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Genetic Analysis of the Touch Response in Zebrafish (*Danio rerio*)

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Both mammals and zebrafish possess mechanosensory neurons that detect tactile sensation via free nerve endings. However, the basis for mechanotransduction and the unique cellular properties of these sensory neurons are poorly understood. We review the advantages of zebrafish for studies of the biological mechanisms involved in touch sensitivity. Importantly, Granato and colleagues (1996) demonstrated that a simple touch assay efficiently recovers mutations that affect sensory neurons.

Within the first five days of development, the zebrafish embryo acquires the ability to respond to a diverse set of sensory inputs. The availability of a wide range of genetic, molecular, cellular and physiological techniques enable analysis of the underlying molecular mechanisms. Moreover, the transparency of the zebrafish embryo combined with transgenic lines expressing fluorescent proteins in specific populations of neurons as well as advances in optical imaging and stimulation methods allow detailed measurement at the cellular level of behavioral mechanisms. Importantly, the findings from zebrafish studies have had implications for human disease. Several zebrafish mutants serve as models for human diseases that involve abnormal behaviors such as seizure conditions, autism, and Down syndrome (Baraban, 2009; Berghmans, Hunt, Roach, & Goldsmith, 2007; Yimlamai, Konnikova, Moss, & Jay, 2005).

Swimming and responses to sensory stimuli are behaviors that have been extensively studied in zebrafish embryos and larvae (e.g., Bang, Yelick, Malicki, & Sewell, 2002; Brockerhoff, Hurley, Janssen-Bienhold, Neuhauss, Driever, & Dowling, 1995; Budick & O'Malley, 2000; Emran, Rihel, & Dowling, 2008; Gahtan, Tanger, & Baier, 2005; Granato et al., 1996; Lindsay & Vogt, 2004; Neuhauss et al., 1999; Nicolson, Rusch, Friedrich, & Nüsslein-Volhard, 1998; O'Malley, Kao, & Fetcho, 1996; Ritter, Bhatt, & Fetcho, 2001; Saint-Amant & Drapeau, 1998). In this review, we use the touch response as the specific example to highlight the advantages of the zebrafish model for study of the biological underpinnings of behavior. Importantly, the methods considered are applicable to the study of other behaviors in zebrafish.

Advantages of the Zebrafish Model for Study of Vertebrate Behavior

The well-characterized responses and simple nervous system of zebrafish provide key advantages for mechanistic studies of vertebrate behavior (for reviews, see Burgess & Granato, 2008; Gahtan & Baier, 2004; Lewis & Eisen, 2003; McLean & Fetcho, 2008). Single pair breedings produce large numbers of progeny

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(typically > 100). Embryos develop quickly from a single cell to complex animals with a diverse behavioral repertoire. Moreover, embryonic development occurs externally allowing easy access to stages that are more difficult to study in mammals.

Many powerful methods have been used successfully to identify the molecular basis of behavioral mechanisms in zebrafish. Importantly, forward genetic methods are possible and genetic screens have identified genes with functions that are essential for specific behaviors (e.g., Granato et al., 1996). In addition, reverse genetic strategies allow targeted knock-down or overexpression and study of a specific gene's role in a biological process (Nasevicius & Ekker, 2000).

Transgenic lines exist that express a fluorescent protein, such as green fluorescence protein (GFP), under control of well-characterized promoter elements allow identification of specific cells *in vivo* in live embryos and larvae (e.g., Higashijima, Hotta, & Okamoto, 2000). The transparency of zebrafish embryos and larvae enables not only optical imaging of anatomy but also of neuronal activity as well as stimulation of individual neurons (e.g., Arrenberg, Del Bene, & Baier, 2009; Brustein, Marandi, Kovalchuk, Drapeau, & Konnerth, 2003; Douglass, Kraves, Deisseroth, Schier, & Engert, 2008; Higashijima, Masino, Mandel, & Fetcho, 2003; Szobota et al., 2007; Wyart et al., 2009).

Touch Response

The zebrafish embryo acquires the ability to respond to tactile stimuli at 24-27 hours post fertilization (hpf) (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; Pietri, Manalo, Ryan, Saint-Amant, & Washbourne, 2009; Saint-Amant & Drapeau, 2000). The touch response is easily elicited by applying tactile stimulation to the tail or head. The response occurs rapidly and varies developmentally, ranging from a twitch in 1 day embryos to a rapid escape response in older larvae. These rapid and unambiguous responses make touch sensitivity an ideal behavior to score in genetic screens.

