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Ear Manipulations Reveal a Critical Period for Survival and Dendritic Development at the Single-Cell Level in Mauthner Neurons

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Abstract

Second-order sensory neurons are dependent on afferents from the sense organs during a critical period in development for their survival and differentiation. Past research has mostly focused on whole populations of neurons, hampering progress in understanding the mechanisms underlying these critical phases. To move toward a better understanding of the molecular and cellular basis of afferent-dependent neuronal development, we developed a new model to study the effects of ear removal on a single identifiable cell in the hindbrain of a frog, the Mauthner cell. Ear extirpation at various stages of *Xenopus laevis* development defines a critical period of progressively-reduced dependency of Mauthner cell survival/differentiation on the ear afferents. Furthermore, ear removal results in a progressively decreased reduction in the number of dendritic branches. Conversely, addition of an ear results in an increase in the number of dendritic branches. These results suggest that the duration of innervation and the number of inner ear afferents play a quantitative role in Mauthner cell survival/differentiation, including dendritic development.

Keywords

mauthner cell; cell survival; dendritic branching; *Xenopus laevis*

INTRODUCTION

The ablation of a primary sensory organ results in hypoplasia of second-order neuronal centers (Harrison, 1935). Early studies in amphibians demonstrated that removal of an eye or nasal pit results in reduced size of the opposite midbrain tectum and in reduced olfactory centers in the forebrain, respectively (Harrison, 1935). In addition, several studies have shown that removal of the ear and associated ganglion neurons prior to axonal outgrowth alters the development of various target neurons in the hindbrain (Levi-Montalcini, 1949; Parks, 1979, 1981; Goodman and Model, 1988; Fritzscht, 1990). Removal of an ear during

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development led to stage specific reduction in the volume and number of viable neurons in the auditory nuclei (Levi-Montalcini, 1949; Parks, 1979; Ryugo and Parks, 2003), apparently correlating with afferent activity (Jackson et al., 1982). Blocking afferent activity with tetrodotoxin resulted in a reduction of volume and number of neurons in the auditory nuclei, similar to ear ablation (Pasic and Rubel, 1989; Sie and Rubel, 1992). Together, these data would suggest that most of the initial neuronal development occurs without excitatory activity (Verhage et al., 2000; Varoqueaux et al., 2002) and that later, without this activity and/or activity related neurotrophin release, cell death, and atrophy of remaining neurons occurs (Rubel and Fritzsche, 2002). This dependence on a presynaptic neuron for survival is not permanent, implying there is a critical period in which input is necessary for cell survival (Rubel and Fritzsche, 2002). In gerbils, cochlea removal before postnatal day (P) 7 resulted in cell death in 45–88% of cochlear nucleus neurons, whereas removal after P9 resulted in virtually no cell death (Tierney et al., 1997). Other species also have critical periods but with varying critical time points and degrees of cell loss (Born and Rubel, 1985; Mostafapour et al., 2000). In addition, while early removal of the ear resulted in death of most cochlear nuclei and severe atrophy of the remaining neurons (Levi-Montalcini, 1949; Parks, 1979), virtually no cell death occurred in the vestibular nuclei (Levi-Montalcini, 1949). Vestibular nuclei neurons exit the cell cycle much earlier than auditory nuclei neurons (Altman and Bayer, 1980), suggesting that the vestibular nuclei neurons may be past the critical period at the time these manipulations took place in mammals whereas others explanations have been proposed for birds (Levi-Montalcini, 1949; Peusner and Morest, 1977). In addition, the vestibular nucleus, unlike the auditory nucleus, receives multisensory inputs and a loss of vestibular inputs does not negatively affect other inputs, such as somatosensory inputs, and has even been shown to result in an increase in these other inputs after adult (Dieringer et al., 1984) or embryonic ear ablation (Fritzsche, 1990). Therefore, it is also possible that these additional inputs stabilize the vestibular nucleus. Comparable to age related differential cell death (Lopez-Otin et al., 2013), the causality for survival of some and death of other second-order neurons following deafferentation remains unclear (Durham and Rubel, 1985; Steward and Rubel, 1985; Born and Rubel, 1988; Durham et al., 1993; Garden et al., 1994; Hyde and Durham, 1994a,b; Garden et al., 1995; Kelley et al., 1997).

