

Interactions with identified muscle cells break motoneuron equivalence in embryonic zebrafish

J. S. Eisen and E. Melançon

Institute of Neuroscience, 1254 University of Oregon, Eugene, Oregon 97403-1254, USA

Correspondence should be addressed to J.S.E. (eisen@uoneuro.uoregon.edu)

Published online: 15 October 2001, DOI: 10.1038/nn742

Two zebrafish motoneurons, CaP and VaP, are initially developmentally equivalent; later, CaP innervates ventral muscle, whereas VaP dies. Current models suggest that vertebrate motoneuron death results from failure to compete for limited, target-derived trophic support. In contrast, we provide evidence that zebrafish ventral muscle can support both CaP and VaP survival. However, VaP's growth cone is prevented from extending into ventral muscle by CaP-dependent interactions with identified muscle fibers, the muscle pioneers; this interaction breaks the initial equivalence of CaP and VaP. Thus, the processes mediating VaP death are more complex than failure to compete for trophic support, and may be important for correct spatial patterning.

Cell death is a widespread phenomenon during nervous system development. In some cases, for example, in *Caenorhabditis elegans*, specific cells within particular lineages cell-autonomously undergo programmed cell death¹. In contrast, most cell death in vertebrate nervous systems apparently results from failure of neurons to obtain appropriate trophic support. In amniotes and amphibians, about half the initial population of somatic motoneurons dies during embryonic development². Death occurs after growth cones have reached their targets; increasing target size decreases cell death whereas reducing target size increases cell death. Although trophic support may be available from other sources³, muscle elimination suggests that nearly all trophic support is target-derived⁴. Thus, the current model is that vertebrate motoneuron death results from competition for target-derived trophic support^{2,3} that may be available only at a limited number of synaptic sites⁵. Several trophic factors transiently support embryonic motoneuron survival, although none of them alone supports long-term survival⁶. Moreover, targeted mutations of mouse genes encoding individual trophic factors or their receptors have revealed only moderate motoneuron depletions, suggesting that motoneurons have complex trophic factor requirements⁶⁻¹⁰.

To investigate whether other interactions might also influence motoneuron survival, we asked if death of an identified motoneuron in embryonic zebrafish fits the current model of vertebrate motoneuron death. In adult zebrafish, each ventral myotome is innervated by a single, identified primary motoneuron (PMN), CaP, and several later-developing secondary motoneurons¹¹. During development, either one or two PMNs arise in the CaP position in each spinal hemisegment¹²⁻¹⁴; the second, variably present cell is called VaP. Initially, CaP and VaP appear identical by morphology¹², gene expression (ref. 14; B. Appel and J.S.E., unpublished data) and developmental potential¹³. However, they can be distinguished later because

CaP's axon but not VaP's axon extends ventrally from the nascent horizontal myoseptum (HM) (Figs. 1a and 3a; also see ref. 12), a choice point or intermediate target where PMN growth cones pause before selecting cell-specific axonal pathways¹⁵⁻¹⁸. In contrast, VaP growth cones never extend beyond the HM, but instead remain there; most die by 36 h.

What causes VaPs to die? CaP and VaP form an equivalence pair¹³; ablation of either cell before axogenesis results in the remaining cell becoming CaP, defining CaP as the preferred fate and VaP as the non-preferred fate of both cells¹³. Consistent with this notion¹⁹, VaP can be rescued from death by ablating CaP before VaP normally dies¹³, whereas ablation of VaP does not effect CaP development or survival (ref. 20 and J.S.E., unpublished data). Thus, interactions between these two cells determine which one becomes VaP. These interactions might regulate VaP death, or VaP might die because it fails to obtain adequate trophic support. For example, ventral muscle might not produce sufficient trophic support for two PMNs or it might have only enough postsynaptic sites to accommodate a single PMN. Alternatively, ventral muscle might have sufficient trophic support that VaP is unable to obtain because its growth cone remains at the HM. We tested these possibilities in four ways: first, by creating a situation in which two CaPs extended growth cones into ventral muscle and determining whether they survived past 36 h; second, by testing whether something at the HM prevented extension of VaP's growth cone into ventral muscle; third, by determining whether direct interactions between CaP and VaP somata were required for VaP to die; and fourth, by investigating whether the interactions between CaP and VaP were unique to these PMNs, by creating a situation in which MiP, another PMN, was artificially duplicated. Our results suggest that muscle pioneers, cells at the HM that are an intermediate target of CaP^{16,17}, act together with CaP to cause VaP to die.