By combining the touch assay with observation of swimming ability, one can identify mutations that affect the sensory but not motor side of the underlying circuit (Granato et al., 1996). Such screens have the potential to identify factors essential for function of mechanosensory primary neurons, as we discuss further below.

Touch response circuit

Several studies have identified conserved pathways of the circuits underlying response to tactile stimulation of the skin in zebrafish and *Xenopus* (Clarke, Hayes, Hunt, & Roberts, 1984; Clarke & Roberts, 1984; Eaton, Farley, Kimmel, & Schabtach, 1977; Li, Perrins, Soffe, Yoshida, Walford, & Roberts, 2001; Li, Soffe, & Roberts, 2002, 2003; Pietri et al., 2009). In addition, in both

vertebrate models, development modulates circuitry as well as the cellular properties of relevant neurons.

Evidence for Mauthner cell involvement in older larvae exists (Eaton et al., 1977; Liu & Fetcho, 1999; O'Malley et al., 1996). In contrast, for embryos, the touch response does not require supraspinal input (Downes & Granato, 2006; Pietri et al., 2009; but see, Saint-Amant & Drapeau, 1998). Pietri et al. (2009) presented a consensus view of the circuit underlying the touch response in the early embryo.

Application of tactile stimuli to the zebrafish trunk leads to contraction of contralaterally located muscle. For the early embryo, Pietri et al. (2009) emphasized the critical roles of the primary mechanosensory Rohon-Beard cell (RB) and the primary ascending commissural interneuron (CoPA) in the touch response circuit. They found that the touch response required the rostral spinal cord, corresponding to somites 1-10, but not the hindbrain. In sum, tactile stimulation activates RBs, that then synapse onto CoPAs, in turn contacting contralateral descending interneurons that activate motor neurons located contralaterally to the site of tactile stimulation (Pietri et al., 2009).

Touch assay

By performing a touch assay, Granato et al. (1996) identified several mutations that affected the touch response but not the ability to swim. The observation that swimming was not affected implicates defects in sensory neurons/processing.

Swimming occurs spontaneously. However, bath application of NMDA (e.g., 100 μM) to intact embryos also elicits swimming (Cui, Saint-Amant, & Kuwada, 2004; McDearmid & Drapeau, 2006). Incorporation of bath application of NMDA in behavioral screens might lead to more efficient observation of swimming ability.

Primary mechanosensory neurons

RBs function as mechanosensory neurons mediating the zebrafish touch response. RBs innervate the skin and sense touch via their free nerve endings (Clarke et al., 1984). In mammals, mechanosensory neurons with similar free nerve endings exist as well as ones with associated structures such as Merkel cells or Meisner corpuscles (Lewin & Moshourab, 2004).

RBs provide mechanosensory function to the zebrafish prior to differentiation of dorsal root ganglion neurons. The dorsal root ganglion neurons begin to contribute to sensory function at about 60 hpf. RBs undergo apoptotic cell death and are largely absent by 120 hpf (Reyes, Haendel, Grant, Melancon, & Eisen, 2004; Svoboda, Linares, & Ribera, 2001; Williams, Barrios, Gatchalian, Rubin, Wilson, & Holder, 2000). Thus, between ~24-60 hpf, RBs mediate tactile sensitivity. Later, between ~60-120 hpf, both RBs and dorsal root ganglion neurons provide this function. After 120 hpf, tactile sensitivity is essentially dependent upon dorsal root ganglion neurons.

In contrast to dorsal root ganglion neurons, RB cell bodies reside within the central nervous system in the spinal cord. Similar to dorsal root ganglion mechanosensory neurons, RBs have peripheral processes that innervate the skin as well as central ascending and descending axons. The peripheral cutaneous processes extend over several segments resulting in a broad RB receptive field. The central ascending axon makes *en passant* connections with the CoPA interneuron (Pietri et al., 2009).

Existing questions about mechanosensory neurons mediating vertebrate touch sensitivity

Mechanosensory neurons exist in both invertebrates (e.g., *Drosophila*, *C. elegans*) as well as vertebrates. Genetic screens in *Drosophila* and *C. elegans* have provided the majority of the available information about molecular determinants of mechanosensitivity (for reviews see Chalfie, 2009; Kernan, 2007). Such work has helped contributed to identifying mammalian orthologues with similar function (Welsh, Price, & Xie, 2002). A major focus of such work concerns members of the degenerin/epithelial sodium channel (DEG/ENaC) family. Beyond identifying these channels *per se*, many questions exist about how these channels interact with both the extracellular and intracellular environments to transduce tactile sensation.

