Coexpression of auxiliary $K_{\beta 2}$ subunits with $K_{\beta 1.1}$ channels is required for developmental acquisition of unique firing properties of zebrafish Mauthner cells

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Zebrafish Mauthner (M) cells, a pair of giant reticulospinal neurons (RSNs) located at the fourth segment of the teleost hindbrain, are bilaterally paired neurons that are required for controlling behavior. The single-spiking property of M cells contrasts with the repetitive firing of their two paired RSNs, MiD2cm and MiD3cm, which are located in the fifth and sixth hindbrain segments, respectively, and which generate repetitive APs at frequencies depending on input intensity (Nakayama and Oda 2004). Because of their similar development and morphology (Hanneman et al. 1988; Mendelson 1986; Metcalfe et al. 1986), these three paired neurons are collectively called the “Mauthner (M) series” (Lee and Eaton 1991). Because the M-series neurons receive auditory inputs (Nakayama and Oda 2004; Szabo et al. 2007), they may code the onset and intensity of sound, respectively, like central auditory neurons in higher vertebrates (Oertel 1999). In this study we focused on how the single-spiking property of M cells is acquired among the homologous neurons and its underlying molecular mechanisms.

The single-spiking property of the auditory neurons in avian and mammalian vertebrates requires low-threshold $K^+$ currents, which are blocked by dandetroxin-I (DTX) (Brew and Forsythe 1995; Fukui and Ohmori 2004). A previous study in adult goldfish demonstrated that M-cell intrinsic excitability is attributable to DTX-sensitive $K^+$ currents (Nakayama and Oda 2004). DTX-sensitive voltage-gated $K^+$ ($K_v$) channels are formed by homo- or heterotetramers of $K_\alpha$-subunits (Gutman et al. 2005; Johnston et al. 2010) containing at least one of $K_{\beta 1.1}$, $K_{\beta 1.2}$, or $K_{\beta 1.6}$ (Harvey 2001). Therefore, it can be asked whether a unique expression of $K_\alpha$ channels underlies the single-spiking property. Furthermore, the gating threshold, kinetics, targeting, or trafficking of the channels can be modified by associative expression of auxiliary $\beta$-subunits ($K_{\beta 1}$, $K_{\beta 2}$, and $K_{\beta 3}$) (Pongs and Schwarz 2010). This possibility has not been clarified in any single-spiking neurons.

In the present study, in vivo whole cell recordings of zebrafish embryos and larval zebrafish revealed that $K_{\beta 2}$ expression in larval zebrafish larvae reveal that $K_{\beta 1.1}$-mediated currents enhanced by $K_{\beta 2}$
subunits are essential for the single-spiking property and that the delayed expression of $K_{\beta2}$ is a crucial step in the acquisition of this unique M-cell trait.

**MATERIALS AND METHODS**

**Animals.** Wild-type zebrafish (*Danio rerio*) and transgenic strains *Tol-026* and *Tol-056*, both of which express green fluorescent protein (GFP) in M cells (and in the case of *Tol-026* in some other RSNs) (Kohashi et al. 2012; Satou et al. 2009; Tanimoto et al. 2009), were reared at 28.5°C and staged according to standard procedures. All procedures were carried out in compliance with protocols approved by the Animal Care and Use Committee of Nagoya University.

**In vivo whole cell recording.** *Tol-026* and *Tol-056* strains were used for 28–60 h postfertilization (hpf) [1–2 days postfertilization (dpf)] and for >60 hpf (2–6 dpf), respectively, to visually guide the electrophysiological approach to the M cells and the homologs. Wild-type fish, retrogradely labeled with Oregon Green 488 dextran (GFP) in M cells (and in the case of other RSNs) (Kohashi et al. 2012; Satou et al. 2009; Tanimoto et al. 2009), were identified by a blast search of a zebrafish genomic database (Zv9) using protein sequences of their mammalian counterparts. Because of the ancestral genomic duplication, we found two paralogs of $K_{\alpha1.1}$, $K_{\alpha1.2}$, $K_{\beta1}$, $K_{\beta2}$, and $K_{\beta3}$ and distinguished each of them as “a” and “b” [i.e., $zKv1.1a$ (*kcnal4*) and $zKv1.1b$ (*kcnalb*)]. We performed polymerase chain reaction after reverse transcription (RT-PCR) using total RNA extracted from embryos (2 dpf) or larvae (5 dpf) and then cloned full-length cDNA in pGEM-T-easy vector (Promega) or pCR4Blunt-TOPO vector (Life Technologies). After sequencing of cDNA, the amino acid sequences were aligned across vertebrates to construct the phylogenetic trees (Chastal W method). The primers for the cloning of each gene are listed in Table 1.

