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Genetic ablation, sensitization and isolation of neurons using nitroreductase and tetrodotoxin-insensitive channels

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Summary

Advances in genetic technologies enable the highly selective expression of transgenes in targeted neuronal cell types. Transgene expression can be used to non-invasively ablate, silence or activate neurons, providing a tool to probe their contribution to the control of behavior or physiology. Here we describe the use of the tetrodotoxin (TTX)-resistant voltage-gated sodium channel Na_v1.5 for either sensitizing neurons to depolarizing input, or isolating targeted neurons from surrounding neural activity, and methods for selective neuronal ablation using the bacterial nitroreductase NfsB.

Keywords

ablation; silencing; sensitization; isolation; tetrodotoxin; nitroreductase; NfsB; SCN5a; Na_v1.5; zebrafish

1. Introduction

A fundamental objective in the study of neuronal circuits is to determine how activity in a given set of neurons contributes to a behavioral response. Key techniques that shed light on such questions are to either selectively inactivate or activate subsets of neurons, and determine how this influences circuit function or behavior. Here we describe transgenic methods to manipulate targeted neurons *in vivo* that allow for analysis of their contribution to behavioral responses in larval zebrafish.

Several techniques have been used in zebrafish to selectively silence or ablate genetically labeled neurons. Neuronal signaling can be constitutively blocked by expression of the tetanus toxin light chain [1] or the inward-rectifier potassium ion channel KiR2.1 [2]. Light-activated membrane channels and pumps allow for reversible neuronal silencing [3–5]. These sophisticated techniques are best paired with electrical recordings to verify the extent of silencing which in many cases is partial or strongly variable between individuals [1–2]. Ablation, which can be visually confirmed, is therefore an experimentally more tractable approach in many cases. Cell-specific ablation in zebrafish has been achieved using

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diphtheria toxin-A [6], the bacterial Kis/Kid system [7] and a modified caspase gene [8]. However the best characterized system relies on expression of the bacterial nitroreductase, *NfsB*, which metabolizes bath applied metronidazole into a cell-impermeant cytotoxin [9–11]. This system allows temporal control of ablation through metronidazole exposure and has recently been optimized to yield robust neuronal ablation within 24 hours [12–13]. A simple way to verify that metronidazole treatment induces apoptosis is to use PhiPhiLux G1D2, a live fluorescent reporter of caspase 3-like activity [14].

Optogenetic techniques have been widely used in zebrafish to acutely depolarize and thereby activate targeted neurons upon light exposure (for example [3,15–17]). These are powerful methods for decoding neuronal circuits, but require illumination with intense light that may interfere with behavioral assays. Moreover, induced firing patterns may not reproduce normal patterns of neuronal activation. An alternate method is to over-express a voltage-gated sodium channel in neurons of interest, thereby increasing their sensitivity to endogenous patterns of depolarizing input [13]. Specific changes in behavioral measures in transgenic fish can thus reveal the contribution of sensitized neurons. The cardiac voltage-gated sodium channel α -subunit ($\text{Na}_v1.5$, encoded by the *SCN5a* gene) is ideally suited for this purpose, as it opens at relatively low membrane voltage and inactivates slowly [18–19]. Finally, $\text{Na}_v1.5$ is resistant to tetrodotoxin, which blocks most central nervous system voltage-gated sodium channels. Injection of tetrodotoxin into the brain to globally suppress neuronal activity, therefore spares neurons that express $\text{Na}_v1.5$. This feature of $\text{Na}_v1.5$ expression enables measurement of the spontaneous or stimulus evoked activity in neurons that have been isolated from synaptic input [13].

2. Materials

2.1. Embryo rearing

1. E3 stock (60 $\times$, 5 L): Add 87.6 g NaCl, 3.8 g KCl, 14.55 g CaCl_2 (MW 147.02) and 24.4 g MgSO_4 (MW 246.48) to 4 L water. Mix, make up to 5 L with water and store at room temperature.
2. E3h medium (1 $\times$, 10L): Dilute E3 stock 1:60 by adding 167 mL E3 stock to 9 L water, add 15 mL 1M HEPES pH 7.3, mix thoroughly. Add water to 10 L and store at room temperature in a carboy with a bubbling stone for aeration. We do not add methylene blue for larvae that will be tested for behavior (see Note 1).
3. Pronase: Dissolve 300 mg in 10 mL water. Make 500 μL aliquots and store at -20°C .
4. Milked plates: Prepare a 2 % solution of non-fat dry milk powder in water. Pour into clean plastic Petri dishes, leave for 10 seconds, then discard milk, rinse twice with purified water then once with E3h.