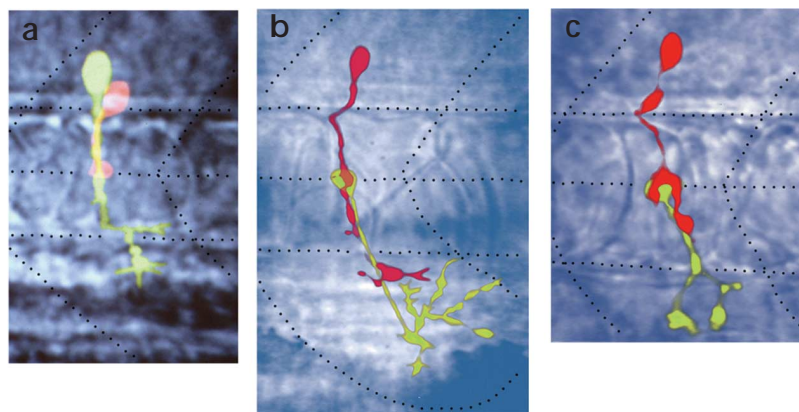


Fig. 1. Ventral muscle can support two CaPs. (a) In wild-type embryos, whenever there are two motoneurons in the CaP position, one becomes CaP (yellow, long axon) and the other becomes VaP (red, short axon). (b, c) Two cases in which a single CaP was transplanted from the spinal cord of a fluorescentin-dextran-labeled donor to the level of the HM of an unlabeled host. After the transplanted CaP extended an axon, the native CaP was labeled by intracellular iontophoresis of rhodamine-dextran. (b) At 48 h, both CaPs had extended axons and formed non-overlapping arbors in ventral muscle. The native CaP had a prominent varicosity and a small branch on the soma of the transplanted one. (c) At 30 h, the axon of the native CaP had nearly enveloped the soma of the transplanted cell. Horizontal dotted lines show spinal cord/notochord boundary (top), HM (middle), ventral aspect of notochord (bottom); diagonal dotted lines show myotome boundaries.

RESULTS

Ventral muscle can support survival of two CaPs

To learn whether ventral muscle can support survival of two CaPs, we based our experiment on earlier observations²¹ that following removal of native CaPs, CaPs transplanted to the HM form normal CaP arbors in ventral muscle. Here we transplanted single CaPs from labeled donor embryos to the HM of unlabeled hosts, labeled the native CaP in the same segment with another vital dye and followed development of both cells for 48 h. Both the transplanted and native CaPs survived and formed extensive, non-overlapping arbors in ventral muscle ($n = 5/6$; Figs. 1b and 3b), arguing that ventral muscle can support innervation by two PMNs, but that they select distinct synaptic sites.

Identified muscle cells prevent VaP axon extension

When both CaP and VaP are in the spinal cord, VaP's growth cone never extends beyond the HM. Thus, it was surprising that both native and transplanted CaPs did so in the experiments described above. This probably resulted from novel interactions between these cells in which the native CaP's growth cone elaborated varicosities on the transplanted CaP's soma at the HM ($n = 11$; Fig. 1b and c), often wrapping it extensively. This suggests that contact with the transplanted CaP facilitated extension of the native CaP's growth cone beyond the HM and into ventral muscle. To learn whether interactions between the native and transplanted CaPs depended on gap junctions, we tested whether they were dye-coupled

using the low molecular weight dye, Lucifer yellow²²; we found no dye-coupling between the two cells ($n = 3$; data not shown).