While the importance of primary sensory input on second-order neuron survival and differentiation is clearly established, all studies have looked thus far only at whole populations of cells when determining the critical period of cell survival following ear removal. Furthermore, technical limitations restrict ear removal in chicken to embryos and in mice to postnatals, preventing a comprehensive analysis of the loss of afferent input over a longer period of time in either species. While afferents or ears can be genetically removed in mice, the resulting mutants are typically not viable, blocking investigations on long term auditory nucleus viability (Ma et al., 2000). More recent work using combined conditional deletion of several genes has worked to overcome this problem for a study of long term effects of innervation on hair cells of the ear (Kersigo and Fritzsche, 2015), but no data on central defects are currently available. To overcome these limitations, we have studied the effects of early otic placode/ear extirpation at various developmental stages, focusing on a single cell in the hindbrain of the externally developing frog, the Mauthner cell.

Mauthner cells are a pair of large, easily identifiable, reticulospinal neurons at the level of the ear in the hindbrain of many aquatic vertebrates (Herrick, 1914; Bartelmez, 1915). Mauthner cells are important for the escape reflex (Korn and Faber, 2005). Inner ear vestibular and auditory neurons of premetamorphic amphibians and fish form synapses on the lateral dendrite of the ipsilateral Mauthner cell, which in turn form synapses on contralateral spinal motor neurons to activate the C-start escape response (Korn and Faber, 2005; Sillar, 2009). A study in axolotls has shown the absence of the Mauthner cell in one-third of embryos following otic vesicle ablation at Stage 27 (Piatt, 1969); however, all Mauthner cells were present when the ear was extirpated at a later stage (Stage 34, roughly comparable to Nieuwkoop and Faber Stage 34 for *Xenopus laevis*). Goodman and Model also reported an occasional absence of the Mauthner cell (1988). While Piatt (1969) suggested that the inner ear afferents are important for, and often a decisive factor for, the development of the Mauthner cell, Goodman and Model (1988) suggested that surgical perturbations may be the cause of the occasional Mauthner cell absence. Since early ear removal, before the critical period, results in significant loss of the cochlear nuclei neurons (Levi-Montalcini, 1949; Parks, 1979; Born and Rubel, 1985; Tierney et al., 1997; Mostafapour et al., 2000), we reason that removal of the ear at the earlier stages (Piatt, 1969; Goodman and Model, 1988) may have prevented the survival and/or differentiation of the Mauthner cell, indicating a yet to be defined critical period for a single cell, in line with variable effects of denervation on hair cells (Kersigo and Fritzsche, 2015).

The inner ear not only affects the development of the Mauthner cell but may also have a role in the development of the lateral dendrite. Removal of the ear in axolotl resulted in an obvious, but unquantified, reduction of dendritic branching of the Mauthner cell lateral dendrites in areas normally receiving vestibular input (Kimmel et al., 1977; Goodman and Model, 1988; Fritzsche, 1990). Likewise, removal of otic vesicles in zebrafish resulted in reduced branching of the Mauthner cell lateral dendrites (Kimmel, 1982) and ablation of the otic vesicle in Stage 38 *X. laevis* embryos resulted in reduced Mauthner cell lateral dendrites (Fritzsche, 1990) but also expansion of ventral dendrites. Blocking nerve impulse activity in axolotls by grafting them to tetrodotoxin-containing newts did not affect Mauthner cell dendritic branching patterns, suggesting that innervation itself, rather than neural activity, is important (Goodman and Model, 1990), the latter being in stark contrast to other experiments using other means to block activity (Pasic and Rubel, 1989; Sie and Rubel, 1992). Taken together, these data suggest that axons from the ear stimulate growth and development of the dendrites. This is further supported by a study in axolotls where an additional ear was transplanted rostral to the native ear. The Mauthner cells in these animals displayed unspecified “enhanced branching” of the lateral dendrite (Goodman and Model, 1988).

In the present study, we explore the effects of otic placode/vesicle extirpation on developing Mauthner cells and their lateral dendrites by removing the ear at both early stages (Stages 24–26), when the ear is a placode, and later stages (Stages 27–40), when the ear has become a closed vesicle, in the frog *X. laevis*. We also explore the effects of additional otic placode transplantations on the developing Mauthner cell and lateral dendrite. This study identifies a critical period during which the Mauthner cell depends on some form of input from the ear for cell survival, either physical input or a diffusible morphogen. In addition, this study

quantifies increased or decreased lateral dendrite development following ear extirpation or addition at various stages.

METHODS

Animals

X. laevis embryos were obtained through induced ovulation using an injection of human gonadotropin and fertilized with a sperm suspension in 0.1X Marc's Modified Ringer's Solution (MMR). Embryos were kept at 18°C in 90 mm Petri dishes containing 0.1X MMR (diluted from 1X MMR, see below) until they reached Stage 46 (Nieuwkoop and Faber, 1994). Once they reached Stage 46, animals that were to be used for unmanipulated controls were anesthetized in 0.2% Benzocaine and fixed in 4% paraformaldehyde (PFA) by immersion.