Genetic Screens

Forward genetic screens serve as a powerful, unbiased approach to identify genes that play essential, nonredundant roles in biological processes. In contrast to reverse genetic approaches, forward genetic strategies seek to mutate genes in an unbiased manner and thus have the potential to reveal previously undiscovered genes or novel functions of known genes. After introduction of mutations, the biological process of interest, for example behavior or morphology, focuses subsequent analyses (e.g., Driever et al., 1996; Haffter et al., 1996).

Although the majority of genetic screens performed so far in zebrafish have used chemicals to introduce mutations, retroviral-based insertional mutagenesis has also been possible (Amsterdam, Nissen, Sun, Swindell, Farrington, & Hopkins, 2004; Gaiano, Amsterdam, Kawakami, Allende, Becker, & Hopkins, 1996; Golling et al., 2002; Petzold et al., 2009). Behavioral genetic screens in zebrafish have identified mutants by looking at phenotypes such as vision (e.g., Brockerhoff et al., 1995; Neuhauss et al., 1999), swimming behavior (e.g., Granato et al., 1996) and touch response (e.g., Granato et al., 1996).

Mutant isolated – now what?

Identify gene. Once mutations have been identified that produce phenotypes of interest, the next step is to identify the gene harboring the mutation. For mutations introduced by chemicals such N-ethyl-N-nitrosourea, this typically entails positional cloning (for reviews see Talbot & Hopkins, 2000; Talbot &

Schier, 1999). Whereas positional cloning often involves considerable effort, the advances in zebrafish genomic resources greatly facilitate the process.

The identification of the responsible gene by positional cloning or retroviral insertional sequences, however, does not suffice. The possibility exists that an additional closely linked mutation or insertion has occurred. As a result, one usually performs several additional tests, as outlined below.

A common starting point is to ask if the gene is expressed at the right time and place in order to play a role in the biological process of interest. Whole mount *in situ* hybridization is extremely feasible in zebrafish allowing rapid examination of this question. If the gene acts cell autonomously, (i.e. the gene function is required in the cell in which it is expressed), it should be straight forward to determine what is an appropriate expression pattern. However, genes that act noncell autonomously (i.e. the gene function is required in cells that do not express it) may not have predictable expression patterns.

Another important point to consider is how the mutation affects the function of the gene. For example, does the mutation result in loss-of-function (null), gain-of-function (new), diminished or altered function? In this regard, having several noncomplementing alleles provides more information about the possible function of the gene, especially if one is considering a novel gene. If the mutation results in loss-of-function, then elimination or reduction of the protein by other means should replicate the mutant phenotype. Antisense morpholinos serve as efficient and effective tools for knock-down approaches (Nasevicius & Ekker, 2000).

Alternatively, if the mutation produces a gain-of-function phenotype, one might seek to knock-down the endogenous protein encoded by the lesioned gene and then overexpress either RNA for the wildtype or mutated version of the gene. The former should rescue while the latter should replicate the mutant phenotype. For the specific case of touch-insensitive mutants, one would score for recovery of touch sensitivity. However, rescue experiments require that one have a reliable assay for genotyping embryos so that one can be sure that both mutant and wild type embryos show touch sensitivity.

For mutations that result in reduced or loss of gene function, the gold standard is to achieve rescue of the phenotypes by overexpression of wildtype RNA into 1-cell stage embryos. Although RNA overexpression appears as a conceptually straight-forward method, it has several technical complications. It can be challenging or impossible to determine the correct concentration to inject because injected RNAs can be toxic to the developing embryo, especially at high concentration. Further, injected RNAs degrade, each with a characteristic half-life. In addition, many genes might be expressed too late or at high levels, making RNA injection often an ineffective approach. In addition, early misexpression of a gene expressed at late times might produce a complicating phenotype. The take-home message is that successful rescue by RNA injection is informative but lack of rescue is not.