**In situ hybridization.** DIG-labeled antisense RNA probes were synthesized by in vitro transcription with linearized plasmids containing full-length cDNA described above, followed by alkaline hydrolysis at 70°C for 10 min to generate 0.5- to 1.0-kb probes, facilitating probe penetration into the brain tissue.

Whole mount in situ hybridization followed by immunostaining was carried out with 2 dpf embryos. Wild-type embryos were raised in 0.003% (5 mM) 2-phenylthiourea (Sigma-Aldrich)-containing fish tank water after 24 hpf to prevent pigmentation. Embryos were fixed at 48–54 hpf in 4% (wt/vol) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 28.5°C for 8 h and treated with methanol. After proteinase K treatment (10 μg/ml; Roche) for 40–60 min at room temperature, embryos were refixed and hybridized with RNA probes at 65°C for 12–16 h. After washout of excess probes at 65°C, embryos were incubated with horseradish peroxidase-conjugated anti-DIG antibody (1:2,000; Roche) and monoclonal 3A10 antibody (M-cell marker, 1:50; Developmental Studies Hybridoma Bank) for 12–16 h at 4°C. RNA probes were detected with a TSA kit with Alexa Fluor 488 (Life Technologies) according to the manufacturer’s instructions, while M cells were visualized after incubation with antimouse IgG secondary antibody conjugated with Alexa Fluor 568 (1:2,000; Roche). After removal of the yolk, the dorsal images of whole-mounted embryos were obtained with confocal microscopy (FV300; Olympus).

For 5 dpf larvae, in situ hybridization was performed on larval brain sections. Injection of 10% (wt/vol) Alexa Fluor 568-fixable dextran (molecular weight 10,000; Life Technologies) into the caudal spinal cord of wild-type larvae at 4 dpf retrogradely labeled RSNs, and they were fixed in 4% PFA-PBS at 120–125 hpf. After methanol treatment, larvae were incubated in 30% sucrose for 1 h and embedded in Tissue-Teq O.C.T. compound (Sakura). A cryostat (Leica CM1850) was used to create 20-μm-thick horizontal sections of larvae, which were treated with proteinase K (5 μg/ml) for 15 min, followed by rinsation for 20 min. Sections were then processed with the same protocol as the whole mount in situ hybridization.

**Two-electrode voltage-clamp recording in Xenopus oocyte.** In vitro transcription of full-length cRNA from linearized plasmid was performed with a mMESSAGE mMACHINE Kit (Life Technologies). *Xenopus laevis* oocytes were collected and treated as previously described (Hopkins et al. 1994) and injected with 46 nl of cRNA (250 ng/ml) with a Drummond microinjector. The volume ratio of $zKv1.1a$ and $zKv1.2b$ cRNA solution was 1:5. K⁺ currents were recorded with two-electrode voltage clamp using an Axoclamp 2B amplifier (Molecular Devices). Microelectrode resistance filled with 3 M KCl ranged from 0.5 to 1.0 MΩ. Oocytes were perfused in the chamber during recording with a bath solution containing (in mM) 96 NaMeSO₄, 2 KCl, 1 MgCl₂, 1 CaCl₂, and 5 HEPES, adjusted to pH 7.6 with NaOH. Niflumic acid (0.3 mM) was added to the bath solution to block the endogenous chloride current (Hopkins et al. 1994). Transient capacitance and linear leakage currents were subtracted by a P4 procedure. A standard Boltzmann equation ($G = G_{\text{max}}/[1 + \exp(V_{1/2} - V_k)]$, where $V_{1/2}$ is the voltage of half-maximal activation and $k$ is the slope factor) was fitted to the conductance-voltage plots. Data were analyzed and fitted with Clampfit 10 software (Molecular Devices).
### Table 1. RT-PCR primers used to amplify DTX-sensitive K*vr1.1* and K*vr1.2* in and out of zebrafish

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<th>Gene</th>
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<th>Reverse Primer</th>
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**kcnab2b knockdown.** The sequences of antisense morpholino oligonucleotides (MOs) synthesized by Gene Tools are as follows: kcnab2bMO: 5’-TTTTTCCGAGGTTCCTAAACACAG-3’; kcnab2bMO2: 5’-GAGCGTCTGGTCCTCCTCATCAGA-3’; CtrlMO: 5’-CTCTAACCTCAGTTACATTTATA-3’. kcnab2bMO and kcnab2bMO2 were designed against the splicing acceptor of the third exon and 5’-UTR before the start of kcnab2b (−44 to −20), respectively. Standard control MO (CtrlMO, Gene Tools) was used as a control. Embryos were injected with 5–10 ng of MO at 1–2 cell stages as described previously (Nasevicius and Ekker 2000). The efficiency of knockdown using kcnab2bMO in each larva was examined by RT-PCR using two primers (primer 1: 5’-TCTCCA-CACGCAGAGTTG-3’; primer 2: 5’-ACGCGTTCTCATACTGCCC-3’) and was detected as a splicing defect.