Removal of Ears

Ear removals were performed in 1X MMR pH 7.6–7.8, diluted from 10X stock (1 MNaCl, 18 mM KCl, 20 mM CaCl₂, 10 mM MgCl₂, 150 mM HEPES) at room temperature under a dissecting microscope. Single otic placodes or otic vesicles were removed from Stages 24 to 40 embryos. Single otic placodes or vesicles were removed from Stages 24 to 26 embryos and then immediately replaced to serve as a surgical control. Embryos were kept in 1X MMR for approximately 15 min following ear removal to promote healing prior to their transfer to 0.1X MMR. Healing was confirmed visually as a fusion of ectoderm. Once embryos reached Stage 46, they were anesthetized in 0.2% Benzocaine, their spinal cords were labeled with dextran amine dyes (see below), and were fixed in 4% PFA.

Dextran Amine Label

Stage 46 embryos were anesthetized in 0.2% Benzocaine. Their spinal cords were cut with dissecting scissors and a small amount of 3000 MW Texas red dextran amine dye was placed onto the cut spinal cord (Fritsch, 1993). The embryos were kept for 3 h to allow for dye diffusion into the hindbrain prior to embryo fixation in 4% PFA.

Immunohistochemistry

Heads were immunostained with antibodies against acetylated tubulin (Farinas et al., 2001) to label all nerves and with 3A10 to label reticular neurons, including the Mauthner cell(s) (Liu et al., 2003; Patten et al., 2007). In addition, myosin (Myo) VI was used to confirm absence of inner ear hair cells on the ablated side. Concentration used for acetylated tubulin (Cell Signaling Technology) was 1:800, for 3A10 (Developmental Studies Hybridoma Bank, University of Iowa) was 1:250, and for Myo VI (Proteus Biosciences) was 1:400. Species-specific secondary antibodies (Alexa) were used at 1:500. Brains were removed following immunohistochemistry. Brains and heads were mounted separately on a slide in glycerol and imaged with a Leica TCS SP5 confocal microscope. Since the 3A10 antibody did not label all of the dendrites filled with dextran amine dye [Fig. 3(A–A'')], this antibody was used only to confirm the presence or absence of the Mauthner cell.

Three-Dimensional Reconstruction of Mauthner cells

Embryos injected with dextran amines into the spinal cord were used for three-dimensional (3D) reconstruction of Mauthner cells (Kopecky et al., 2012). Briefly, brains were mounted ventral-side up in glycerol on a microscope slide. Confocal z-series images at 1.5 μm were taken of the hind-brain using a Leica TCS SP5 confocal microscope. Z-series stacks were loaded into Amira Version 5.4 software for manual segmentation as described previously (Kopecky et al., 2012).

Dendritic Branching Analysis

The total number of terminal branches for each Mauthner cell lateral dendrite was counted from the 3D reconstructed Mauthner cells. The difference between left and right Mauthner cells was calculated by subtracting the number of branches on the right from the left Mauthner cell.

Sholl analysis (Sholl, 1953) was performed to determine the branching pattern of the lateral dendrite of Mauthner cells. Concentric circles spaced 25 μm were drawn around the cell soma. The number of lateral dendritic branches that crossed each circle was counted for the right Mauthner cell.

Statistical Analysis

The data are represented as the mean $\pm$ SEM if not stated otherwise. Nonparametric statistical tests were used to determine significance for all terminal branch count analyses. A Wilcoxon signed-rank test was conducted to compare the left and right terminal branch counts for the Mauthner cells within each group (control, remove-replace, remove placode, remove early otic, remove late otic, and extra ear). Using the actual differences, a Kruskal-Wallis test was conducted to compare the median difference among the six groups. Pairwise *post hoc* comparisons were conducted using Mann-Whitney *U* tests and adjusted for all pairwise comparisons using the Bonferroni adjustment. A Students' *t* test was used for comparing mean number of crossings between Mauthner cells from ear removed or added animals and control animals following Sholl analysis. All statistical tests were performed at the 0.05 significance level.

