Coexpression of auxiliary \( K_\beta 2 \) subunits with \( K_\gamma 1.1 \) channels is required for developmental acquisition of unique firing properties of zebrafish Mauthner cells

Takaki Watanabe, Takashi Shimazaki, Aoba Mishiro, Takako Suzuki, Hiromi Hirata, Masashi Tanimoto, and Yoichi Oda

Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, Japan; and Center for Frontier Research, National Institute of Genetics, Mishima, Japan

Submitted 22 August 2013; accepted in final form 10 December 2013

Coexpression of auxiliary \( K_\gamma \beta 2 \) subunits with \( K_\gamma 1.1 \) channels is required for developmental acquisition of unique firing properties of zebrafish Mauthner cells. J Neurophysiol 111: 1153–1164, 2014. First published December 11, 2013; doi:10.1152/jn.00596.2013.—Each neuron possesses a unique firing property, which is largely attributed to heterogeneity in the composition of voltage-gated ion channel complexes. Zebrafish Mauthner (M) cells, which are bilaterally paired giant reticulospinal neurons (RSNs) in the hindbrain and induce rapid escape behavior, generate only a single spike at the onset of depolarization. This single spiking is in contrast with the repetitive firing of the M cell’s morphologically homologous RSNs, MiD2cm and MiD3cm, which are also involved in escapes. How the unique firing property of M cells is established and the underlying molecular mechanisms remain unclear. In the present study, we first demonstrated that the single-spiking property of M cells was acquired at 4 days postfertilization (dpf), accompanied by an increase in dendrotoxin-I (DTX)-sensitive low-threshold \( K^+ \) currents, to which the M cell repetitively fires as its homologs. Second, in situ hybridization showed that among DTX-sensitive \( K_1 \) channel \( \alpha \)-subunits, \( zKv1.1a \) was unexpectedly expressed even in the homologs and the bursting M cells at 2 dpf. In contrast, \( zKv\beta 2b \), an auxiliary \( \beta \)-subunit of \( K_1 \) channels, was expressed only in the single-spiking M cells. Third, \( zKv1.1a \) expressed in Xenopus oocytes functioned as a low-threshold \( K^+ \) channel, and its currents were enhanced by coexpression of \( zKv\beta 2b \) subunits. Finally, knockdown of \( zKv\beta 2b \) expression in zebrafish larvae resulted in repetitive firing of M cells at 4 dpf. Taken together, these results suggest that associative expression of \( K_\gamma \beta 2 \) subunits with \( K_\gamma 1.1 \) channels is crucial for developmental acquisition of the unique firing properties of the M cells among homologous neurons.

zebrafish; Mauthner cell; potassium channel; \( K_\gamma 1.1; K_\gamma \beta 2 \)

