Development of techniques to deliver transgenes into neurons of amphibians

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Abstract

Vocalizations of the Xenopus laevis are an exceptionally well suited model system to understand neural mechanisms underlying behavior. In this species, neural mechanisms of calling can be studied in vitro because fictive vocalizations can be elicited in the isolated brain. Application of optogenetic tools to the in vitro, fictive preparation will allow us to analyze how neurons function to generate sex-specific motor programs in real time. To this end, we explored techniques to express transgenes in the neurons of X. laevis. Specifically, we used targeted electroporation of plasmids and targeted injection of recombinant adeno-associated virus (rAAV), lentiviral vector based on equine infectious anemia virus (EIAV), and recombinant vesicular stomatitis virus (rVSV) into the brains and cranial nerves of X. laevis. The efficiency of the transfection and transduction depends on the age of the animals and the target location within the CNS. Although electroporation was an effective method of delivering plasmids into the neurons in tadpoles, the transgene expression in adult brains was largely restricted to glial cells. Targeted injection of rAAV or EIAV into adult brains resulted in highly variable transduction efficiency or no infection, respectively. In contrast, rVSV with VSV glycoprotein injected into the forebrain and the midbrain of adult X. laevis yielded fast, high, and reliable transduction efficiency, although injection into cranial nerves and brainstem resulted in lower yield. We conclude that rVSV can be used as a vector to deliver transgenes into adult Xenopus neurons.

Methods

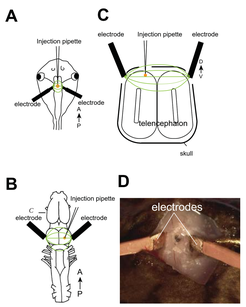

Figure 1. Electroporation setup used for tadpoles *in vivo* (A), for adult brain *in vitro* (B), and adult brain *in vivo* (C and D). A glass pipette containing plasmid suspension was lowered into the brain (A, B, and C), and plasmid was pessure-injected. Following the injection, a pair of platinum electrodes (D) were lowered to the two sides of the injection area, a small amount of Ca2+ free steinberg solution (tadpoles) or frog saline (adults) were applied to cover the tips of the two electrodes along with the injection area, and electrical pulses were applied. C: transverse section of the telencephalon at the level indicated as C in Fig B.

Plasmid	Promoter	Transgene
pPB.CAG.GFP	CAG	GFP
pCS2.EGFP(CAAX)	sCMV	EGFP
pCS2FA.ChR2YFP	sCMV	ChR2YFP

Table 1. Types of plasmids used for electroporation

Virus type	recombinant virus name	promoter	transgene
AAV	AAV9.hSYn.hChR2(H134R)-eYFP	hsynapsin	hChR2-YFP
EIAV	EIAV-TLoop-ChR2-YFP	CMV	ChR2-YFP
VSV	VSV-G VSV-Venus 2 (pl 21)	n.a.	Venus 2
VSV	RABV-G VSV-eGFP	n.a.	eGFP
VSV	G-Deleted VSV-eGFP	n.a.	tdTomato

Table 2. Types of viruses used as a vector to deliver transgenes

Electroporation

Electroporation of plasmid into tadpole ventricle resulted in neurons expressing fluorescent markers, but the same treatment in adult brains mostly resulted in labeled radial glial cells.

Probability of detecting any transgene expression in adult brains was very low (25%, n=8), and even in these brains, most expression was restricted to radial glial cells; the probability was much higher in tadpoles.

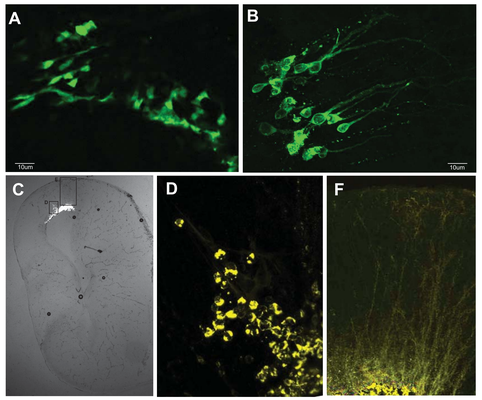

Figure 2. Cells labeled with fluorescent proteins after electroporation. A. GFP-labeled tadpole neurons one day after plasmids were electroporated *in vivo*. B. One of the rare GFP-labeled optic tectum neurons of adult X. laevis five days after electroporation *in vitro*. C. Transverse section of left telencephalon of adult X. laevis five days after plasmids were electroporated. D. YFP-expressing radial glial cell somas (inset shown in Fig 2C) that seem to form the ependymal lining of the ventricle. F. YFP-labeled long radial processes that span the entire width of the telencephalon that ends in the pial surface (inset shown in Fig 2C).