RESULTS

Effect of Stage of Ear Removal on the Degree of Ear Regeneration

Because of the regulative nature of otic placode development, we considered that ears removed early might reform or regenerate from the placodal field, we examined the amount of ear regeneration at the various stages. Removal of ears at otic placode stages (Stages 24–26) led to regrowth of a part or of nearly all of the ear by Stage 46 in over one-third of the embryos [Fig. 1(a)], the remaining embryos did not have ear regrowth [Fig. 1(d)], as confirmed by studying the otic region innervation with tubulin and myo VI staining [Fig. 1(d')]. The amount of ear regrowth in these early stages varied, ranging from an endolymphatic duct, to a small otic vesicle, or to a nearly complete ear. Removal of ears at otic vesicle stages (Stages 27–40) resulted in fewer instances in which the ear regrew in embryos by Stage 46 as compared to ears removed at placode stages (Stages 24–26); most had no ear

regrowth when the ear was removed at Stage 27 and older. In these later stages, if there was any regrowth, it was nearly always just the endolymphatic duct. No regrowth of any part of the ear was detected in embryos in which the ear was removed at Stages 38–40. These data confirm previous work indicating placodal induction in amphibians extends over a lengthy period (Yntema, 1950). Only animals in which there was no regeneration of the ear were used for subsequent studies investigating the effects of ear removal on the Mauthner cell.

Effect of Stage of Ear Removal on the Presence of the Mauthner Cell

Prior to determining the effect of early ear removal on the Mauthner cell, we first wanted to determine the extent that our microdissections might also eliminate Mauthner cell progenitors in some cases, as had been previously suggested (Goodman and Model, 1988). To test this, ears were removed between Stages 24 and 26, and immediately replaced. The replaced ear developed normally in 23 of 25 embryos [Compare Fig. 1(c) with Fig. 1(b)]. In one of these abnormal cases, the replaced ear developed a single otoconia, and the other formed only a vesicle. Fourteen animals, in which their ears were removed and replaced immediately, were selected at random and the presence of Mauthner cells was assessed by retrograde labeling using dextran amine labeling. The Mauthner cell was present and morphologically similar to controls at Stage 46 in all embryos ($n=14$) [Fig. 2(a,b)]. We therefore reasoned that any loss of a Mauthner cell following ear removal would be due to the absence of the ear, rather than the procedure for ear removal.

We next removed ears over a range of stages to determine the effect on Mauthner cell formation. Our results showed that the stage of ear removal had a clear effect on the development and/or survival of the Mauthner cell. The earlier the stage of ear removal, the less likely it was that the ipsilateral Mauthner cell would be present at Stage 46 [Fig. 2(c)]. To confirm that cases of absent Mauthner cells were not artifacts of incomplete Dextran amine dye application, an antibody against reticular neurons, including the Mauthner cell, 3A10, was also used [Fig. 2(d–d'')]. In every animal in which the Mauthner cell was not detected with Dextran amine dye, there was also no antibody recognition of the Mauthner cell. In addition, we looked for the crossing axons near the boundary of rhombomeres 4 and 5, as the Mauthner cell is the only pair of large neurons to cross here [Fig. 2(e–g)]. When the ipsilateral Mauthner cell body was not detected, its respective axon was also absent [Fig. 2(g)], arguing against incomplete filling. The percentage of Mauthner cell survival was calculated for each group [Fig. 2(c)]. When the ear was removed at otic placode stages, between Stages 24 and 26, the Mauthner cell was present on the ablated side 38% of the time ($n=91$), whereas this frequency increased with removal at later stages, between Stages 27 and 30 [Mauthner cell present 64% of the time ($n=39$)]. Removal at even later stages caused little effect on Mauthner cell development, which formed on the ablated side 95% of the time ($n=40$). The Mauthner cell was always present when the ear was removed between Stages 36 and 40 ($n=24$). These data indicate that ear removal has a stage-specific effect on Mauthner cell viability and/or differentiation along typical Mauthner cell characteristics.

Effect of Stage of Ear Removal on the Dendritic Branching of Surviving Mauthner Cells

Prior to determining the effect ear removal has on dendritic branching of the lateral dendrite of surviving Mauthner cells, we wanted to establish the innate variability in dendritic

branching between left and right Mauthner cells within the same animal as well as variability in dendritic branching between animals for control animals and for those whose ear was removed and then immediately replaced. The total numbers of dendritic branch terminals were counted for 3D reconstructed left and right Mauthner cells [Fig. 3(b–d)] obtained from confocal images of dextran amine dye-labeled cells. 3D reconstructions were made from dextran amine-labeled Mauthner cells, as opposed to 3A10-labeled cells, due to a more complete labeling of the dendrites [Fig. 3(a–a'')]. The mean number of dendritic branches in the lateral dendrite of control animals was 22.2 ± 1.8 for the left Mauthner cell and 21.1 ± 1.4 for the right ($n=14$) [Fig. 3(f)], with a mean difference of 1.1 ± 1.2 ($n=14$). As expected, this difference between left and right Mauthner cell dendritic branch number in controls was not significant ($p>0.05$, $n=14$). With respect to variation, there was more variation in the number of dendritic branches across different control animals than within the same animal. The range of dendritic branches across control animals was from 11 branches to 33 branches for a single Mauthner cell. The greatest difference between the dendritic branch number of left and right Mauthner cells within the same animal was seven branches.