The ability of a transplanted CaP to facilitate extension of a native CaP growth cone into ventral muscle suggested that something at the HM normally prevents extension of a second CaP growth cone beyond this choice point, forcing a second CaP to become VaP. The HM is formed by a small set of 4 – 6 identified muscle cells, called muscle pioneers (MPs)²³. MPs are a transient, early target for CaP; later CaP innervates no muscle cells in this region¹⁶. To test whether MPs prevented VaP growth cone extension, we ablated them by laser irradiation about 1–3 hours before they would normally be contacted by CaP and VaP growth cones, then labeled CaP and VaP with different colored vital dyes. Under these circumstances, both cells extended growth cones beyond the HM and formed non-overlapping arbors in ventral muscle ($n = 12/13$; Fig. 2a); both cells developed as CaPs. These results provide evidence that MPs normally prevent extension of VaP's growth cone beyond the HM and into ventral muscle.

Muscle pioneers promote VaP death

To learn whether VaP died because it failed to extend a growth cone beyond the HM, we monitored survival of CaP/VaP pairs following MP ablation. In half the cases ($n = 4/8$), an HM formed (data not shown) and one motoneuron of the pair died; in the other cases, both motoneurons survived past 36 h. Although only half the VaPs survived in this experiment, this is very different from the normal situation, in which only 10–15%

Fig. 2. Both CaP and VaP extend axons into ventral muscle and survive after MP ablation and in mutants lacking MPs. (a) MPs were ablated by laser irradiation before axon outgrowth, in myotomes overlying spinal hemisegments containing both CaP and VaP. CaP and VaP were labeled by intracellular iontophoresis of vital fluorescent dyes and were observed until between 36 and 48 h. By 39 h, both cells extended normal-appearing CaP axons and formed non-overlapping arbors in ventral muscle. Arrows indicate axon branches extending laterally, through muscle. (b) CaP and VaP were labeled by intracellular iontophoresis of vital fluorescent dyes and followed to 36 h in *helix* mutant embryos. Both cells extended axons and formed non-overlapping arbors in ventral muscle. The apparent overlap is due to the two-dimensional representation of the three-dimensional axon branches, some of which extend laterally through the muscle. Horizontal dotted lines show spinal cord/notochord boundary (top), ventral aspect of notochord (bottom); diagonal dotted lines show myotome boundaries.

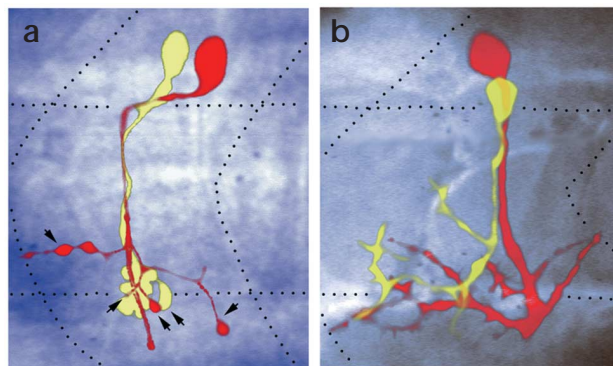
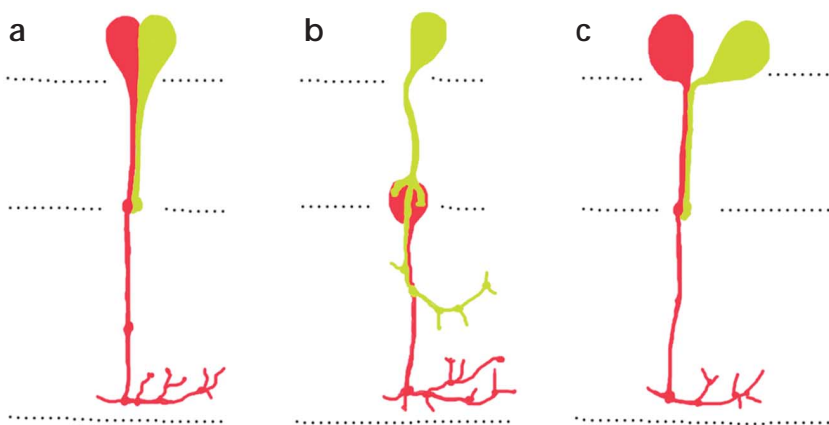