¹Although methylene blue is widely used in embryo medium to reduce fungal growth, it has potent biochemical effects, producing behavioral changes in mice [20] and molecular changes in zebrafish [21–22].

2. 2. Ablation and isolation reagents

1. Metronidazole working solution (10 mM): weigh 85.6 mg of metronidazole, add to 50 mL E3h, protect from light by wrapping tube in aluminum foil and mix thoroughly at room temperature until fully dissolved (see Note 2).
2. PhiPhiLux G1D2 (8 μ M): 8 μ M solution is supplied by the distributor (OncoImmunin, Gaithersburg, MD). Store at 4°C, protected from light.
3. Tetrodotoxin stock solution (500 μ M): Wear personal protective equipment including gloves, mask and eye protection when handling tetrodotoxin. Weigh 0.16 mg tetrodotoxin citrate (MW 319.27) in a chemical hood, add to 1 mL H₂O and mix thoroughly.
4. Tetrodotoxin working solution (1 μ M): dilute TTX stock solution 1:500 with water and mix thoroughly. Use within 12 h. Dispose of all solutions containing TTX in hazardous materials waste following Institute guidelines (see Note 3).
5. Lysis buffer: 10mM Tris pH 7.5, 50mM KCl, 0.3% Tween20, 0.3% TritonX, 1mM EDTA. Store at 4°C.
6. Proteinase K: Dissolve 10 mg powder in 1 mL water immediately before use. Store powder at 4°C.

2. 3. Fish stocks

1. For ablation studies use *Tg(UAS-E1b:BGi-epNTR-TagRFPT-oPre)y268* (UAS:epNTR) which contains the highly active variant of nitroreductase epNTR fused to TagRFPT (see Note 4)[13].
2. For activation and isolation experiments use *Tg(UAS-E1b:BGi-SCN5a-v2a-TagRFPT)y266* (UAS:SCN5) which co-expresses the human voltage-gated sodium channel Na_v1.5 and TagRFPT [13].
3. Maintain UAS:epNTR and UAS:SCN5 together with a Gal4 line that has an easily recognized expression pattern (Note 5).

² 10 mM is the maximal solubility for metronidazole in water. We attach the solution to a benchtop rotator and vigorously mix for about 1 h at room temperature to fully dissolve. We make metronidazole solution fresh for each application and use within 2 h of dissolving. The powder can be stored at 4°C, but in our laboratory we replace the stock every 12 months.

³ Tetrodotoxin is extremely hazardous. Stock powder should be stored at -20°C in a locked container. In our laboratory we prepare stock and working solutions at BSL-2. We handle solutions and dishes that contact TTX with gloves and handle materials as medical pathological waste. Leftover TTX solution is inactivated with a 30 minute exposure to 1% bleach and disposed of as chemical waste. In the United States, TTX is classified as a Select Agent toxin. Institute guidelines for using TTX will vary so we suggest you seek guidance before use.

⁴ Several variants of nitroreductase are available: (1) the original bacterial NfsB fused to mCherry or CFP [9–10], (2) wildtype NfsB with amino acid mutations T41Q, N71S, F124T (mutNTR) [12] and (3) zebrafish codon optimized NfsB with amino acid mutations T41L, N71S, F124W fused to TagRFP (epNTR-TagRFP) [13]. Both mutNTR and epNTR produce more rapid and complete ablation than the original NfsB, are readily available as UAS lines and are therefore currently the best options.

⁵ UAS reporter lines are sensitive to transgene silencing, leading to variegated reporter expression that worsens across generations [23]. To mitigate this, UAS lines should be propagated together with a Gal4 driver, raising only individuals with strong, complete UAS reporter expression (generally on the order of 10–20% of larvae). Stock maintenance is facilitated by keeping the UAS and Gal4 transgenes as single-copy heterozygotes in each generation, so that strong expression is due to non-silenced transgenes, rather than the presence of multiple copies of UAS and/or Gal4.