Another potential problem with RNA injection is that the exogenous RNA will be distributed to many cells, some of which normally express the gene and others that do not. Ectopic expression of RNA may produce phenotypes that

preempt analysis of rescue. Transgenic approaches, greatly advanced by methods using Tol2 transposons and the GAL4/UAS system, provide alternative approaches and allow expression of desired gene in a controlled manner by selection of an appropriate promoter (Asakawa & Kawakami, 2008; Davison et al., 2007; Kawakami, Shima, & Kawakami, 2000; Kwan et al., 2007).

Identify gene's essential function in biological process. Identification of the gene responsible for the mutant phenotype is often the "tip of the iceberg". There will be instances in which the mutation occurs in a well-characterized gene that has a known function in the biological process of interest. On the surface, this may seem very satisfying. But, will one have learned anything new, beyond proof-of-principle for mutagenesis methods? The alternative scenario of identifying a gene with no known function, at least in the biological process under study, offers the possibility of discovering a new biological mechanism or previously unidentified player in a known process.

For the case of touch sensitivity, an interesting example is the *touchtone* mutants that show reduced touch sensitivity between ~35-72 hpf (Arduini & Henion, 2004; Cornell et al., 2004). Another allele of the gene harboring the touchtone mutation is *nutria*, isolated on the basis of the reduced size of the mutants (Elizondo et al., 2005). Subsequent positional cloning revealed that the gene encoding transient receptor potential melastatin-7 (TRPM7) carries the *touchtone* and *nutria* mutations (Elizondo et al., 2005). TRPM7 functions as a channel permeable to divalent cations such as magnesium and calcium. Interestingly, growth in media containing elevated magnesium or calcium, but not sodium chloride, produced a rescue of the touch insensitivity (Elizondo et al., 2005). However, despite the identification of the touchtone/nutria gene, the cellular mechanism responsible for the touch insensitivity is still not known. Further study of TRPM7 function, therefore, has the potential to reveal a novel mechanism required for function of mechanosensory neurons.

Mutations isolated so far

The neurons involved in the touch response circuit differentiate appropriate excitable membrane properties. Moreover, the neurons need to form correct synaptic connections so that the circuit functions properly. Defects in neurogenesis, mechanosensory channels/complexes, excitable membrane properties, axonal outgrowth/pathfinding, synapse formation, or dendrite development could each suffice to produce touch insensitivity.

Tübingen touch-insensitive mutants. The 1996 Tübingen large-scale chemical mutagenesis zebrafish screen identified touch-insensitve mutants, comprising six different complementation groups (Granato et al., 1996; Haffter et al., 1996). These mutants have a reduced touch response but are able to swim. The observation that swimming was relatively normal indicated that neither muscle function nor circuits underlying patterned motor output were impaired, suggesting that the mutations affected the sensory side of the touch response circuit.

Four of the touch-insensitive mutants have reduced voltage-gated sodium current (I_{Na}) amplitudes in RBs: *alligator* (*ali*), *macho* (*mao*), *steifftier* (*ste*), and *crocodile* (*cro*). We consider the *mao* mutant in detail below. In brief, among the touch insensitive mutants, *mao* homozygotes displayed the most severe reductions in touch sensitivity. Eectrophysiologcal analysis of RBs revealed a drastic reduction in I_{Na} amplitude, resulting in failure to fire an action potential. As a result, RBs do not communicate sensory information to the nervous system.

ali and *ste* mutants share many characteristics. Both have a reduced touch response by 32-33 hpf, 5-6 hours later than *mao* (Ribera & Nüsslein-Volhard, 1998). They also both have a subset of RB cells that are similar to *mao* RB cells, having severely reduced I_{Na} amplitudes and an inability to fire overshooting action potentials; other *ali* and *ste* RBs, however, had I_{Na} of normal amplitude and fired sodium-dependent impulses (Granato et al., 1996; Ribera & Nüsslein-Volhard, 1998).

In contrast, two other touch-insensitive mutants, *cro* and *schlaffi* (*sla*), have normal touch responses at 2 but not 3 dpf, when they show reduced sensitivity to tactile simuli. At 3 dpf, *cro* mutants had RB cells with reduced I_{Na} amplitudes. In contrast, RBs in *sla* mutants had normal I_{Na} amplitudes at 3 dpf. The cellular basis for touch insensitivity in *sla* mutant has not yet been identified**.**

Another touch-insensitive mutant reported by the 1996 Tübingen screen, *touchdown* (*tdo*), was initially isolated on the basis of a pigmentation defect due to a reduction in the number of melanophores (Kelsh et al., 1996). However, *tdo* RBs have normal sodium currents and action potentials. Similar to *trpm7* and *sla* mutants, the cellular basis for the touch insensitivity *tdo* phenotype is not yet known.

