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Optogenetic stimulation of neuronal repair

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Environmental insult, disease or trauma can affect the physical integrity of neuronal circuits, and the inability of many neurons to regenerate injured axons invariably leads to irreversible neural dysfunction [1]. The conserved second messenger cyclic adenosine monophosphate (cAMP) can promote axonal re-growth [2–5]. Widely used pharmacological or genetic approaches to increase intracellular levels of cAMP are often inadequate for precise neural-circuit reconstruction because their activity cannot be easily timed to specific target cells. These shortcomings have prevented the controlled repair of pre-defined neurons at selected time points in whole specimens. Thus, technologies to guide neuronal repair in time and space would enable studies of neural-circuit recovery with unprecedented resolution. Towards this aim, we have implemented a proof-of-principle optogenetic method to promote the selective regeneration of refractory axons in a living vertebrate.

Our approach is based on the blue-light activatable adenylyl cyclase bPAC from the soil bacterium *Beggiatoa* [6]. As an experimental paradigm we used the afferent neurons of the zebrafish lateral line, which have one central axon that projects along the hindbrain, and one peripheral axon that innervates superficial mechanosensory receptors called neuromasts (Figures 1A–B and S1A–C in Supplemental Information, published with this article online). Transgenes can be specifically expressed in lateralis neurons using the 'SILL' enhancer, either in single neurons by plasmid injection or in the entire neuronal population from chromosome-integrated DNA [7] (Figure S1D). Using SILL, we generated zebrafish expressing a bPAC-Myc-V2A-mCherry fusion gene (hereafter bPAC-mCherry) alone or together with the presynaptic marker Synapsin1-GFP [8] (Figure S1H–J). Control fish co-expressed Synapsin1-GFP and membrane-targeted mCherry (Figure S1E–G). bPAC-mCherry expression did not affect the development, synaptogenesis, or stability of these neurons (Figure S1K–M). To promote neuronal damage at selected locations and probe repair in living zebrafish larvae, we employed a nanosecond ultraviolet laser to cut the entire lateralis nerve using the stable transgenic line *Tg [HGn39D]*, or individually identified axons in fish with mosaic expression of mCherry (Figures 1C–N,P–R and S2A–F) [7]. All types of cuts led to a rapid distal degeneration (Figures 1D,G,J,M and S2A–F). Post-injury quantifications showed that nearly 100% of the severed peripheral axons regenerated and re-innervated neuromasts within 72 hours (Figure 1O). However, only 5% of the central axons regenerated within the same period, regardless of the presence of uncut axons (Figure 1O). Although it is currently unknown what prevents lateralis central axons from re-growing, central glial may have an inhibitory effect. These results indicate that similarly to many mammalian neurons, lateralis afferent neurons regenerate efficiently their peripheral axons but very poorly their central projections to the brain [1].

Using ELISA we confirmed that photostimulation of bPAC-mCherry at 450–490nm raised cAMP levels in 5-day post fertilization (dpf) zebrafish that resulted from injecting bPAC-mCherry mRNA into fertilized eggs (Figure S2G). Next, we photostimulated neurons expressing mCherry or bPAC-mCherry in 5-dpf fish after severing peripeheral or central axons. Quantifications revealed that the onset and speed of peripheral-axon re-growth did not differ significantly between control and bPAC-mCherry(+) neurons (Figure S2H–K). Remarkably, however, the regeneration of severed central axons of bPAC-mCherry(+) neurons dramatically increased after photostimulation (Figure 1P–T). We also achieved similar success rates of central-axon regeneration using the *Tg[SILL:bPAC-mCherry]* stable transgenic line (Figure S2L–Q). Pharmacological inhibition assays revealed that central-axon regenerative success was dependent on cAMP-dependent protein kinase-A [2] (Figure 1S). Imaging Synapsin1-GFP expressed in individually identified neurons before severing and after bPAC stimulation showed that regenerated central axons re-targeted the correct area of the brain, and re-formed fewer but mature presynaptic structures (Figure 1U–V). It will be interesting to test the ability of bPAC to promote the repair of other neurons, including the Mauthner cell [4]. Towards this end, we have generated a stable transgenic line that can express *bPAC-V2A-mCherry* under control of GAL4 drivers (data not shown).

The biological novelty that arises from these results is that under permissive conditions given by elevation of intracellular cAMP there is no critical period for lateralis-neuron axonogenesis and synaptogenesis. Additionally, our results indicate that individual regenerating axons are able to actively pathfind in the absence of scaffolding axons. Yet, the most salient aspect of this work is the demonstration of a simple and versatile optogenetic approach that achieved the desired aim of promoting the regeneration of refractory axons in the whole animal. This strategy is superior to classical pharmacological or genetic methods to increase cAMP levels because of the higher spatiotemporal resolution of light. It is also generally applicable because bPAC-mediated elevation of cAMP works across species and does not need additional factors. In its current implementation, this approach will be immediately suitable to probe the capacity of defined neural circuits to reclaim functionality after the time-selected repair of individual or multiple neurons. Its potential may further increase by using adenylyl cyclases sensitive to longer wavelengths for better penetration and lower toxicity. Additionally, adaptation of this method to molecular-screening platforms may help to identify cell-autonomous and non-autonomous effectors of axonal re-growth downstream of cAMP signaling, to find molecules with therapeutic potential [9]. In summary, the control of neuronal repair by optogenetics represents a powerful tool that could find broader uses in neurobiological research and biomedicine.

SUPPLEMENTAL INFORMATION

Supplemental Information contains two figures and experimental procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.09.038.