Fig. 3. Diagrammatic representation of the effect of CaP and VaP somata juxtaposition on VaP survival. (a) Normally, the CaP (red, long axon) and VaP (yellow, short axon) somata are in close contact in the spinal cord. The axons of both cells fasciculate as they extend ventrally. Both cells typically form varicosities at the HM. Only CaP innervates ventral muscle, whereas VaP dies. (b) Transplantation of a CaP (red) to the HM facilitates axonal outgrowth of a native CaP (yellow) past the HM, probably because the growth cone of the native CaP extends along the transplanted CaP, rather than contacting the MPs. Under these conditions, both cells survive. (c) When a CaP (yellow) is transplanted outside the spinal cord so its soma is not in contact with the native CaP (red), the axons fasciculate just as they do normally (see a). Under these conditions, one of the two cells becomes CaP and survives while the other one becomes VaP and dies. Top line, ventral aspect of spinal cord; middle line, HM; bottom line, ventral aspect of myotome.



of VaPs survive and innervate dorsal muscle, not the ventral muscle innervated by CaP¹². It seemed possible that new MPs were induced to form by notochord signaling^{24–26} following ablation, or that ablation was incomplete. Thus, we examined survival of VaPs in mutants that lack MPs.

Both *no tail* (*ntl*)²⁴ and *helix* (*hlx*)²⁷ mutants lack MPs. In these mutants, both cells of most labeled CaP/VaP pairs survived ($n = 4/5$ for *ntl*^{-/-}; $n = 7/8$ for *hlx*^{-/-}) and formed non-overlapping, CaP-like arbors in ventral muscle (Fig. 2b). VaP's growth cone remained able to extend into ventral muscle when it did not interact with MPs and this extension promoted survival. Thus, by preventing VaP's growth cone from reaching ventral muscle, MPs caused VaP to die. However, MPs do not simply prevent ventral extension by CaP growth cones; a CaP extends beyond them in every myotome, and it is only a second CaP that cannot extend a growth cone and thus becomes VaP. This argues that both CaP and MPs are required to prevent VaP growth cone extension into ventral muscle.

CaP soma contact seems unnecessary for VaP death

CaP and VaP somata are always in close contact with one another early in normal development^{12,13}. However, trans-

plantation of one of the two cells to the HM allowed both cells to survive, suggesting that normal contact between CaP and VaP somata is important for VaP death. To address this issue, we transplanted a second CaP into segments containing only a single cell in the CaP/VaP position. CaPs transplanted even many cell diameters away from their normal locations typically migrate back (ref. 28 and unpublished), so we transplanted the second cell outside the spinal cord basal lamina²⁹ (Fig. 3c). A CaP soma remaining in this position would not have normal contact with VaP, although we cannot eliminate the possibility of other contacts, like cytonemes³⁰.

Surprisingly, most CaPs transplanted outside the spinal cord moved back to their normal positions ($n = 24/28$). There were only four cases in which the transplanted soma remained outside the spinal cord. In three cases, the transplanted cell became VaP and died; in the remaining case, both cells survived past 36 h. The proportion of cells that became VaP and died in this experiment is similar to controls in which the somata of CaP and VaP are in contact within the spinal cord. Thus, contact between CaP and VaP somata seems unnecessary for VaP death, suggesting that if direct interactions with CaP participate in VaP death, they probably occur along the axons or growth cones.