### Statistical analysis
Developmental changes in M-cell firing properties were analyzed by using a Kruskal-Wallis test with post hoc Steel-Dwass test for multiple comparisons. Slopes of regression properties were analyzed by using a Kruskal-Wallis with post hoc Steel-Dwass test for multiple comparisons. A paired Student’s t-test was used to compare two independent data sets. A Mann-Whitney U-test was used to compare two independent data sets. A paired Student’s t-test was used to compare the effects of DTX treatment in the same M cell. All data are represented as means ± SE.

## RESULTS

**M cells acquire single-spiking property during larval development.** In zebrafish, a pair of M cells are formed as early as 7.5 hpf and are maintained to adulthood (Mendelson 1986). To investigate the development of M-cell excitability, we examined the firing elicited by step currents injected into embryonic and larval M cells, using an in vivo whole cell patch-clamp technique (Fig. 1, A and B). We focused on intrinsic neuronal excitability by pharmacologically blocking recurrent inhibition and other synaptic inputs (Furukawa and Furshpan 1963; Nakayama and Oda 2004). At 1 dpf (28–39 hpf), the M cells repetitively generated APs with a slight adaptation in frequency above the threshold current (T) (n = 21; Fig. 1C). This repetitive firing was promoted until 2 dpf (50–63 hpf, n = 27) with an increase in the firing frequency. The number of APs during 100-ms depolarization by a 2T current increased from 9.8 ± 0.8 APs at 1 dpf to 14.3 ± 1.2 APs at 2 dpf (P < 0.05, Steel-Dwass test; Fig. 1, D and E). However, between 2 and 3 dpf (74–90 hpf, n = 21) the M-cell firing became less repetitive (2T: 6.0 ± 0.6 APs, P < 0.01, Steel-Dwass test), and it changed into a typical single AP at 4 dpf (100–166 hpf, n = 31; 1.5T: 1.2 ± 0.1 APs; 2T: 2.6 ± 0.2 APs). The firing rate of the M cells at 4 dpf was comparable to that of mature M cells of adult goldfish (Nakayama and Oda 2004). Thus the firing property of the M cells developed from a tonic mode of firing at 1 dpf to phasic bursting at 2–3 dpf and finally to unitary AP discharge at 4 dpf. The threshold current required for generating an AP in the M cells progressively increased during early development (Fig. 1F). It also accompanied the reduction of passive input resistance (Fig. 1G) and the increase of membrane capacitance (Fig. 1H) as well as the morphological maturation of the M cells (Fig. 1A).

In addition to the change in firing rate, we found that the latency from the step-depolarization onset to AP initiation decreased and the AP waveform became narrow. The decrease in the AP onset latency (Fig. 1, I and J) with decrease in jitter (Fig. 1, I and K) may be attributed to a smaller time constant of M cells and a higher rate of membrane potential change (dV/dt), which was indicated by a steeper pre-AP slope (Fig. 1, L and M) defined as dV/dt at the inflexion point prior to AP.
initiation (Li et al. 2011). Because the depolarizing phase before spike generation is strongly regulated by voltage-gated conductance, these data suggest that the development of active membrane properties also refines the temporal precision of the spike onset in M cells. The AP waveform developed from wide to narrow as follows (Fig. 2, A–C): AP amplitude, the voltage difference from threshold potential to the AP peak, gradually increased until 2 dpf and slightly decreased at 4 dpf (Fig. 2, A and B). AP half-width, the duration at AP half-amplitude, decreased until 4 dpf (Fig. 2, A and C) in association with an increase in the maximum rate of rise and fall of the AP (Fig. 2, D and E). Moreover, the threshold potential for AP initiation and the afterhyperpolarization peak shifted to more hyperpolarized levels until 2 dpf without a change in the resting membrane potential through development (Fig. 2F).

In contrast to M cells, MiD2cm and MiD3cm retained the tonic firing property with the frequency proportional to the amplitude of the injected depolarizing current, as shown in adult goldfish (Nakayama and Oda 2004). MiD2cm and MiD3cm increased the frequency of the firing elicited by suprathreshold currents during larval development between 2 and 4–6 dpf (MiD2cm, $P < 0.01$; MiD3cm, $P < 0.05$, ANCOVA; Fig. 3).