Fig 2A: pPB.CAG.GFP, 3.5ug/ul, 100nL injected into tadpole ventricle. Via electrodes 1mm apart, 20V, 5 pulses (70msec) delivered at 1Hz. Fig 2B: pCS2FA.ChR2YFP, 1.7ug/ul, 2nL injected into adult optic tectum in vitro. Via electrodes 1.5mm apart, 100V, 12 pulses (70msec) delivered at 1Hz. Fig 2C, D, and E: pCS2FA.ChR2YFP, 1.7ug/ul, 700nL injected into adult telencephalon in vivo. Using electrodes 1mm apart, 100V, 14 pulses (70msec) delivered at 1Hz.

AAV and EIAV

Recombinant adeno-associated virus (rAAV) was a slow and unreliable vector, and EIAV did not work at all for X. laevis nervous system.

Success rates of expressing transgenes in the Xenopus neurons using rAAV and EIAV were 4% (n=47 adult X. laevis) and 0% (n=9 adult X. laevis and 9 tadpolesa) respectively.

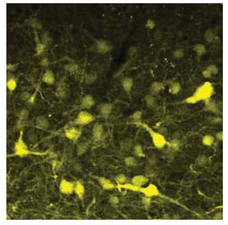

Figure 3. One of the rare examples of *Xenopus* adult telencephalic neurons expressing reporter gene (EYFP) three weeks after injection of pAAV-hSyn-hChR2(H134R)-EYFP.

VSV

1. Recombinant vesicular stomatitis virus (rVSV) is a fast and reliable vector for X. laevis nervous system.

Reporter gene expression was detected in almost all tadpoles and adult Xenopus injected with rVSV within 24 hours.

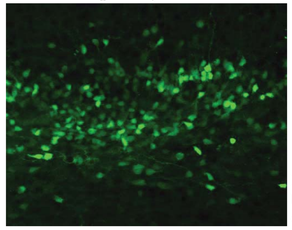

Figure 4. *Xenopus* adult neurons in olfactory bulb expressing Venus 2 24 hrs after injection of VSV-G VSV-Venus 2 (pl 21).

2. Transduced neurons show normal physiological function indicating that reporter gene expresses without obvious toxicity.

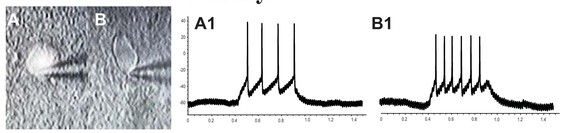

Figure 5. DIC image of telencephalic neurons of adult Xenopus brain slice preparation and the current-clamp recordings. A. DIC image of an adult Xenopus telencephalic neuron expressing reporter gene (Venus 2) in a whole-cell patch-clamp configuration. A1, Spikes shown in response to 10pA current injection. B. DIC image of a telencephalic neuron near the neuron shown in A on the same slice preparation. B1. Spikes of neuron B in response to 10pA current injection. The brain slices were obtained one day after VSV-G VSV-Venus2 was injected into the telencephalon.

3. Injection of rVSV(VSV-G) into motor nerves or rVSV(RABV-G) into muscles does not result in transduction of motoneurons, but rVSV(VSV-G) can be taken up by axon terminals, at least in tadpoles.