Other motoneurons forced to compete do not die

Do the types of interactions occurring between CaP, VaP and the MPs also occur between other PMNs? MiP also extends a growth cone to the MPs, pauses for several hours, sprouts a dorsal collateral and then retracts the process in contact with the MPs. Ablation of CaP prevents normal retraction of MiP's ventral process³¹ and ablation of MPs, or MPs plus CaP permit this process to extend further ventrally than normal¹⁷, suggesting that important interactions occur between MiP and MPs. This raised the possibility that two MiPs might interact

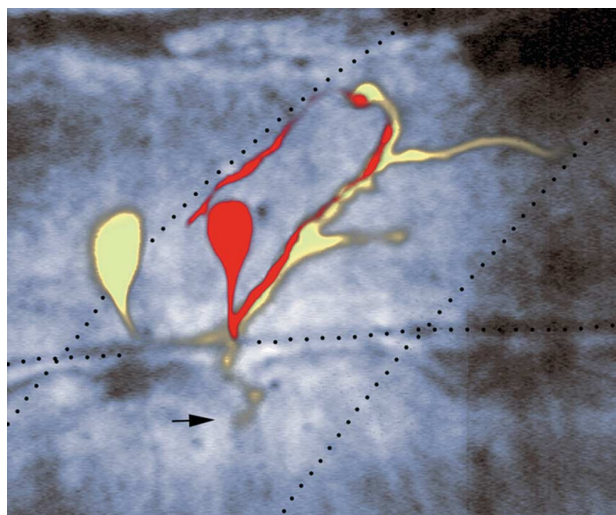


Fig. 4. Two MiPs divide the dorsal innervation territory. A single MiP (red) was transplanted from a labeled donor to an unlabeled host before axogenesis, and the adjacent native MiP was labeled by intracellular iontophoresis with fluorescein-dextran. Both MiPs extended normal axons and formed normal arbors in dorsal muscle. By 36 h, the two cells divided up the innervation territory so that their axon branches were non-overlapping; in this case, the transplanted cell arborized on lateral myotome whereas the native cell arborized medially in caudal dorsal myotome and was still in the process of retracting its ventral process (arrow) when this image was taken. Horizontal dotted line shows spinal cord/notochord boundary; diagonal dotted lines show myotome boundaries.

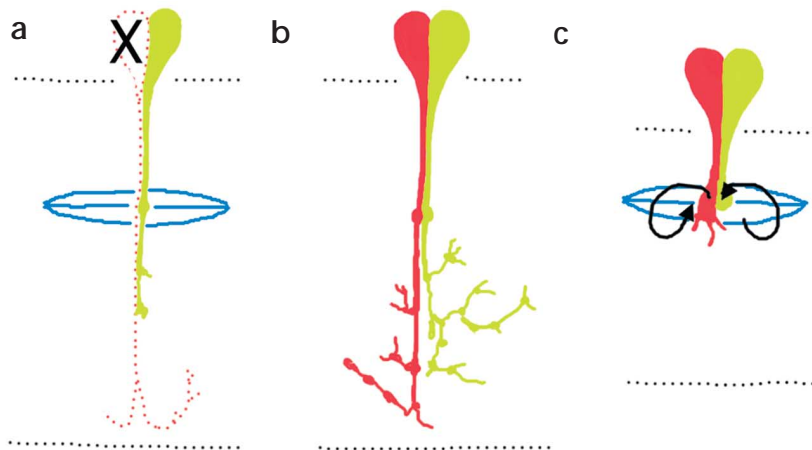


Fig. 5. Model of interactions between CaP, MPs and VaP. (a) Ablation of CaP (indicated by X and dotted line) allows VaP (yellow) to develop into CaP and extend its axon ventral of the MPs (blue). (b) In the absence of MPs, both cells of the CaP/VaP pair become CaPs. (c) We suggest that normally when CaP (red) contacts the MPs (blue), it signals to them (left arrow) and they in turn signal (right arrow) to VaP (yellow) to prevent it from extending its growth cone further.

with MPs similarly to CaP and VaP, resulting in death of one MiP. To test this possibility, we transplanted a second MiP into the spinal cord adjacent to the native MiP and followed development of both cells. Both MiPs formed normal morphologies, including retracting the ventral process, and survived through 36 h ($n = 5$); in three cases, both MiPs arborized in non-overlapping territories in dorsal muscle (Fig. 4). These results provide evidence that the interactions we observed between CaP, VaP and the MPs are restricted to these cells.