Taken together, these results demonstrated that zebrafish M-cell firing properties developed through the following three stages: immature repetitive firing at 1 dpf, phasic bursting at 2–3 dpf, and single spiking at 4 dpf. After 2 dpf the M cell reduced the bursting frequency and the spike onset latency and increased the threshold current for spiking, and finally generated a single or at most two APs only at depolarization by 4 dpf, which makes the M cell functionally different from the M-cell homologs.

Developmental increase in DTX-sensitive low-threshold $K^+$ currents is required for single-spiking property of M cells. We next investigated the molecular basis of the developmental acquisition of the single-spiking property of M cells. By application of DTX, a blocker to assess the low-threshold voltage-gated $K^+$ channels, the single-spiking M cell at 4–6 dpf...
dpf was altered to exhibit phasic bursting as shown in adult goldfish (Nakayama and Oda 2004) (Fig. 4A). The number of APs elicited by suprathreshold currents at 1.5T or 2T increased significantly after DTX treatment (P < 0.01, paired Student’s t-test; Fig. 4C, Table 2). The threshold current decreased simultaneously, the spike onset latency and jitter increased, and the pre-AP slope decreased (each P < 0.01, paired Student’s t-test; Fig. 4A, B and C, Table 2). It should be noted that the firing frequencies at 1.5T and 2T after DTX treatment were similar to that at 3 dpf (P > 0.05, Mann-Whitney U-test) but slightly different from that at 2 dpf (P < 0.01, Mann-Whitney U-test; see DISCUSSION). In contrast, DTX did not significantly affect the AP waveform (Table 2). Thus DTX-sensitive currents are required to suppress the bursting activity, to increase threshold currents, and to reduce spike onset latency without changing the AP waveform. In contrast, the repetitive firing of M cells at 2 dpf was almost unchanged by DTX treatment (n = 7; Fig. 4D, Table 2), which indicated no significant change in the firing frequency and the spike onset timing (Fig. 4, D–F), whereas a small, yet significant reduction in threshold current was observed (P < 0.05, paired Student’s t-test; Fig. 4F).

Fig. 2. AP waveform of M cells during development. A: APs elicited by threshold current at each developmental stage are superimposed (magenta, 1 dpf; red, 2 dpf; green, 3 dpf; blue, 4 dpf). Dashed lines show half-width at AP half-amplitude. Inset: APs including afterhyperpolarization. B: AP amplitude increased between 1 and 2 dpf but moderately decreased between 3 and 4 dpf as shown in A. C: half-width significantly decreased from 1 to 2 dpf and slightly between 3 and 4 dpf. D: phase-plane plot, dV/dt vs. membrane potential (V_m), for a single AP of each developmental stage. E: maximum rate of rise and fall of an AP increased during development. F: threshold potential for spiking and antipeak of afterhyperpolarization shifted to more hyperpolarized levels, whereas the resting membrane potential was relatively stable (1–6 dpf: −84.2 ± 0.5 mV, P = 0.88, Kruskal-Wallis test). n = 21, 27, 21, 14, 17 at 1, 2, 3, 4, 5–6 dpf, respectively. *P < 0.05, **P < 0.01, Steel-Dwass test.

Fig. 3. Firing patterns of M-cell homologs MiD2cm and MiD3cm. A: representative firing responses of MiD2cm (left) or MiD3cm (right) at 2 and 4 or 6 dpf (top) elicited by step current injection with different intensities (1T, 1.5T, 2T) (bottom). MiD2cm and MiD3cm fired upon injection of much smaller threshold currents denoted above the traces and produced regular spiking with frequencies proportional to the injected current intensity. B: linear relationship between injected current intensity represented as times of threshold current (T) and the mean number of APs during 100 ms of depolarization in MiD2cm and MiD3cm at 2 dpf and 4–6 dpf. Slopes became steeper during larval development. *P < 0.05, **P < 0.01, ANCOVA. MiD2cm: 50–64 hpf, n = 7, 103–126 hpf, n = 9; MiD3cm: 52–60 hpf, n = 7, 99–152 hpf, n = 12.
Therefore, we hypothesized that the DTX-sensitive currents in zebrafish M cells were enhanced during larval development. To test this possibility, we measured voltage-dependent outward currents under a voltage-clamp configuration in the presence of a voltage-gated Na+ channel blocker tetrodotoxin (1 μM) (Fig. 5). Step depolarization above −70 mV elicited sustained currents (I_sustain in Fig. 5) with initial transient currents above −50 mV (2 df, n = 10, 4–6 df, n = 7; Fig. 5, A, top, and B). The initial transient currents were presumably a rapid-activating and fast-inactivating K+ current called the A-type current (I_A), evoked additionally to sustained currents. The sustained low-threshold currents increased in amplitude between 2 and 4–6 df (at −65, −55, −45 mV, P < 0.01, Mann-Whitney U-test; Fig. 5C). As expected, DTX significantly suppressed the sustained but not the initial transient outward currents (2 df, n = 5, 4–6 df, n = 4; Fig. 5, A, middle, and C). Furthermore, DTX-sensitive low-threshold currents, which are obtained by subtraction of currents between before and after DTX treatment, were elevated during larval development (2 df, 0.5 ± 0.2 nA; 4–6 df, 1.8 ± 0.3 nA at −55 mV, P < 0.05, Mann-Whitney U-test; Fig. 5, A, bottom, and C).