Neurons exhibit a wide variety of intrinsic firing properties required for information processing in the nervous system. The Mauthner (M) cells, a pair of giant reticulospinal neurons (RSNs) located at the fourth segment of the teleost hindbrain, induce fast escape in response to noxious stimuli such as loud sounds (Burgess and Granato 2007; Kohashi and Oda 2008; Korn and Faber 2005; Zottoli 1977). M cells typically generate only a single action potential (AP) in response to a suprathreshold depolarizing step, and it occurs immediately after the onset of the stimulus (Nakayama and Oda 2004). This intrinsic property, in combination with the recurrent inhibition of the M cell itself, ensures a single AP and plays a central role in triggering the initial phase of escape behavior with minimal latency in goldfish and zebrafish (Furukawa and Furshpan 1963; Kohashi et al. 2012; Koyama et al. 2011; Nissanov et al. 1990; Takahashi et al. 2002). Thus there is a tight link between the unique firing property and the role of the M cell in 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 dendrotoxin-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 \( K^+ \) (\( K_\gamma \) ) channels are formed by homo- or heterotetramers of \( K_1 \) \( \alpha \)-subunits (Gutman et al. 2005; Johnston et al. 2010) containing at least one of \( K_1.1, K_1.2, \) or \( K_1.6 \) (Harvey 2001). Therefore, it can be asked whether a unique expression of \( K_1 \) 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 larvae show that M-cell intrinsic excitability changes from repetitive firing to single spiking during larval development. Expression analysis of \( K_1 \) \( \alpha \)- and \( \beta \)-subunits in single cells, electrophysiological assessment in Xenopus oocytes, and knockdown of \( K_\gamma \beta 2 \) expression in zebrafish larvae reveal that \( K_1.1 \)-mediated currents enhanced by \( K_\gamma \beta 2 \)
subunits are essential for the single-spike property and that the delayed expression of Kβ2 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 (molecular weight 10,000; Life Technologies), were used for the recordings from MDr2cm and MDr3cm at 4–6 dpf. After anesthesia with 0.02% tricaine methanesulfonate (MS-222; Sigma-Aldrich) and immobilization with 1 mM d-tubocurarine (an acetylcholine receptor blocker; Sigma-Aldrich) for ~15 min, embryos or larvae were rinsed and pinned with tungsten wires on a silicon dish filled with extracellular solution containing (in mM) 134 NaCl, 2.9 KCl, 1.2 MgCl2, 2.1 CaCl2, 10 HEPES, and 10 glucose, adjusted to pH 7.8 with NaOH. The head skin was peeled off, and a thin layer of dorsal hindbrain was removed by suction through a glass pipette (diameter >10 μm) to allow access to M cells or M-cell homologs. Whole cell recordings from M cells or homologs were obtained with a MultiClamp 700B amplifier (Molecular Devices) and were collected on a computer using a digitizer (Digitdata 1440A, Molecular Devices) at a sampling rate of 20 or 50 kHz. Patch-clamp electrodes were pulled from borosilicate glass (GD-1.5; Narishige) with a pipette resistance of 3–8 MΩ when filled with extracellular solution containing (in mM) 119 K-glucerate, 6 KCl, 2 MgCl2, 10 HEPES, 10 EGTA, and 4 Na2ATP at 290 mosM and adjusted to pH 7.2 with KOH. The liquid junction potential (15 mV) was calculated and corrected for. The recorded cells were labeled, for identification purposes, with 0.005% Alexa Fluor 594 (Molecular Probes) or pCR4Blunt-TOPO vector (Life Technologies). After preparation, they were fixed in 4% PFA-PBS at 120–125 hpf. After methanol 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 refixation 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 zKvl1a and zKvβ2 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 KC1 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 KC1, 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 = Gmax/[1 + exp(V1/2 – V/k)]) was fitted to the conductance-voltage plots. Data were analyzed and fitted with Clampfit 10 software (Molecular Devices).

Cloning of DTX-sensitive Kα and β-subunits. The nucleotide sequences of zebrafish Kα.1, Kα.2, Kβ.1, Kβ.2, and Kβ.3 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 Kα.1, Kα.2, Kβ.1, and Kβ.2 and distinguished each of them as “a” and “b” [i.e., zKv1.1a (kcnal1u) and zKv1.1b (kcnal2)]. 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 (Clustal W method). The primers for the cloning of each gene are listed in Table 1.

In situ hybridization. Digoxigenin (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.

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 zKvl1a and zKvβ2 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 KC1 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 KC1, 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 = Gmax/[1 + exp(V1/2 – V/k)]) 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 Kᵥ1 subunits