DISCUSSION

What interactions mediate motoneuron death?

Overproduction and subsequent death of motoneurons has been well described in amniote vertebrates where it is ascribed primarily to limited availability of trophic support (for review, see ref. 3). Such a mechanism would match the size of a motoneuron pool with its target. Additionally, neuronal elimination might adjust fine-grained motoneuron patterning in subtle ways we cannot yet appreciate because we do not have markers that distinguish all the different types of spinal motoneurons³². The idea that motoneurons compete for limited trophic support originally came from experiments in which target removal augmented and target addition diminished motoneuron death (for review, see ref. 2). Although many potential trophic factors can provide transient support for motoneurons in gain-of-function experiments, loss-of-function experiments reveal that most participate in survival of only a subset of motoneurons; thus, the trophic requirements of motoneurons may be complex^{3,7-10}. Alternatively, other types of signals might also be involved. For example, motoneuron death may result from failure of target-induced transformation of motoneurons from growing cells to transmitting cells⁶. Commissural interneurons change their trophic dependence during axonal extension, initially depending on intermediate target-derived trophic support and later on target-derived trophic support³³. Our studies provide evidence that signals from intermediate targets also affect vertebrate motoneuron survival.

Interactions with an intermediate target prevent VaP growth cone extension into target muscle and mediate VaP death. The growth cones of all zebrafish PMNs contact a specific intermediate target, MPs at the HM choice point, and typically pause there for up to several hours before extending further¹⁵. Thus, MPs may produce a 'stop' signal that inhibits PMN growth cones. Consistent with this idea, ablation of MPs allows

MiP¹⁷ and VaP growth cones to extend further ventrally than usual. For MiP, this is only a few muscle fiber diameters, whereas for VaP, the cell essentially becomes a second CaP. Normal extension of CaP's growth cone into ventral muscle requires *stumpy* gene function¹⁸. In *stumpy* mutant embryos, CaPs that do not extend growth cones into ventral muscle die¹⁸, as do VaPs in wild types^{12,13}, whereas CaPs extending even a short distance into ventral muscle survive¹⁸. This observation, together with our finding that VaPs are rescued from death in the absence of MPs provides strong evidence that interactions with this intermediate target are necessary for VaP death. Although choice points such as the crural and brachial plexus have been described for chick limb motoneuron growth cones³⁴, it is currently uncertain whether there are muscle fibers that provide interactions similar to those we ascribe to zebrafish MPs. Indeed, such intermediate targets may be cryptic in other species that have many more motoneurons and muscle fibers whose development cannot be followed easily in living embryos.

VaP may ultimately die due to lack of target-derived trophic support. Although expression of several neurotrophic factors and their receptors has been described in zebrafish³⁵, we currently have little understanding of the trophic requirements of zebrafish motoneurons. The studies described here and our analysis of CaP motoneurons in *stumpy* mutants¹⁸ are consistent with the idea that VaP dies because its growth cone cannot extend into a region where appropriate trophic support is available. Further tests of this possibility await a more complete description of motoneuron trophic requirements.

What governs equivalence pair cell fate choice?

CaP and VaP form an equivalence pair by the formal definition established in *C. elegans*¹⁹. Ablation of CaP causes VaP to adopt the CaP fate¹³, whereas ablation of VaP has no effect on CaP (ref. 20 and J.S.E., unpublished data). It seems surprising that the interactions governing cell fate choice do not occur exclusively between these two cells, but require another cell type. However, this situation seems more common among invertebrates. In leech, the P and O blast bandlets form an equivalence group in which P is the preferred fate³⁶, but interactions between these two cell populations depend on position-dependent signals from cells outside the equivalence group^{37,38}. Similarly, in *C. elegans*, equivalence between the two daughters of the anterior blastomere is broken by signals from P1-descendants, which are, again, cells outside the equivalence pair³⁹. Here we describe this type of interaction among vertebrate neurons; the commonality of mechanisms used during vertebrate motoneuron development^{32,40,41} suggests that similar interactions might govern cell fate choice by other vertebrate motoneurons.