DTX-sensitive K_\alpha channel zkV1.1a α-subunit is expressed in repetitive-firing M cells and homologous neurons from an early developmental stage. The nanomolar range of DTX as used in this study blocks the low-threshold K+ channels comprising K_\alpha1.1, K_\alpha1.2, or K_\alpha1.6 α-subunits (Harvey 2001). We assessed their expression in developing M cells. In accordance with the ancestral genomic duplication in teleosts (Amores et al. 1998), there were six DTX-sensitive K_\alpha α-subunit genes, kenα1a, kenα1b, kenα2a, kenα2b, kenα6a, and kenα6b, in the zebrafish database that encode counterparts of mammalian K_\alpha1.1, K_\alpha1.2, and K_\alpha1.6, respectively (Fig. 6A). In situ hybridization showed that among these genes only kenα1a mRNA was expressed in M cells (Fig. 6, B and C). The zKv1.1a channel expressed in Xenopus oocytes by injection of kenα1a mRNA showed sustained outward currents with short rise time at command voltages above −60 mV (n = 40; see Fig. 8A), which were suppressed by 100 nM DTX (data not shown). These results confirmed that zebrafish K_\alpha1.1 α-subunits function as DTX-sensitive low-voltage-activated channels.

Therefore, we speculated that kenα1a mRNA is expressed by single-spiking mature M cells but not by repetitive-firing early M cells. However, our temporal expression analysis revealed that kenα1a mRNA was expressed at both stages. In addition, kenα1a mRNA was unexpectedly expressed in the M-cell homologs, MiD2cm and MiD3cm (Fig. 6B), both of which show repetitive firing (Fig. 3). Therefore, the expression of kenα1a mRNA alone does not account for either the differ-

**Table 2. Effects of DTX treatment on M cells**

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<th>4–6 df (n = 7)</th>
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<td>At 2T</td>
</tr>
<tr>
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<tr>
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<tr>
<td>Latency, ms</td>
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<td>Amplitude, mV</td>
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<td>Half-Width, ms</td>
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<td>0.49 ± 0.04</td>
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Values are means ± SE. A comparison of the parameters of M-cell firing in DTX experiments is shown. T, threshold current; AP, action potential; df, days postfertilization. *P < 0.05, **P < 0.01, paired Student’s t-test.

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M cell-specific and delayed expression of auxiliary β-subunit zKvβ2b among M-series cells during late developmental stage. It is known that Kv channel function is modified by auxiliary K,β subunits (Pongs and Schwarz 2010). Hence, we next assessed the expression to identify molecules regulating Kv currents in RSNs. We found zebrafish genes encoding two K,β1 (kcnab1a and kcnab1b), two K,β2 (kcnab2a and kcnab2b), and one K,β3 (kcnab3) (Fig. 7A). In situ hybridization revealed that among the five transcripts only kcnab2b mRNA was expressed in the M cells. It is noteworthy that kcnab2b mRNA was expressed at 5 dpf but not at 2 dpf (Fig. 7, B and C). Furthermore, neither MiD2cm nor MiD3cm cells expressed kcnab2b mRNA (Fig. 7B). These data suggest that zKvβ2b, encoded by kcnab2b, may be the critical determinant that enables the M cell-specific acquisition of the single-spiking property.

To determine whether zKvβ2b actually influences zKv1.1a channel function, we coexpressed these proteins in Xenopus oocytes and measured Kv currents, using a voltage-clamp configuration. Coexpression of zKvβ2b with zKv1.1a doubled the amplitude of the sustained currents elicited by step depolarization compared with oocytes expressing only zKv1.1a (n = 34; Fig. 8, A and B). The threshold for activation and open probability of the channels, which are estimated from fitting parameters of conductance-voltage plots to a Boltzmann equation, were almost unchanged because there was no difference in half-activation voltage (V1/2): zKv1.1a, 39.2 ± 0.5 mV; +zKvβ2b, 39.6 ± 0.8 mV, P = 0.95, Mann-Whitney U-test) with only a slight difference in the slope values (k: zKv1.1a, 7.5 ± 0.2 mV; +zKvβ2b, 9.0 ± 0.3 mV, P < 0.01, Mann-Whitney U-test; Fig. 8C). These data show that zKvβ2b subunits enhance the zKv1.1a-mediated current and suggest that zKvβ2b contributes to repression of the firing by M cells.