3. Methods

3. 1. Raising larvae for behavior testing

1. Cross Gal4 transgenic fish to UAS:epNTR fish for ablation studies, or UAS:SCN5 for sensitization and isolation studies (see Note 6).
2. Collect embryos from crosses within 1-2 hours post fertilization (hpf) and combine all individual clutches. Remove abnormal or unfertilized embryos.
3. At 6-12 hpf, sort fertilized embryos into dishes at a density of 15-25 per 6 cm Petri dish in a volume of 10 mL of E3h media. Raise and maintain larvae in a 28.5°C light cycle incubator (see Note 7).
4. At 1 dpf remove all dead or deformed embryos and debris.
5. At 2 dpf hatch embryos by adding 10 μ L of pronase to each dish. Incubate embryos for 3 hours, then pipette embryos lightly up and down to ensure all embryos are removed from their chorions. Change E3h media completely after dechoriation.
6. Sort larvae for transgene expression, keeping both transgene positive and negative groups (see Notes 8, 9). If the UAS stocks are maintained with a different Gal4 line in the background, this pattern will need to be separated. After dechoriation, larvae tend to stick to clean plastic dishes, sometimes resulting in damage and should be sorted into milked plates (see Note 10).
7. Maintain transgenic and control non-transgenic larvae at the same density, changing medium every 2 days (see Note 11).

3. 2. Ablation using nitroreductase and metronidazole

1. Raise larvae for metronidazole treatment to 3 dpf (see Note 12).

⁶.Alternatively epNTR or Na_v1.5 can be expressed in target neuron populations by direct expression using a characterized promoter. However because Gal4 amplifies expression, this is the recommended strategy.

⁷.For the light cycle incubator, we use a 14/10 light/dark period. During the light cycle we have used light intensities between 10 and 500 μ W/cm².

⁸.The UAS:epNTR and UAS:SCN5a lines co-express TagRFPT so that transgenic larvae can be readily recognized for sorting. As silencing of the UAS reporter leads to variegated expression [23], during sorting select only larvae with robust transgenic expression in the complete expected pattern and be careful to avoid sorting larvae with weak expression into the negative control group. When sorting lines with dim fluorescence it can be helpful to screen larvae using a camera with long exposures.

⁹.When sorting embryos or larvae for neuronal ablation and subsequent behavioral analysis, avoid using tricaine. Exposure to tricaine, especially prolonged exposure, can alter behavior and influence subsequent analysis. When possible, sorting prior to dechoriation may be helpful, avoiding the difficulty in detecting fluorescence in moving larvae. Motile larvae can be placed in small individual drops to facilitate screening. Sorting before inflation of swim bladders is easier, as larvae do not float to the surface.

¹⁰.If embryos are re-sorted into the dishes they were raised in, the debris from the hatching process is sufficient to coat the bottom of the plate and prevent sticking. Otherwise, sort into milked plates.

¹¹.Ideally maintain 15-25 larvae per 6 cm Petri dish, but in any case be sure to raise controls and experimental larvae at the same density because this can significantly alter larval behavior [24].

¹².Metronidazole treatment can be applied at any time and the optimal time point to initiate ablation will need to be empirically determined for each new line. We find that starting the metronidazole treatment just before the onset of transgene expression often gives the most complete ablation. The maximal dose and treatment duration with metronidazole is 10 mM for 48 hours, however if nitroreductase is highly expressed, robust ablation may be achieved with lower doses and shorter exposure times. Treatment with 10 mM for 48 hours is a good starting point, because if ablation is not effective under these conditions, stronger nitroreductase expression will be needed.