Retroviral insertion mutants. Retroviral insertion mutagenesis has also isolated touch-insensitive mutants. In contrast to the majority of chemical mutagenesis lesions, genes perturbed by the retroviral insertion producing touch insensitivity have already been identified (Amsterdam et al., 2004; Gaiano et al., 1996; Golling et al., 2002). The results have yielded expected as well as unexpected genes. For example, the hi1059 insertion occurs in neurogenin related protein-1 and thereby probably affects differentiation of sensory neurons. In contrast, hi577a lesions the vacuolar ATP synthase subunit E gene. Future studies will identify the biological basis for the touch insensitivity phenotype of hi577a.

Narrowminded. Artinger, Chitnis, Mercola, and Driever (1999) isolated a touch-insensitive mutant, *narrowminded* (*nrd*), in a genetic screen that assayed gene expression patterns by *in situ* hybridization. The *nrd* mutation resides in the *prdm1* gene that encodes a SET/zinc finger transcription factor (Hernandez-Lagunas et al., 2005). This mutation results in loss of RBs, and subsequent loss of the sensation that they mediate – touch. In addition, *prdm1* mutants have an initial loss of neural crest cells. However, older larvae do have neural crest and their derivatives (e.g., dorsal root ganglion neurons). Consequently, the touch insensitivity phenotype recovers. Study of *prdm1* function indicates that it plays an important role at the neural plate– neural crest border in mechanisms that specify RB and neural crest cells.

Macho. In wildtype embryos, RB sodium currents increase in amplitude between 16 and 48 hpf (Pineda et al., 2006; Ribera & Nüsslein-Volhard, 1998). Interestingly, the developmental increase in I_{Na} coincides with appearance of touch sensitivity, supporting an essential role for this current in RB function (Kimmel et al., 1995; Saint-Amant & Drapeau, 2000; Ribera & Nüsslein-Volhard, 1998). The *mao* mutation maps to linkage group 2 but the identity of the lesioned gene is not yet known (Geisler, Rauch, Geiger-Rudolph, Albrecht, van Bebber, & Nüsslein-Volhard, 2007). Nonetheless, the *mao* mutant has permitted studies of the role of activity in RB development. RB cells normally undergo programmed cell death and are largely gone by 5 days post fertilization (dpf) (Reyes et al., 2004; Svoboda et al., 2001; Williams et al., 2000). Dorsal root ganglion cells, that develop after 36 hpf, are not required for RB programmed cell death (Reyes et al., 2004). However, RB death occurs more slowly in the absence of sodium current dependent activity (Svoboda et al., 2001).

Although *mao* mutant RB cells have a drastic reduction in sodium current amplitudes, I_{Na} is not completely suppressed (Ribera & Nüsslein-Volhard, 1998; Pineda et al., 2006. The *mao* mutation produces a similar potent but incomplete suppression of retinal ganglion cell sodium current (Gnuegge, Schmid, & Neuhauss, 2001).

RBs express more than one type of sodium channel (Novak et al., 2006; Pineda et al., 2006). Thus, the *mao* gene may incompletely suppress the function of all sodium channel types. Alternatively, the *mao* gene may affect the function of a subset or just one sodium channel. Further study of this mutant will resolve this issue and potentially discover new biological mechanisms relevant to sensory neurons.

Future Perspectives

The zebrafish has provided significant insights into biological mechanisms underlying an essential behavior for the early embryo, the touch response. Some mutations that result in touch insensitivity have a general effect on sensory neuron development such that the basis for touch insensitivity is understood (e.g., *hi1059*, *nrd*). In contrast, other mutations affect biological mechanisms that are either at present unknown or poorly understood (e.g., *mao*).

Analysis of the Tübingen touch-insensitive mutants revealed the unexpected finding that screening for touch insensitivity efficiently recovers mutations that affect sensory neuron sodium current. Electrophysiological analysis of RBs revealed defects in sodium currents in four of six touch insensitive mutants: *ali, cro, ste and mao* but not *sla* or *tdo*.

Many touch-insensitivity mutations lesion as yet unidentified genes. Future study of these mutants has the potential to provide new information about the poorly understood transduction mechanisms underlying vertebrate tactile sensation.

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