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Zv Access Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>kcna1a</td>
<td>zKv1.1a</td>
<td>ENSDARG00000062942</td>
<td>5'-GGATGACAGTTGTGGCCACGGGTGACC-3'</td>
<td>5'-GCTCGAGATGACAGTTGTGGCCACGGGTGACC-3'</td>
</tr>
<tr>
<td>kcna1b</td>
<td>zKv1.1b</td>
<td>ENSDARG00000017108</td>
<td>5'-GCTATACATCTGTTAGACGCTTCCCC-3'</td>
<td>5'-GCTCGAGATGACAGTTGTGGCCACGGGTGACC-3'</td>
</tr>
<tr>
<td>kcna2b</td>
<td>zKv1.2b</td>
<td>ENSDARG00000002241</td>
<td>5'-GCTATACATCTGTTAGACGCTTCCCC-3'</td>
<td>5'-GCTCGAGATGACAGTTGTGGCCACGGGTGACC-3'</td>
</tr>
<tr>
<td>kcna6a</td>
<td>zKv1.6a</td>
<td>ENSDARG00000046014</td>
<td>5'-GCTCGAGATGACAGTTGTGGCCACGGGTGACC-3'</td>
<td>5'-GCTATACATCTGTTAGACGCTTCCCC-3'</td>
</tr>
<tr>
<td>kcna6b</td>
<td>zKv1.6b</td>
<td>ENSDARG00000091755</td>
<td>5'-GCTATACATCTGTTAGACGCTTCCCC-3'</td>
<td>5'-GCTCGAGATGACAGTTGTGGCCACGGGTGACC-3'</td>
</tr>
<tr>
<td>kcnab1b</td>
<td>zKv1.2b</td>
<td>ENSDARG00000046014</td>
<td>5'-GCTATACATCTGTTAGACGCTTCCCC-3'</td>
<td>5'-GCTCGAGATGACAGTTGTGGCCACGGGTGACC-3'</td>
</tr>
<tr>
<td>kcnab2a</td>
<td>zKv1.2b</td>
<td>ENSDARG00000046014</td>
<td>5'-GCTATACATCTGTTAGACGCTTCCCC-3'</td>
<td>5'-GCTCGAGATGACAGTTGTGGCCACGGGTGACC-3'</td>
</tr>
<tr>
<td>kcnab3</td>
<td>zKv1.2b</td>
<td>ENSDARG00000046014</td>
<td>5'-GCTATACATCTGTTAGACGCTTCCCC-3'</td>
<td>5'-GCTCGAGATGACAGTTGTGGCCACGGGTGACC-3'</td>
</tr>
</tbody>
</table>

kcnah2b knockdown. The sequences of antisense morpholino oligonucleotides (MOs) synthesized by Gene Tools are as follows: kcnah2bMO: 5'-TTTTCTCAGAGTCCCTAAACACACAG-3'; kcnah2bMO2: 5'GACGAGTCGAGTGGCTTCTCAGAGTCCCTAAACACACAG-3'; CtrlMO: 5'-TCTCTACCTAGGTTCAATTTATA-3'. kcnah2bMO and kcnah2bMO2 were designed against the splicing acceptor of the third exon and 5'-UTR before the start codon of kcnah2b (−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 kcnah2bMO in each larva was examined by RT-PCR using two primers (primer 1: 5'-TCTCTACCTAGGTTCAATTTATA-3'; primer 2: 5'-ACGCCGGTTCTTCATACGCG-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 lines were compared by analysis of covariance (ANCOVA). 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. 1A 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 2 T 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. 1D and E). However, between 2 and 3 dpf (74–90 hpf, n = 21) the M-cell firing became less repetitive (2 T: 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
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, n = 7; 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. 4, 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 ($I_{\text{sustain}}$ in Fig. 5) with initial transient currents above −50 mV (2 dpf, $n = 10$, 4–6 dpf, $n = 7$; 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 dpf (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 dpf, $n = 5$, 4–6 dpf, $n = 4$; 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 dpf, 0.5 ± 0.2 nA; 4–6 dpf, 1.8 ± 0.3 nA at −55 mV, $P < 0.05$, Mann-Whitney U-test; Fig. 5, A, bottom, and C).

DTX-sensitive K⁺ 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 Kv1.1, Kv1.2, or Kv1.6 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⁺ α-subunit genes, kcnal1a, kcnal1b, kcnal2a, kcnal2b, kcnal6a, and kcnal6b, in the zebrafish database that encode counterparts of mammalian K⁺,1.1, K⁺,1.2, and K⁺,1.6, respectively (Fig. 6A). In situ hybridization showed that among these genes only kcnal1a mRNA was expressed in M cells (Fig. 6, B and C). The zKv1.1a channel expressed in Xenopus oocytes by injection of kcnal1a 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⁺,1.1 α-subunits function as DTX-sensitive low-voltage-activated channels.