Interactions between VaP and MPs are complex. When there is a single CaP in a spinal hemisegment, it always extends a growth cone ventrally from the MPs. Thus, MPs do not simply produce a signal that prevents motoneuron growth cone extension. However, when there is a CaP/VaP pair, only one cell's growth cone extends beyond the MPs; this cell becomes CaP. Ablation of CaP allows the other cell of the pair to extend its growth cone beyond the MPs and become CaP (Fig. 5a) and absence of the MPs allows both cells to become CaP (Fig. 5b). These observations suggest that CaP and the MPs act together to prevent VaP from extending its growth cone beyond the HM. How might this work? Whichever cell of the CaP/VaP pair that first contacts the MPs might induce them to express ligands or receptors that allow only that cell to extend a ventral axon and become CaP. In effect, CaP would signal to the MPs and they, in turn, would signal to the other cell of the pair (Fig. 5c), effectively breaking the equivalence between them and preventing the second cell from extending its growth cone ventrally and causing it to become VaP. The availability of mutations in a large number of zebrafish genes^{27,42,43} as well as ongoing screens for genes involved in motoneuron development^{44,45} should provide insights into the molecular mechanisms governing the choice of VaP fate and the course of VaP differentiation, and should reveal whether similar interactions are involved in motoneuron death and survival in other vertebrate species.

METHODS

Animals. Zebrafish adults were maintained and embryos were raised as previously described¹⁷; embryos were staged by hours (h) or days post-fertilization at 28.5°C⁴⁶. Embryos homozygous for mutations in the *no tail* or *helix* genes were obtained by crossing heterozygous carriers. Animal use was approved by University of Oregon Institutional Animal Care and Use Committee.

Single cell labeling. Individual PMNs were labeled with rhodamine-dextran or fluorescein-dextran (3K; Molecular Probes, Eugene, Oregon) or with Lucifer yellow²² by impaling them with micropipettes pulled on a Flaming Brown micropipette puller (Sutter Instruments, San Rafael, California) and ringing the negative capacitance of a Biodyne AMP-2 electrometer amplifier, as previously described^{13,47}.

Single cell transplantation. Individual PMNs were transplanted from rhodamine dextran or fluorescein dextran-labeled donor embryos into unlabeled host embryos as previously described^{13,48}. Briefly, donors were labeled at the 1–4 cell stage, allowed to develop until PMNs could be recognized under a 40× water immersion objective (Zeiss, Thornwood, New York), mounted next to unlabeled hosts in 1.2% agar in physiological saline. Individual PMNs were removed by gentle suction through a micropipette broken off to 10–20 μm, transferred to hosts and released by gentle pressure.

Single cell ablations. Individual neurons or muscle cells were ablated by focusing the beam of a laser (Laser Science, Newton, Minnesota) onto the cell through the objective of a Zeiss Standard microscope as previously described^{17,47}.

Image processing. Images were acquired using Axovideo (Axon Instruments, Union City, California), stored on an optical disk recorder (Panasonic, Secaucus, New Jersey) and processed using Photoshop (Adobe, San Jose, California) to add images from different focal planes, enhance background to noise and add pseudocolor.

ACKNOWLEDGEMENTS

Special thanks to B. Eisen. We thank M. Westerfield, C. Kimmel and D. Armstrong for discussions and comments on the manuscript and the University of Oregon Zebrafish Facility staff for animal husbandry. Supported

by NIH NS23915 and HD22486. Renovation and expansion of the University of Oregon Zebrafish Facility supported by NIH RR11724, NSF 9602828, the M.J. Murdock Charitable Trust and the W.M. Keck Foundation.

RECEIVED 20 JULY; ACCEPTED 25 SEPTEMBER 2001

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