Expression of zKvβ2b is required for developmental acquisition of single-spiking property of M cells. Finally, to test the possibility that coexpression of zKvβ2b and zKv1.1a after 2 dpf is a key feature of the unique excitability of M cells, we examined the effects of knockdown of expression levels of zKvβ2b protein using an antisense MO, kcnab2bMO, designed against the splice-acceptor site of exon 3 (Fig. 9A). RT-PCR analysis showed that the injection of kcnab2bMO resulted in a frame shift on kcnab2b mRNA caused by skipping exons 3 and 4 because of aberrant splicing (Fig. 9, B and C) (Eisen and Smith 2008), suggesting that nonfunctional proteins were expressed in kcnab2bMO-injected larvae instead of zKvβ2b proteins, at least until 4 dpf. The M cells in the larvae injected with kcnab2bMO exhibited phasic bursting with long spike onset latency at 4 dpf (100–111 hpf, n = 12 cells/9 fish; Fig. 9, D and E), similar to DTX-treated M cells at 4–6 dpf (Fig. 4A). The control MO (CtrlMO)-injected larvae (100–105 hpf, n = 6 cells/6 fish) showed no change in M-cell excitability (Fig. 9, D and E). The kcnab2bMO treatment significantly increased the number of APs elicited by step depolarization and spike onset latency (no. of APs at 1.5T, CtrlMO: 1.2 ± 0.2 APs, kcnab2bMO: 2.4 ± 0.3 APs; onset latency, CtrlMO: 2.5 ± 0.2 ms, kcnab2bMO: 3.3 ± 0.2 ms; each P < 0.01, Mann-Whitney U-test). The same results were obtained by injecting kcnab2bMO2, which was designed against 5′-UTR to inhibit translation (103–110 hpf, n = 10 cells/7 fish; data not shown). Moreover, low-threshold sustained outward currents of the M cell at 4 dpf decreased in kcnab2bMO-injected larvae compared with that of the M cell injected with CtrlMO (CtrlMO: 104–110 hpf, n = 8 cells/5 fish, 3.0 ± 0.5 nA; kcnab2bMO: 102–111 hpf, n = 13 cells/10 fish, 1.5 ± 0.3 nA at −55 mV, P < 0.01, Mann-Whitney U-test; Fig. 10). Thus
these results confirmed that the expression of zKvβ2b is required for developmental acquisition of the single-spiking property of M cells associated with the enhancement of low-threshold outward currents.

Taken together, these results revealed that the developmental expression of zKvβ2b subunits after 2 dpf enhances zKv1.1α-mediated currents specifically in M cells, but not in the homologs, resulting in enhanced suppression of bursting to acquire the single-spiking property of M cells at 4 dpf.

DISCUSSION

The present study demonstrates the developmental change in firing patterns and provides molecular basis underlying this process in zebrafish M cells. M cells develop firing properties from immature tonic firing at 1 dpf to phasic bursting at 2 dpf, and finally at 4 dpf acquire the single-spiking mode, which is in contrast to the M-cell homologs, MiD2cm and MiD3cm. During the first 2 dpf, both M cells and their homologs express zKv1.1α subunits. However, after 2 dpf an auxiliary β-subunit, zKvβ2b, is expressed in larval M cells but not in the homologs. In parallel with this, M cells burst more phasically with shorter duration at the onset of depolarization. Coexpression of zKvβ2b and zKv1.1α subunits enhances zKv1.1α-mediated low-voltage-gated currents in M cells, which is essential for the acquisition of the unique single-spiking property in M cells by 4 dpf. These results implicate the common expression of K1.1 as a feature of the ancestral “original” neurons, while the M cell-specific and delayed expression of K1.2 may be a factor in the differentiation of firing properties.

