a homooligomeric

channel protein, is the only neuronal AChR in the mammalian brain with an appreciable affinity for α Btx (Lindstrom, 1996). (In the avian brain another subunit, α 8, also binds α Btx.) In the cerebellum, lynx1 is associated with the cell bodies and proximal dendrites of Purkinje cells, which are sites of α 7 localization and cholinergic innervation from mossy fibers (Barmack et al., 1992; Caruncho et al., 1997). This provocative colocalization prompted Miwa et al. (1999) to look for a functional interaction by expressing the α 7 subunit in oocytes. They found by electrophysiological recording that addition of recombinant lynx1 increased the response of the α 7 AChR to acetylcholine. These interesting findings suggest that lynx1 might have a role in modulating ACh activity in the nervous system and might do so by direct interaction with the AChR.

Several questions remain, however. First, direct interaction of lynx1 with the AChR has yet to be demonstrated. Miwa et al. (1999) found that lynx1 facilitated the action of acetylcholine not only on the α 7 AChR but also on the α 4 β 2 receptor, a neuronal AChR that is not sensitive to α Btx (Lindstrom, 1996). Although lynx1 may have a different specificity for AChRs than α Btx, this finding at least raises the possibility that lynx1 affects the AChR indirectly by acting through an entirely different receptor. Direct interaction, if present, should be easily demonstrable by showing that lynx1 can block α Btx binding to the AChR, or that α Btx can block the binding of the lynx1/Fc fusion protein used to demonstrate lynx1 binding sites in cerebellar tissue sections.

Second, if lynx1 interacts directly with the AChR, how does it act? There are few examples of peptides that directly modulate neurotransmitter receptor function. The effect of substance P on desensitization of the nAChR (Stallcup and Patrick, 1980) is a rare case. According to current ideas, α Btx binds at a site that is close to, but distinct from, the ACh binding site (Unwin, 1993). Does lynx1 bind at the same site, and does it alter the binding of ACh or alter the channel properties of the AChR?

Third, the location of lynx1 on the postsynaptic cell surface is unusual for a neuroeffector peptide. One possibility is that it could act as a retrograde signal to nicotinic or other receptors on the presynaptic terminal to modulate transmitter release. If true, this mechanism would be novel and interesting, as the peptide would presumably act only after proteolytic cleavage or, less likely, by direct membrane contact. Alternatively, lynx1 might play a different role at the synapse, perhaps more akin to Ly-6 in mediating adhesive or recognition functions in lymphocytes.

Finally, one would like to know if lynx1 is found in muscle cells or motor neurons, and if it acts at the neuromuscular junction. A physiological role for lynx1 at this synapse would bring the α neurotoxin story full circle in a historically fitting and entirely satisfying way.

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