For animals in which the right ear was removed and immediately replaced, the mean number of dendritic branches in the lateral dendrite was 23.9 ± 1.9 for the left Mauthner cell and 19.7 ± 1.3 for the right ($n=14$) [Fig. 3(f)]. The mean difference between left and right Mauthner cell dendritic branch number for embryos in which the right ear was removed and immediately replaced was 4.1 ± 2.1 ($n=14$). Although the Mauthner cell on the side in which the ear was removed and immediately replaced had a tendency toward fewer dendritic branches compared with the control side, there was no significant difference between left and right mean Mauthner cell dendritic branch numbers when one ear was removed and immediately replaced ($p>0.05$, $n=14$). These data would suggest that the surgical procedure itself to remove an ear has no significant effect on the number of lateral dendrites that develop in the Mauthner cell.

When ears were completely removed at any of the stages, the dendritic branching of the remaining ipsilateral Mauthner cell lateral dendrite was always reduced compared to the control side. For each of the stages of ear removal: otic placode, early otic vesicle, and later otic vesicle, the mean number of dendritic branches for the ipsilateral Mauthner cells was significantly less than the mean number of dendritic branches for the contralateral control Mauthner cell ($p<0.001$, $n=14$ for each stage of ear removal). For animals in which the right ear was removed at placode stages (Stages 24–26), the mean number of dendritic branches in the right lateral dendrite was 4.9 ± 0.6 compared with 23.1 ± 3.1 branches on the control side ($n=14$) [Fig. 3(f)]. For animals in which the right ear was removed at early otic vesicle stages (Stages 27–30), the mean number of dendritic branches in the right lateral dendrite was 7.3 ± 1.0 compared with 21.3 ± 2.5 branches on the control side ($n=14$) [Fig. 3(f)]. For animals in which the right ear was removed at later otic vesicle stages (Stages 31–40), the mean number of dendritic branches in the right lateral dendrite was 8.8 ± 0.9 compared with 23.8 ± 2.2 branches on the control side ($n=14$) [Fig. 3(f)]. These data suggest that the lateral dendrite of surviving Mauthner cells may depend over a lengthy period on vestibular input for normal development.

To determine significant differences between groups, the difference in branch number between left and right Mauthner cells was calculated by subtracting the number of dendritic branches of the right Mauthner cell from the left Mauthner cell. Using the Kruskal–Wallis test to compare the medians of these differences among the groups, a significant group effect was found ($p < 0.001$). Following a Bonferroni multiple-comparison adjustment, no significant difference was found between the control and the remove and replace groups ($p > 0.05$) [Fig. 3(g)]. While there was a trend showing a more severe reduction in dendritic branch numbers at the earliest stage of ear removal compared with later stages [Fig. 3(f)], no stage of ear removal was significantly different from each other ($p > 0.05$) [Fig. 3(g)]; however, all stages of ear removal were significantly different from the control and remove and replace groups ($p < 0.005$ for each).

Sholl Analysis was performed to compare branching patterns. The numbers of dendritic crossings for Mauthner cells from animals in which the ipsilateral ear had been removed and replaced were significantly less than control Mauthner cells for 25 μm and 50 μm distances away from the soma ($p < 0.05$) but were similar 75 μm away from the soma and beyond [Fig. 3(h)]. The number of crossings for Mauthner cells from animals in which the ipsilateral ear had been removed was significantly fewer than the number of crossings for control Mauthner cells at 25 μm and at 50 μm distances away from the soma for both removal at otic placode stages (Stages 24–26) and early otic vesicle stages (Stages 27–30; $p < 0.05$), and significantly fewer at only 50 μm away from the soma for removal at later otic vesical stages (Stages 31–40; $p < 0.05$). While there were no crossings in control right side Mauthner cells beyond 100 μm away from the soma, four animals with removed ears had crossings at 125 μm away from the soma, although only by one dendrite branch.