Kv1.1 channels required for single spiking. Single-spiking neurons in the brain stems of avian and mammalian species exhibit DTX-sensitive low-threshold K+ currents, which are mediated by heteromeric channel complexes comprising K1.1 with K1.2 or K1.6 α-subunits (Dodson et al. 2002; Fukui and Ohmori 2004). The medial nucleus of trapezoid body neurons in Kcnal-null mice exhibits defects in temporal precision of spiking and increased firing frequency (Brew et al. 2003; Gittelman and Tempel 2006). A recent study in zebrafish showed that K1.1 channels are expressed in M cells and may contribute to the firing (Brewster and Ali 2013). Here, we revealed that M cells acquire the single-spiking property during development by functionally differentiating from bursting neurons as their homologs and clarified the role of K1.2 on K1.1 channels in producing the single-spiking property. When the M cell fires, K1.1 channels are opened just above the resting membrane potential. The outward currents suppress postsynaptic depolarization and reduce membrane resistance, resulting in elevating threshold currents for firing and simultaneously decreasing the time constant of depolarization before spiking. The rapid activation of K1.1 currents may counteract inward currents through voltage-gated Na+ channels. Thus high Na+ conductance, which overcomes the K+ conductance, leads to a high membrane dV/dt before AP initiation. Finally, the sustained large K+ conductance inhibits the subsequent firing in cooperation with strong recurrent inhibition of the M cell itself, which appears before 4 dpf (Takahashi et al. 2002). These
mechanisms account for single spiking with short onset latency and minimal jitter in response to a large depolarizing input, suggesting that the Kv1.1 currents play a role in determining the threshold and the timing of initiation of fast escape behavior.

We found that kcnal1a mRNA is expressed not only in single-spiking M cells but also in repetitive-firing M cells as well as their repetitive-firing homologs (Fig. 6). In mammals, Kv1.1 channels contribute to the production of fast spiking of GABAergic interneurons in the neocortex (Goldberg et al. 2008; Li et al. 2011) and repetitive firing of pyramidal neurons in the hippocampus (Golding et al. 1999; Smart et al. 1998). Thus a wide range of firing patterns is produced possibly by modulating the conductance, kinetics, or expression of the Kv1.1 channels but not by the gene expression alone. This possibility is indicated in the ventral cochlear and trigeminal nuclei, where low-threshold K⁺ currents in repetitive-firing neurons exhibit a smaller conductance or faster inactivation than those in single-spiking neurons (Catacuzzeno et al. 2008; Rothman and Manis 2003a, 2003b). Conversely, large sustained low-threshold K⁺ currents are required to produce the single-spiking property observed in M cells. Actually, the level of Kv1.1 channel gene expression correlates with the excitability in auditory neurons of mouse and chick (Barnes-Davies et al. 2004; Brew et al. 2003; Fukui and Ohmori 2004; Hoffpaurer et al. 2010). Chick nucleus magnocellularis neurons expressing Kv1.1 at higher density require larger threshold currents to fire and exhibit less firing in response to step depolarization than neurons expressing the subunit at lower density (Fukui and Ohmori 2004). Thus the higher level of Kv1.1 expression may be more effective in inhibition of repetitive firing. In addition, we describe here for the first time the importance of auxiliary β-subunits that may enhance the cell surface expression of Kv1.1 channels.

Coexpression of Kβ2 subunits modifies properties of Kv1.1 channels. The coexpression of the auxiliary Kβ2 subunits with Kv1.1 α-subunits in M cells provides further insights into a role of the β-subunits for development and the establishment of specific neuronal excitability characteristics, particularly in single-spiking neurons. It should be noted that M cells do not express either Kvβ1 or Kvβ3 subunits, which are known to rapidly inactivate Kv1.1 channels in Xenopus oocytes and cultured mammalian cells (Bahring et al. 2004; Rettig et al. 1994). In contrast, zebrafish Kvβ2 subunits do not inactivate but instead enhance the amplitude of Kv1.1 currents (Fig. 8), as do those of rat and Xenopus (Akhtar et al. 1999; Lazaroff et al. 1999). Therefore, the interaction with homomeric Kvβ2 subunits rather than with Kvβ1 or Kvβ3 subunits is likely required for Kv1.1 channels to generate large low-threshold K⁺ currents without any rapid inactivation in M cells. Indeed, the amplitude of DTX-sensitive currents in the M cells increased in parallel with the expression of Kvβ2 subunits. The kcnab2b-knockdown experiments (Fig. 9 and Fig. 10) confirmed that expression of Kv1.1 channels alone is not sufficient to generate the outward currents that effectively suppress M-cell bursting. Consistent with these results, knockdown of Kvβ2 reduces Kv1.1 currents in Xenopus embryos (Pineda et al. 2008), and amygdala neurons in Kcnab2 (Kβ2)-knockout mice exhibit hyperexcitability (Perkowski and Murphy 2011).