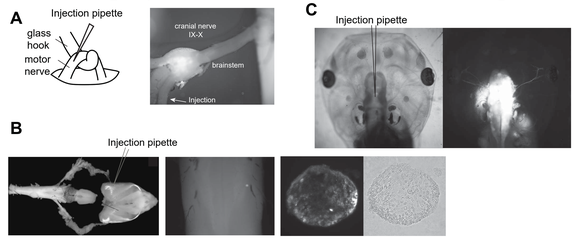

Figure 6. VSV entry to the nervous system was explored. A. rVSV(VSV-G) was injected into cranial motor nerve (left). Twenty-four days post injection, the nerve showed no reporter gene expression (right). B. rVSV(RABV-G) was injected into the laryngeal muscles (left, Fig from Zornick & Kelley 2008). Twenty-eight days post injection, the brainstem (right, dorsal view, middle) nor laryngeal nerve (right) showed no reporter gene. C. rVSV(VSV-G) was injected into the ventricle of a tadpole (left). Twenty-four hours post injection, reporter gene expression was seen not only in the optic tectum, but also in the optic nerve, indicating that the VSV was taken up by the axon terminals of retinal ganglion cells.

4. rVSV(VSV-G) does not travel transsynaptically in anterograde direction.

Contrary to reports that showed that rVSV with VSV glycoprotein travels transsynaptically in the anterograde direction, we did not see trans-synaptic transduction of neurons in tadpoles or in adult X. laevis.

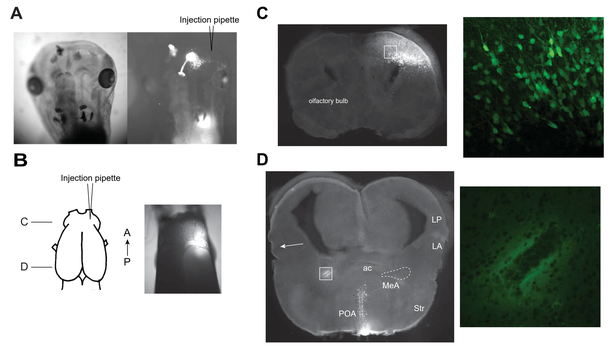

Figure 7. VSV injected in the nose of tadpoles and olfactory bulb of adults did not transduce neurons transsynaptically. A. VSV was injected into the right nose of a tadpole *in vivo* (left). Three day after the injection, reporter gene expression was seen in olfactory receptor neurons, but not in the neurons of olfactory bulb (right). B. VSV was injected into the right olfactory bulb of adult *in vivo* (left). Five days later, reporter gene expression was detected from the surface of the brain (right). C. Transverse sections of olfactory bulb (left, at the level shown in Fig B). Confocal image of the inset (right). Labeled neurons in preoptic area (POA) are thought to be transduced by injected virus leaked into the ventricle. D. Transverse section of caudal telencepalon (at the level shown in Fig B). No labeling was seen in contralateral lateral amygdala (LA, white arrow). Confocal image of the inset on right shows that there are no labeled neurons in contralateral medial amygdala either. ac, anterior commissure, LP, lateral pallium, LA, lateral amygdala, MeA, medial amygdala, Str, striatum.

5. Neurons in the central vocal pathways can be transduced by rVSV(VSV-G) without disrupting circuit function.

Injection of rVSV(VSV-G) into adult laryngeal motor nucleus resulted in successful transduction of vocal neurons without disrupting the function of the vocal circuitry, as evident in the fictive vocalizations elicited in response to 5-HT application to the isolated brain.

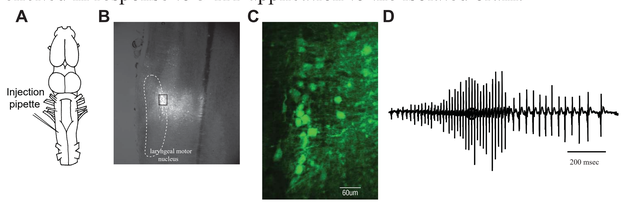

Figure 8. rVSV(VSV-G) injected into laryngeal motor nucleus of adult X. laevis *in vivo*. A. Dorsal view of the brain indicating the injection location. B. Horizontal section of the brainstem showing neurons labeled with Venus 2 two days after injection. C. Confocal image of the labeled neurons in inset of Fig B. D. Fictive advertisement call recorded from the laryngeal nerve of the transduced brain.

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