Effect of Increasing Afferent Input on the Dendritic Branching of Mauthner Cells

The addition of an ear rostral to the native ear [Fig. 1(e)] lead to growth of the vestibular nerve fibers of these ears into the brain that mostly end in the vestibular fiber tract and nuclei with the unmanipulated control ear [Fig. 1(e')]. Dependent on the orientation of the grafted ear, the afferents may form vestibular-dominance column like segregations (Elliott et al., 2015). Again, the total numbers of dendritic branch terminals were counted for 3D reconstructed left and right Mauthner cells [Fig. 3(e)] obtained from confocal images of dextran amine dye-labeled cells. In those cases, where afferents of the “third” transplanted ear reached into the brain, there was a significant increase in the number of dendritic branches in the ipsilateral Mauthner cell ($p < 0.001$, $n = 10$) of approximately 30% more branches. The mean number of dendritic branches in the right lateral dendrite was 31.1 ± 1.9 compared with 22.5 ± 2.3 branches on the control side ($n = 10$) [Fig. 3(f)]. Following the significant Kruskal–Wallis test for a group effect, the *post hoc* test comparing the median difference for the three-eared frogs and the controls was found to be significant ($p < 0.005$) [Fig. 3(g)]. These data suggest that the upper limit of lateral dendrite branching is determined by yet to be understood interactions of all vestibular afferent fibers with the growing lateral dendrite.

Sholl Analysis was also performed. The numbers of dendritic crossings for Mauthner cells from animals in which an additional ear was transplanted rostral to the native ear were

significantly more than Mauthner cells from control animals between 75 μm and 100 μm away from the soma, at which point there were no additional crossings in control Mauthner cells [Fig. 3(h)] ($p < 0.05$). In one animal with an extra ear, crossings were detected 175 μm away from the soma. Five additional animals with an extra ear had crossings 150 μm away from the soma.

In summary, data on lateral dendrite development of Mauthner cells show a clear influence of vestibular afferents on dendritic growth (Fig. 4): extra afferents through a third ear increase dendrite branches and transient presence or complete absence of afferents following ear removal at various stages results in dendrite branch reduction.

DISCUSSION

The results presented here extend previous work on studying the effects of ear removal on second order neurons in the hindbrain by focusing on a single hindbrain neuron, the Mauthner cell. Ablation of ears in embryos between Stages 24 and 27 had previously been shown to have the capacity to regenerate a new ear or partial ear (Waldman et al., 2007). Similarly, our results showed complete or partial ear regeneration during these stages. Beyond Stage 27 the ear rarely regenerated; if there was any part of the ear present, it was nearly always only the endolymphatic duct. We did not find any instance of regeneration beyond Stage 38. That the ear regenerated more often when removed at the early placode stages indicates that the remaining tissue was competent to regenerate a new ear (Waldman et al., 2007), consistent with earlier suggestions (Yntema, 1950). Alternatively, while care was taken to remove the entire ear, it cannot be ruled out that a small portion was left behind. This may be the likely scenario for endolymphatic ducts being present at later stages of ear removal, when the tissue is not believed to be as likely to regenerate (Waldman et al., 2007).

Using two independent methods to label neurons, dextran amine dye tracers, and immunohistochemistry, and by looking for the crossing of the large axons near the boundary of rhombomeres 4 and 5, we have shown that the development and/or survival of the Mauthner cell is dependent on the presence of the ear. Moreover, the presence or absence of the Mauthner cell depends on the stage at which the ear is removed. The earlier in development that the Mauthner cell is deprived of input from the ear, the more likely it is that the cell does not survive and/or form a recognizable Mauthner cell using our criteria. Our removal of an ear at stages earlier than had been removed before, reveals that previous reports of Mauthner cell loss were underestimated (Piatt, 1969; Goodman and Model, 1988). While the ipsilateral Mauthner cell is completely undetectable by our current methods of detection in some animals, and we presume this means the cell did not survive and/or develop, we acknowledge that we cannot completely rule out the possibility that the cell has either shrunk down and/or has adopted a new fate instead and is no longer recognizable as a Mauthner cell. Although, the lack of an axon of any ipsilateral cell crossing around the boundary of rhombomeres 4 and 5 in these animals do make this latter possibility less likely.

The Mauthner cell was always observed after ear removal beyond Stage 36, indicating that stages earlier than Stage 36 are in a critical period of dependence on the ear for survival.