2. Remove E3h media from dishes and rinse once with 5 mL metronidazole solution. Completely remove the rinse solution and add 10 mL metronidazole solution (see Note 13).
4. Place dishes with metronidazole treated and untreated larvae in a light-cycle incubator with dim light intensity ($0.5 \mu\text{W}/\text{cm}^2$) (see Note 14).
5. Optional (see Note 15): To label apoptotic cells, 12 h after starting metronidazole treatment, remove several larvae and immerse for 1 h in $8 \mu\text{M}$ PhiPhiLux G1D2 (see Note 16). Protect embryos in PhiPhiLux G1D2 solution from light. Perform three washes in E3h (20 min each) before live imaging using a 488 nm confocal laser and 500–550 nm bandpass emission filter. The fluorescence signal of PhiPhiLux G1D2 in apoptotic cells is relatively weak, but easily distinguished from the very low background fluorescence in surrounding tissue, so a relatively high laser power should be used to detect the PhiPhiLux G1D2 label.
6. After 24 h, replace the medium with freshly prepared metronidazole and return dishes to the dimly lit incubator.
7. After 48 h, remove dead or deformed individuals (see Note 17), then discard the metronidazole solution and perform 3 washes with fresh E3h media. Even after ablation conditions have been validated, a few larvae should be set aside and checked for loss of the fluorescent transgene expressing cells (see Note 18).
8. Allow larvae to recover for 24 hours in E3h under normal intensity light-cycle conditions before starting behavioral tests.

3.3. Sensitization of neurons by targeted expression of $\text{Na}_v1.5$

1. Raise groups of larvae, changing media at every 2 days as described in section 3.1.

¹³Metronidazole treatment alone may affect behavior, for example we find that treatment of wildtype larvae sensitizes the acoustic startle response. Non-transgenic siblings, treated with metronidazole, and handled in parallel to the experimental group are an essential control for ablation experiments. We also suggest keeping a group of metronidazole untreated larvae as treatment and quality controls.

¹⁴This can be achieved by placing the treated embryos in a container with a semi-transparent lid in a regular light-cycle incubator. Inexpensive sheets of neutral density plastic can be obtained from GAM Products. Metronidazole is light-sensitive and prolonged exposure will cause degradation.

¹⁵Verification that metronidazole treatment results in complete ablation of nitroreductase-expressing cells is essential, because the effectiveness varies with cell type and levels of nitroreductase expression. Loss of neurons with the co-expressed fluorescent protein should be confirmed by an independent measure such as immunostaining for a cellular marker of the targeted neurons or detection of apoptosis in the targeted cells. For detecting apoptosis, we recommend PhiPhiLux G1D2 because of the low background in brain. However, alternate methods for detecting apoptosis include TUNEL staining [9], hoechst staining for nuclear fragmentation [9], immunolabeling of activated caspase-3 [11] and acridine orange staining [12].

¹⁶Since PhiPhiLux treatment labels cells undergoing active apoptosis, the ideal time for labeling will need to be empirically tested for each line and cell type, however, approximately 12 hours after starting metronidazole treatment is a general starting timepoint. We have used PhiPhiLux to detect apoptotic cells in 3 and 4 dpf larvae.

¹⁷A common effect of metronidazole treatment is over-inflation of the swim bladder which leads to abnormal balance and may preclude behavioral characterization. Larvae with over-inflated swim-bladders float to the surface and congregate in the meniscus. This can be minimized using lower doses of metronidazole, or treating larvae after swim bladder inflation is complete (4 dpf). Viable lines for nitroreductase mediated ablation and subsequent behavioral testing cannot have expression of nitroreductase in tissues including skeletal muscle, heart, skin, or notochord, as the ablation will lead to severe morphological abnormalities. Expression can be suppressed outside the brain by incorporating a Neuronal Restrictive Silencing Element (NRSE) into the transgene [25–26].

¹⁸During post-ablation screening, it is common to observe fluorescent aggregates appearing as puncta that are smaller than cell bodies. Such debris does not indicate unsuccessful ablation.

2. Sort for TagRFPT expression, keeping non-transgenic fish as controls. Due to the large size of the $\text{Na}_v1.5$ mRNA, fluorescence is relatively dim in these fish and post-hoc genotyping may be necessary to identify transgenic larvae.
3. Establish stimulus conditions that avoid floor or ceiling effects for the behavior to be tested.
4. Measure behavior in $\text{Na}_v1.5$ expressing fish compared to transgene negative fish under normal assay conditions. Use a range of stimulus intensities that affect the aspect of behavior measured (Note 19).
5. For post-hoc genotyping to identify $\text{Na}_v1.5$ expressing larvae, first make DNA by placing larva in 30 μL lysis buffer, incubating at 98°C for 10 min then placing on ice. Add 5 μL proteinase K, then incubate at 55°C for 2 h. Incubate at 98°C for 10 min to inactivate proteinase K, then dilute 1:20 with water. Use 5 μL of diluted DNA for each of two PCRs. For the Gal4 transgene conditions will vary according to the line. For the UAS:SCN5-2a-TagRFPT transgene the primers are 5'-TCTGTGCATTGACTTGGTGAG and 5'-GGCGGTTCTACCCTGAATTA. Use standard PCR conditions with 0.2 μM each primer, 0.2 mM dNTPs and 35 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec. Run product on 2% agarose gel. Larvae with the UAS:SCN5 transgene will produce a 279 bp band (see Note 20).