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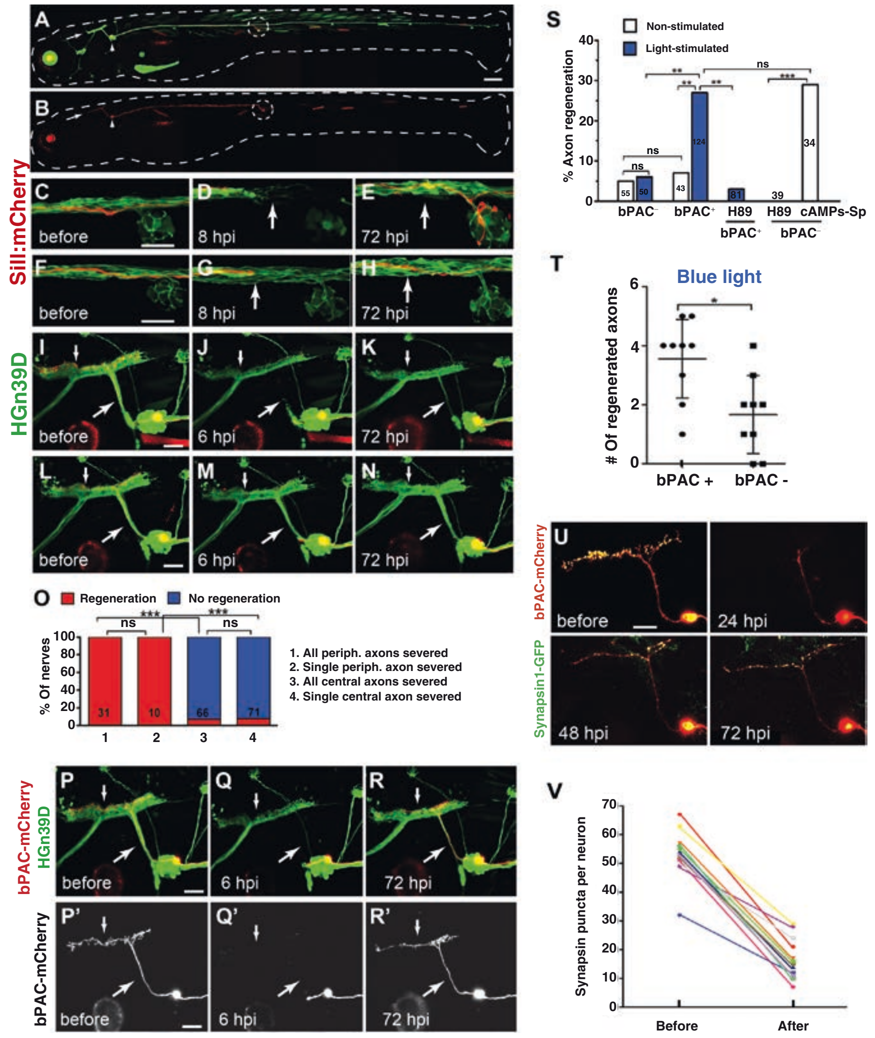

Figure 1. Photoactivation of bPAC stimulates the regeneration of refractory axons.

Tg[HGn39D] stable transgenic line (green) at 5 dpf also expressing mCherry in an individual lateralis neuron (red). The arrow points to the central-axon projections; the arrowhead indicates the posterior lateralis ganglion. Dotted lines outline the fish body, and the circle shows the neuromast innervated by the mCherry-expressing neuron. (B) The same specimen showing the neuron expressing mCherry. (C–E) The peripheral nerves of *Tg[HGn39D]* (green) and a single axon marked by SILL:mCherry before severing (C), 8 hours-post-injury (hpi) (D), and 72 hpi after whole-nerve severing (E). The arrow indicates the injury location. The distal axons degenerated rapidly (D), but regenerated after 72 hrs (E). (F–H) Severing a single peripheral axon expressing mCherry also led to distal degeneration (F–G), and regeneration within 72 hpi (H). (I–K) Central projections of anterior and posterior lateralis neurons in *Tg[HGn39D]* (green) and a mCherry-marked single neuron from the posterior lateral line (red) before transection (I). Upon severing the entire central axon bundle, all distal fragments degenerated 6 hpi (J), and the majority did not re-grow even after 72 hpi (K). Some newborn neurons from a small adjacent ganglion extended axons towards the brain (K). The small white arrow shows the terminal projection along the hindbrain, and the large arrow indicates the site of central-axon transection. (L–N) Severing singly marked axons (red) and leaving most of the other axons intact (green) showed that uncut axons did not promote the regeneration of the severed axons. (O) Quantification of peripheral- and central-axon regeneration in the two axotomized categories. The number of independent axons examined is shown inside the bars. (P–R) A single neuron expressing bPAC-mCherry (red) in the *Tg[HGn39D]* stable line (green). After severing the entire central bundle, all distal fragments degenerated 6 hpi. The sole neuron expressing bPAC-mCherry regenerated its central axon after photostimulation. (S) Quantification of the central-axon regeneration of bPAC(+) neurons after photostimulation (~27%), which was significantly higher than that of bPAC(-) or non-stimulated bPAC(+) neurons (~5%). ~30% of bPAC(-) neurons regenerated central axon in fish treated with the PKA activator cAMPS-Sp, and very few regenerated after treatment with the PKA inhibitor H89. (T) Quantification of central-axon regeneration in the *Tg[SILL:bPAC]* stable transgenic line at 24 hpi. (U) An example of an identified axon expressing bPAC- mCherry (red) and Synapsin1-GFP (green) before severing and after severing upon blue-light stimulation. (V) Counting Synapsin1-GFP puncta before axotomy and after regeneration showed that regenerated axons matured pre-synaptic structures, albeit fewer. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, 'ns' indicates non-significant. Scale bars: 150 µm (A-B), 25 µm (C,F,I,L,P,U).