Stage 36 corresponds with the stage at which afferents from the ear were observed projecting into the hindbrain of axolotls (Fritzsche et al., 2005). As with neurons in the cochlear nucleus (Levi-Montalcini, 1949; Parks, 1979; Ryugo and Parks, 2003), not all Mauthner cells are absent following ear ablation prior to the proposed critical period as suggested for the cochlear nucleus (Garden et al., 1994; Hyde and Durham, 1994a). Clearly, our data are consistent with earlier suggestions of a dependency of the Mauthner cell on the ear. Moreover, our data do not support the suggestion that loss of Mauthner cells is simply a function of ear removal manipulation (Goodman and Model, 1988), since we always found a Mauthner cell when we simply removed and replaced the ear. Given that Mauthner cells are among the first neurons to exit the cell cycle (Lamborghini, 1980), combining early ear ablation with BrdU/EdU labeling could help show that the ipsilateral Mauthner cell indeed died. The Mauthner cell provides a unique future opportunity to study initiation of degeneration as a consequence of partial and delayed denervation in a single cell. Consistent with our data, loss of some vestibular neurons has been observed after ear ablation in chicken (Peusner and Morest, 1977) but no systematic analysis of ear removal at different stages has been conducted in this species, and thus, no critical phase on vestibular neurons has been defined prior to our study.

Our data suggest that the significant reduction in dendritic branching following ear removal was a result of the loss of input from the ear and not from the physical process of ear removal. We base this interpretation on the fact that there was no significant difference in the total number of dendritic branches of the lateral dendrite between left and right Mauthner cells when the ear had been removed and immediately replaced on the right side. Since the numbers of dendritic crossings from the Sholl analysis were less for Mauthner cells in which the ear was removed and immediately replaced than for Mauthner cells from control animals for shorter distances from the soma, we conclude the effect of ear removal itself has a potential effect on dendritic morphology rather than on the total number of dendritic branches. Perhaps the slightly different morphology was due to a short delay in the arrival of sensory afferents from ears that were removed and immediately replaced as compared to sensory neuron afferents from control ears, although this has not been confirmed. It should be noted that our stages were too young to investigate the already described effect of otocyst removal on the ventral dendrites that seem to expand as the lateral dendrite shrinks (Fritzsche, 1990). Moreover, our whole mount preparation makes such an analysis in the Z-axis cumbersome even with the added reduction in focal plane due to the confocality of our laser scanning microscope. To evaluate those features, sections are needed which may not allow us to fully analyze the lateral dendrites due to partial loss in sections.

In all cases in which the ear was removed and not replaced, the dendritic branching on the ablated side was always less than the dendritic branching on the unoperated side. Furthermore, in all cases in which an extra ear was transplanted rostral to the native ear, there was increased dendritic branching on the side with an extra ear compared with the unoperated side. Taken together, these quantitative data suggest that the degree of dendritic branching is related to the number of presynaptic sensory afferents, consistent with earlier exclusively qualitative findings (Kimmel et al., 1977; Kimmel, 1982; Goodman and Model, 1988; Fritzsche, 1990). Our study investigates not only the effect of ear removal in general on dendritic branching but also the effect of the specific timing of ear removal. The earlier the

ear was removed, the fewer dendritic branches were present, suggesting that the degree of dendritic development depends on the time at which the ear was removed. While there was no significant difference between the three stages of ear removal when comparing the difference between left and right Mauthner cells following ear removal, there was a trend in which the number of dendritic branches was slightly higher the later the ear was removed. Since the otic ganglion is only recognized after Stage 31 (Nieuwkoop and Faber, 1994), the slightly more dendritic branches in the earlier otic vesicle stages (Stages 27–30) than in the otic placode stages (Stages 24–26) may indicate that there are other mechanisms for dendritic development in addition to direct innervation from the presynaptic sensory afferents. That any dendrites developed in these two groups in the complete absence of any innervation could support additional mechanisms such as short range diffusible factors released from the ear or alternatively suggest that there is some autonomous development of dendritic branches in the direction of the future sensory neurons, but that contacts between the dendrites and inner ear sensory neurons are necessary for further development and maintenance of the dendritic branches, consistent with previous suggestions (Fritsch, 1990). Follow up work on older tadpoles is needed to understand the impact of the lack or increase of dendritic growth on vestibular compensation (Lambert and Straka, 2012; Peusner et al., 2012; Lambert et al., 2013). It should be noted that most vestibular compensation experiments have dealt with animals having proprioceptive and visual inputs to aid in the adjustment (van der Kooij and Peterka, 2011). In contrast, our tadpoles have no limbs and thus have relied primarily on visual-vestibular interactions for compensation. We are currently testing ways to accelerate compensation with appropriate stimulation.

The increase in dendritic branching following the addition of an extra ear rostral to the native ear was also suggested in axolotls (Goodman and Model, 1988), although not quantified. An increase in dendritic branching indicates that additional sensory neurons entering the hindbrain from the transplanted ear stimulate the growth of additional dendritic branches. That the number of dendritic branches on the side with the extra ear was not double the number on the control side suggests that an upper limit of dendritic branching may occur. This could be tested by grafting additional ears to a single side or by manipulating the ear molecularly (Fritsch et al., 2015) to increase the number of viable neurons.