Therefore, we speculated that kcnal1a mRNA is expressed by single-spiking mature M cells but not by repetitive-firing early M cells. However, our temporal expression analysis revealed that kcnal1a mRNA was expressed at both stages. In addition, kcnal1a 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 kcnal1a mRNA alone does not account for either the differ-

Table 2. Effects of DTX treatment on M cells

<table>
<thead>
<tr>
<th>4–6 dpf ($n = 7$)</th>
<th>2 dpf ($n = 7$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of APs</strong></td>
<td><strong>Threshold Current, nA</strong></td>
</tr>
<tr>
<td>At 1.5T</td>
<td>At 2T</td>
</tr>
<tr>
<td>Control</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>+DTX</td>
<td>2.8 ± 0.1 **</td>
</tr>
<tr>
<td>2 dpf ($n = 7$)</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td>Control</td>
<td>6.0 ± 0.9</td>
</tr>
<tr>
<td>+DTX</td>
<td>6.0 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. A comparison of the parameters of M-cell firing in DTX experiments is shown. T, threshold current; AP, action potential; dpf, days postfertilization. *$P < 0.05$, **$P < 0.01$, paired Student’s t-test.
parameters of conductance-voltage plots to a Boltzmann equation, which are estimated from fitting probability of the channels, which are estimated from fitting. The amplitude of sustained currents evoked by the command voltages of 85 mV. DTX-sensitive currents (iDTX) were obtained by subtraction of the currents in the presence of DTX from the control currents. The amplitude of sustained currents evoked by the command voltages of −65, −55, and −45 mV increased during development (2 dpf, n = 10; 4–6 dpf, n = 7). DTX-sensitive currents are enhanced between 2 and 4–6 dpf (DTX 2 dpf, n = 5; DTX 4–6 dpf, n = 4). *P < 0.05, **P < 0.01, Mann-Whitney U-test.

To determine whether zKvβ2b actually influences zKv1.1a-mediated currents and suggest that zKvβ2b contributes to repression of the firing by M cells.

Expression of zKvβ2b is required for developmental acquisition of single-spinging 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 MO-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

Fig. 5. Developmental increase in DTX-sensitive low-threshold K+ currents in M cells. A: representative voltage-dependent currents at 2 dpf (left) and 4–6 dpf (right) of M cells before (top) and after (middle) DTX treatment evoked by step commands from −95 to −45 mV at 10-mV intervals for 500 ms after pre-voltage clamping at −115 mV for 500 ms as shown in B. Sustained currents (iSustain) were elicited by commands above −70 mV. The fast transient current (iT) was observed above −50 mV. Evoked currents were analyzed after subtraction of leak currents (iLeak), which were estimated from the response between −95 and −85 mV. DTX-sensitive currents (bottom) were obtained by subtraction of the currents in the presence of DTX from the control currents. B: command voltages described above. Holding potential was −85 mV. C: current (I)-voltage (V) relationships of sustained currents measured between 200 and 400 ms after the onset of commands: control currents and DTX-sensitive currents at 2 dpf and 4–6 dpf. The amplitude of sustained currents evoked by the command voltages of −65, −55, and −45 mV increased during development (2 dpf, n = 10; 4–6 dpf, n = 7). DTX-sensitive currents are enhanced between 2 and 4–6 dpf (DTX 2 dpf, n = 5; DTX 4–6 dpf, n = 4). *P < 0.05, **P < 0.01, Mann-Whitney U-test.

J Neurophysiol • doi:10.1152/jn.00596.2013 • www.jn.org
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 zKv1.1-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 the M cells by 4 dpf. These results implicate the common expression of K<sub>1.1</sub> as a feature of the ancestral “original” neurons, while the M cell-specific and delayed expression of K<sub>β2</sub> 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 K<sub>1.1</sub> with K<sub>1.2</sub> or K<sub>1.6</sub> 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 K<sub>1.1</sub> 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 K<sub>β2</sub> on K<sub>1.1</sub> channels in producing the single-spiking property. When the M cell fires, K<sub>1.1</sub> 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 K<sub>1.1</sub> 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 \(K_{\text{v}}1.1\) currents play a role in determining the threshold and the timing of initiation of fast escape behavior.

We found that \(kcnal1\) 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, \(K_{\text{v}}1.1\) channels contribute to the production of fast spiking of GABAergic interneurons in the neocortex (Goldberg et al. 1999; Smart et al. 1998) 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 \(K_{\text{v}}1.1\) 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 \(K_{\text{v}}1.1\) 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 \(K_{\text{v}}1.1\) 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 \(K_{\text{v}}1.1\) expression may be more effective in inhibition of repetitive firing. In addition, we describe here for the first time the importance of auxiliary \(\beta\)-subunits that may enhance the cell surface expression of \(K_{\text{v}}1.1\) channels.