3.4. Isolation of neurons from circuit activity with $\text{Na}_v1.5$ and tetrodotoxin

1. Raise groups of larvae with and without $\text{Na}_v1.5$ transgene expression.
2. If using a transgenic method to monitor neuronal activity, such as the GCaMP calcium sensors or arch voltage sensors, also sort for this transgene. If using a synthetic indicator to monitor neuronal activity, such as Calcium Green-1 dextran, label neurons in both $\text{Na}_v1.5$ positive and negative groups of fish (see Note 21).
3. At 5-6 dpf embed larva in 2 % low melting point agarose in E3h in a chamber suitable for microscopy (see Note 22).
4. Record stimulus evoked changes in neuronal activity in neurons that express $\text{Na}_v1.5$ and in the same neurons in non-transgenic fish.
5. While still embedded in agarose, remove larva from the microscope and move to the injection stage. Backload a pulled glass pipette with 1 μM tetrodotoxin

¹⁹The degree of sensitization will vary according to cell type and $\text{Na}_v1.5$ expression level. As a rough guide, larvae with Mauthner cells expressing sufficient levels of transgene that RFP was visible under epifluorescence, showed a roughly 2-fold increase in short latency escape responses. Larvae with motor neurons expressing the transgene at visible levels showed a 30 % increase in the bend angle during long latency escape responses [13].

²⁰The genotyping protocol for the UAS:SCN5 (y266) line can also be used to maintain adult transgenic fish. The primers bind in the 3'UTR and transposon arm of the transgene and do not distinguish fish with one or two copies of the transgene.

²¹Several options are available to monitor neuronal activity using fluorescent indicators of calcium activity. Reticulospinal neurons can be backfilled by injection of dextran-coupled calcium or oregon green into the spinal cord [27–28]. Injection of acetoxymethyl-esters of calcium indicators into target brain regions and pan-neuronal expression of GCaMP can also be used to monitor activity in any group of neurons (reviewed in [29]).

²²During embedding, agarose should be only slightly warm to touch to prevent damage to the larva. Submerge larva in agarose and position using a plastic pipette tip. Add E3h above the solid agarose in the chamber to prevent the larva from dehydrating.

working solution. Attach pipette to a picospritzer to pressure inject 2-3 nL tetrodotoxin solution into the hindbrain ventricle (Figure 2 shows how to position the needle for injection).

6. Realign embedded larvae in imaging setup and record neuronal activity-dependent fluorescence changes evoked in experimental paradigm (see Note 23).
7. To verify that TTX was active and not lethal, 10 minutes after TTX injection, larvae should be immobilized (completely unresponsive to a body touch) however the heart should remain beating. Larvae should recover sufficiently from TTX injection to resume free-swimming behavior in 3-5 h.

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5. References

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Figure 1.

Detection of apoptosis using PhiPhiLux. RFP expression in spinal cord neurons in *Et(REx2-cfos:Gal4ff)y270, UAS:epNTR-TagRFPT* control larva (left) and larva treated with metronidazole (right). Grey panels show RFP expression every 12 hours from the start of metronidazole treatment at 72 hpf. At 84 hpf, larvae were treated with PhiPhiLux to label apoptotic cells (green, middle panels). Note fluorescent debris from ablated neurons. Scale bar 50 μ m.

Figure 2.

Injection into the brain ventricle. A. Glass injection needle (in this case filled with rhodamine-dextran for demonstration purposes) positioned to inject into the ventricle of 6 dpf larva. Needle is inserted at the midline and at the same anterior-position position as the otic vesicles. B. Larva immediately after dye injection into the ventricle.