The data presented here provide the foundation for future work investigating the molecular basis of dendritic growth and atrophy. We provide evidence that vestibular afferent fibers have a role in the stimulation of dendritic development of a second order neuron, the Mauthner cell. Observations that BDNF is in vestibular hair cells in frogs and other vertebrates (Cristobal et al., 2002; Fritsch et al., 2004; Hallböök et al., 2006), and TrkB receptors are expressed in the sensory neurons of the ear and the region of the hind-brain where the Mauthner cells are located (Panagiotaki et al., 2010), might suggest a role for BDNF in dendritic growth and merit further exploration through effects of BDNF or TrkB knockdown or CRISPR-mediated gene deletion. Beyond that, our model will allow for correlations of alterations in gene expression profiles with increase or decrease in dendritic growth and, ultimately, establish causality beyond the correlative and probabilistic evidence provided by past research using cohorts of neurons instead of single cells.

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Figure 1.

Success of ear removal. (a) Percentage of animals with any form of ear regrowth at each stage of ear removal. (b) Control *X. laevis* at Stage 46. (c) Embryo in which the right ear was removed and replaced. (d) Embryo in which the right ear was removed. (d') Immunohistochemistry for acetylated tubulin showing cranial nerves (roman numerals) and myoVI showing the absence of the ear on the right side as indicated by the absence of hair cells. (e) Embryo in which an additional ear was added rostral to the native right ear. (e') Lipophilic dye labeling showing sensory neuron projections from both the native (red) and transplanted (green) ears into the vestibular nucleus in the hindbrain. Native ears are circled

in black and labeled, Ear. Transplanted ear is circled in white. Scale bar is 0.5 mm in b–d and f; 200 μm in e; 25 μm in g.

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Figure 2.

Mauthner cell survival. (a) Dextran amine dye-labeled Mauthner cells in a control animal. (b) Dextran amine dye-labeled Mauthner cells in an animal in which the right ear was removed and immediately replaced. (c) Percentage of Mauthner cells present on the right side at the different stages of ear removal. (d) Dextran amine dye-labeled and (d') 3A10 antibody immunohistochemistry showing the absence of the ipsilateral Mauthner cell following the removal of the right ear at Stage 28. (d'') Merge of d and d'. (e) Higher magnification image showing bilateral Mauthner cell axon crossing in a control animal. (f) Higher magnification image showing bilateral Mauthner cell axon crossing in an animal in which the ear was removed and immediately replaced. (g) Higher magnification image showing only unilateral Mauthner cell axon crossing in an animal in which the right ear was removed at Stage 27. M, Mauthner cell; arrowheads indicate the crossing of two Mauthner cell axons. Scale bar is 50 μm.

Figure 3.

Dendritic development of Mauthner cells following ear manipulation. (a) Dextran amine dye labeling and (a') 3A10 immunohistochemistry of a Mauthner cell (M) showing filling of dendrites by dextran amine dye. (a'') Merge of a and a'. (b) 3D reconstruction of a pair of Mauthner cells from a control embryo. (c) 3D reconstruction of a pair of Mauthner cells in which the right ear was removed and immediately replaced show little difference in the number of dendritic branches between Mauthner cells. (d) 3D reconstruction of a pair of Mauthner cells from an animal in which the right ear was removed at Stage 26 show a reduction in dendritic branching in the ipsilateral Mauthner cell. (e) 3D reconstruction of a pair of Mauthner cells from an animal in which an additional ear was transplanted rostral to the native ear at Stage 26 show an increase in dendritic branching in the ipsilateral Mauthner cell. (f) Number of dendritic branches following ear removal or ear addition. Dark shaded bars are left (control) Mauthner cells, light shaded bars are right (treated) Mauthner cells.

***, $p < 0.001$. (g) Median differences between left and right Mauthner cells following ear removal or ear addition. **represent significant difference from control, $p < 0.005$. (h) Sholl Analysis of the Mauthner cells on the right (treated) side in f. The number of dendritic branch crossings were counted at 25 μm intervals. Scale bar is 50 μm . Error bars are standard errors of the means.

Figure 4.

Comparison of Mauthner cell dendritic branching following ear manipulation. Overlay of three right Mauthner cells from a control animal (yellow), an animal in which the ear was removed (magenta), and an animal with an extra ear (green). The number of dendritic branches (indicated in parenthesis) for the three representative Mauthner cells is approximately equal to the mean number of dendritic branches for each group as revealed in Fig. 3.