Coexpression of \(K_{\beta2}\) subunits modifies properties of \(K_{\text{v}}1.1\) channels. The coexpression of the auxiliary \(K_{\beta2}\) subunits with \(K_{\text{v}}1.1\) \(\alpha\)-subunits in M cells provides further insights into a role of the \(\beta\)-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 \(K_{\beta1}\) or \(K_{\beta3}\) subunits, which are known to rapidly inactivate \(K_{\text{v}}1.1\) channels in \(Xenopus\) oocytes and cultured mammalian cells (Bahring et al. 2004; Rettig et al. 1994). In contrast, zebrafish \(K_{\beta2}\) subunits do not inactivate but instead enhance the amplitude of \(K_{\text{v}}1.1\) currents (Fig. 8), as do those of rat and \(Xenopus\) (Akhtar et al. 1999; Lazaroff et al. 1999). Therefore, the interaction with homomeric \(K_{\beta2}\) subunits rather than with \(K_{\beta1}\) or \(K_{\beta3}\) subunits is likely required for \(K_{\text{v}}1.1\) 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 \(K_{\beta2}\) subunits. The \(kcnab2b\)-knockdown experiments (Fig. 9 and Fig. 10) confirmed that expression of \(K_{\text{v}}1.1\) channels alone is not sufficient to generate the outward currents that effectively suppress M-cell bursting. Consistent with these results, knockdown of \(K_{\beta2}\) reduces \(K_{\text{v}}1.1\) currents in \(Xenopus\) embryos (Pineda et al. 2008), and amygdala neurons in \(Kcnab2b\) (\(K_{\beta2}\))-knockout mice exhibit hyperexcitability (Perkowski and Murphy 2011).

The enhancement of \(K_{\text{v}}1.1\) 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
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). DTX-treatment (Fig. 5), although the transient currents comprising the old 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 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.

![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 (58 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 (196 bp) instead of normal mRNA (114 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.

![Fig. 10. Knockdown of kcnab2b reduces low-threshold sustained outward currents of M cells at 4 dpf. A: representative outward currents of M cells at 4 dpf in CtrlMO (left) and kcnab2bMO-injected (right) larvae evoked by step commands from −95 to −45 mV at 10-mV intervals, as shown in Fig. 5. Note that knockdown of kcnab2b affected I_sustain, but not I_A, as in the case of DTX treatment (Fig. 5), although the transient currents comprising I_sustain and I_A appeared to decrease. B: I-V relationships of sustained outward currents (I_sustain) after subtraction of leak currents of M cells at 4 dpf in CtrlMO- and kcnab2bMO-injected larvae. The amplitude of sustained currents evoked by the command voltages of −55 and −45 mV significantly decreased in kcnab2bMO-injected larvae (CtrlMO, n = 8 cells/5 fish; kcnab2bMO, n = 13 cells/10 fish). **P < 0.01, Mann-Whitney U-test.**
ACKNOWLEDGMENTS

We thank Dr. Y. Okamura and Dr. R. Sakata of Osaka University for teaching two-electrode voltage-clamp recording and Dr. K. Ohsumi of Nagoya University for providing Xenopus oocytes. We also thank Y. Matsunari for fish care.

REFERENCES

This work was supported by Grants-in-Aid for Scientific Research (KAKENHI 22300126, 23650164, 23115508) and a fellowship from the Japan Society for the Promotion of Science (KAKENHI 09J09220).

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. interpreted results of experiments; 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.

ACKNOWLEDGMENTS

We thank Dr. Y. Okamura and Dr. R. Sakata of Osaka University for teaching two-electrode voltage-clamp recording and Dr. K. Ohsumi of Nagoya University for providing Xenopus oocytes. We also thank Y. Matsunari for fish care.

GRANTS

This work was supported by Grants-in-Aid for Scientific Research (KAKENHI 22300126, 23650164, 23115508) and a fellowship from the Japan Society for the Promotion of Science (KAKENHI 09J09220).

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., T. Shimazaki, A.M., T. Suzuki, and M.T. performed experiments; 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.

REFERENCES