The enhancement of Kv1.1 currents explains the effect of DTX treatment on M-cell firing during development as shown above (Fig. 4). However, the DTX-sensitive current density calculated on the basis of membrane capacitance did not show significant increase because the capacitance of the M cells
Fig. 9. Knockdown of kcnab2b expression prevents M cells from acquiring the single-spiking property. A: schematic showing the genomic structure of kcnab2b. Exons are represented as boxes. Antisense morpholino oligonucleotides (MO) were designed against the splicing acceptor site of exon 3 in kcnab2b to block normal splicing (solid lines), causing skipping of exons 3 (44 bp) and 4 (38 bp) (dashed line). Arrows over exons 1 and 5 indicate the location of primers 1 and 2 for RT-PCR. B: normal splicing in wild-type (WT, top) and kcnab2bMO-induced aberrant splicing (MO, bottom) between exons 1 and 5 of kcnab2b. The kcnab2bMO-induced aberrant splicing generates a frame shift resulting in a stop codon in exon 5. C: RT-PCR products obtained from WT (2 dpf) and kcnab2bMO-injected (2 and 4 dpf) larvae confirmed that injection of kcnab2bMO led to aberrant splicing of kcnab2b mRNA (114 bp) instead of normal mRNA (196 bp). D: representative firing responses of the M cells from larvae injected with CtrlMO (n = 6 cells/6 fish at 4 dpf, left) and kcnab2bMO (n = 12 cells/9 fish at 4 dpf, right), elicited by step-depolarizing currents with the same protocol shown in Figs. 1 and 4. M cells from 4 dpf larvae injected with kcnab2bMO exhibited burst firing with a decrease in intensity of threshold current (1T), similar to the burst firing of M cells after DTX treatment. E: superimposed M-cell spikes elicited by threshold currents that were recorded in larvae injected with CtrlMO (left) and kcnab2bMO (right). Onset latency and jitter of the M-cell spiking were aberrant in larvae injected with kcnab2bMO.

measured with the voltage-clamp procedure increased fourfold from 2 to 4–6 dpf, whereas the amplitude of DTX-sensitive currents increased approximately threefold (Fig. 1 and Fig. 5). This may be caused at least partially by the overestimation of M-cell capacitance with the development of club endings of the auditory afferents, which are a type of gap junction-mediated coupling (Pereda et al. 2013; Tuttle et al. 1986), or by an increase in space-clamp errors because of large M-cell dendrites, resulting in the underestimation of the voltage-dependent currents. Thus the enhancement of current density could not be estimated from capacitance measurement on the basis of the spherical model, in which the channels are uniformly distributed. Instead, the enhancement of Kv1.1 currents by coexpression with K,β2 may occur locally in M cells. It has been shown in cultured cells that Kv1.1 current enhancement is mediated by the interaction between the N-terminal T1 domain of Kv1.1 and K,β2 (Accili et al. 1997; Shi et al. 1996; Vacher et al. 2008) and that K,β2 promotes the axonal targeting of Kv1.1, including the axon initial segment (Gu et al. 2006; Vacher et al. 2011). Therefore, this raises the possibility that the cell surface expression of zKv1.1a is enhanced at the axonal region of the M cells when coexpressed with zKvβ2 during development.

Treatment with DTX or knockdown of kcnab2b expression did not cause the excitabilities of the mature M cells to fully revert to those detected at 2 dpf (Fig. 4 and Fig. 9). M cells at 2 dpf exhibited phasic bursting for a longer duration than DTX-treated M cells at 4 dpf. This indicates that DTX-insensitive K+ currents work together with their DTX-sensitive counterparts to reduce excitability at the developed stage (Fig. 5). Further study is required to identify the DTX-insensitive K+ channel molecules and investigate whether they develop after 2 dpf to reduce firing frequency, increase threshold current, and sharpen AP waveform.

Developmental acquisition of unique firing properties of M cells. We demonstrate that zebrafish M cells exhibit three stages of excitability during maturation. This correlated with the development of auditory inputs in the M cells. At the first stage, the M cells showed immature repetitive firing at 1 dpf. At the second stage, M-cell firing changed into phasic bursting by 2 dpf. This occurred nearly synchronously when the M cells become sensitive to sound (Tanimoto et al. 2009). At the third stage, the M cells acquired the single-spiking property between 3 and 4 dpf. It should be noted that this stage is coincident with the time when the inner ear hair cells and/or auditory afferent...
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.W. and Y.O. conception and design of research; T.W., T. Shimazaki, A.M., T. Suzuki, and M.T. performed experiments; T.W. analyzed data; T.W. interpreted results of experiments; T.W. prepared figures; T.W. drafted manuscript; T.W., H.H., M.T., and Y.O. edited and revised manuscript; T.W. and Y.O. approved final version of manuscript.

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