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A novel cyclic nucleotide-gated ion channel enriched in synaptic terminals of isotocin neurons in zebrafish brain and pituitary

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Abstract

Cyclic nucleotide-gated (CNG) channels are nonselective cation channels opened by binding of intracellular cyclic GMP or cyclic AMP. CNG channels mediate sensory transduction in the rods and cones of the retina and in olfactory sensory neurons, but in addition, CNG channels are also expressed elsewhere in the central nervous system, where their physiological roles have not yet been well defined. Besides the CNG channel subtypes that mediate vision and olfaction, zebrafish has an additional subtype, CNGA5, which is expressed almost exclusively in the brain. We have generated CNGA5-specific monoclonal antibodies, which we use here to show that immunoreactivity for CNGA5 channels is highly enriched in synaptic terminals of a discrete set of neurons that project to a subregion of the pituitary, as well as diffusely in the brain and spinal cord. Double labeling with a variety of antibodies against pituitary hormones revealed that CNGA5 is located in the terminals of neuroendocrine cells that secrete the nonapeptide hormone/transmitter isotocin in the neurohypophysis, brain, and spinal cord. Furthermore, we show that CNGA5 channels expressed in *Xenopus* oocytes are highly permeable to Ca²⁺, which suggests that the channels are capable of modulating isotocin release in the zebrafish brain and pituitary. Isotocin is the teleost homolog of the mammalian hormone oxytocin, and like oxytocin, it regulates reproductive and social behavior. Therefore, the high calcium permeability of CNGA5 channels and their strategic location in isotocin-secreting synaptic terminals suggest that activation of CNGA5 channels in response to cyclic nucleotide signaling may have wide-ranging neuroendocrine and behavioral effects.

Keywords

oxytocin; cyclic nucleotides; calcium entry; neuroendocrine cells; synaptic plasticity; neurohypophysis

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Cyclic nucleotide-gated (CNG) channels are nonselective cation channels that play a central role in sensory transduction in the primary receptor neurons of vision and olfaction. In addition, CNG channels are found in a variety of regions of the brain, where their physiological roles have not yet been well defined. Because they are permeable to Na^+ and Ca^{2+} and are gated by the intracellular messengers cyclic AMP and cyclic GMP, CNG channels have been proposed to participate in synaptic plasticity induced by neural activity or neuromodulators (Bradley et al., 1997; Barnstable et al., 2004). In keeping with this idea, CNG channels have been reported to modulate transmitter release at synaptic terminals of retinal photoreceptors (Rieke and Schwartz, 1994; Savchenko et al., 1997), in addition to their role in phototransduction. Also, in the mammalian olfactory bulb, Murphy and Isaacson (2003) have shown that in response to moderate elevations in cyclic nucleotides, presynaptic CNG channels increase Ca^{2+} entry and thereby enhance spontaneous transmitter release. However, in response to large elevations in cyclic nucleotides, CNG channel opening decreases evoked transmitter release, most likely by depolarizing the presynaptic terminal and thus inducing Na^+ channel inactivation.

CNG channel α subunits in the mammalian brain are the same as those expressed in visual or olfactory receptor neurons (CNGA1, CNGA2, or CNGA3), but in zebrafish, transcripts of a novel CNG isoform, CNGA5, have been detected by RT-PCR almost exclusively in the brain (Tetreault et al., 2006). When expressed in *Xenopus* oocytes, channels formed by CNGA5 exhibit unusual properties (Tetreault et al., 2006), which suggests that this isoform may be specialized for a particular CNS role. The specificity of CNGA5's expression could potentially be useful for unraveling the functions of CNG channels in the CNS, especially since the zebrafish is so amenable to genetic manipulation. However, it is not yet clear what cell types express CNGA5 in the zebrafish CNS, and the potential role of this novel subtype therefore remains uncertain.

To identify the cells that express CNGA5 and to establish the subcellular localization of the channels, we generated CNGA5-specific monoclonal antibodies that do not cross react with α subunits of CNG channels in retinal photoreceptors (CNGA1 and CNGA3) or olfactory receptors (CNGA2) of zebrafish. Because CNG channels are thought to modulate synaptic transmission, we focused on localization of CNGA5 immunoreactivity at CNS synapses and on the identification of a candidate neurotransmitter whose release is likely to be modulated by CNGA5 channels in the zebrafish CNS. We also measured the Ca^{2+} permeability of CNGA5 channels expressed in *Xenopus* oocytes, to determine if the channels are likely to influence transmitter release by supporting calcium influx at presynaptic terminals. Based on our findings, we propose that CNGA5 channels are important presynaptic modulators of neuroendocrine systems that influence reproductive and social behavior in zebrafish.

EXPERIMENTAL PROCEDURES

Production and characterization of anti-CNGA5 antibody

To generate monoclonal antibodies specific for CNGA5, we immunized Balb/c mice with a protein consisting of glutathione S-transferase (GST) fused to the last 106 amino acids of CNGA5, which is a region of high diversity across CNG channel subtypes. We also constructed a fusion peptide of His₆ with the same C-terminal region of CNGA5, which was used to detect positive polyclonal mouse antisera by ELISA. Hybridomas were then produced using standard methods (Bekele-Arcuri et al., 1996), and 68 positive hybridoma cell lines were identified by ELISA immunoreactivity against the His-tagged C-terminus of CNGA5. Forty of these were also positive for immunofluorescence staining of HEK293 cells expressing full-length CNGA5. The 12 strongest clones were then tested for specificity using immunofluorescence staining of COS1 cells expressing full-length CNGA5 or full-length goldfish CNGA3. Figure 1A,B shows specific staining of CNGA5-expressing cells but not CNGA3-expressing cells by clone L55/54, with antibody L36/12 serving as a positive control for CNGA3 expression (Fig.

1C,D). The monoclonal antibody L36/12, which detects both CNGA1 and CNGA3, was obtained from the UC Davis/NIH NeuroMab Facility, supported by NIH grant U24NS050606 and maintained by the Department of Neurobiology, Physiology and Behavior, College of Biological Sciences, University of California, Davis, CA 95616.

Two monoclonal antibodies, L55/10 and L55/54, that were strongly positive for full-length CNGA5 but not goldfish CNGA3 were then subcloned, produced at large scale, purified, and tested by immunostaining on cryosections of zebrafish tissue. Both antibodies showed no staining above background in cryosections of 8-day larval zebrafish retina (arrowheads, Fig. 1E,F), although intense immunoreactivity was present in the adjacent pituitary (arrow, Fig. 1E) and brain (arrows, Fig. 1F) in the same sections. There was also no staining above background with L55 antibodies in adult zebrafish retina, as illustrated in Fig. 1G,H. In addition, no staining was observed in the olfactory epithelium, confirming that the L55 antibodies do not cross react with native CNGA1 (rod photoreceptors), CNGA3 (cone photoreceptors), or CNGA2 (olfactory cilia) in larval or adult zebrafish. All subsequent results shown in this paper were obtained using monoclonal antibody L55/54. As a further test of specificity, L55/54 was preincubated with the antigenic CNGA5 C-terminal protein used to generate the antibodies. As shown in Fig. 2, this preincubation abolished all immunostaining in the brain and pituitary. Based on all these tests, we conclude that L55/54 specifically detects CNGA5 channel protein.

Other antibodies

To characterize cells expressing CNGA5, double-label immunofluorescence experiments were carried out using L55/54 and a variety of rabbit polyclonal antibodies, summarized in Table 1. Primary antibodies were detected with Alexa-488-conjugated goat anti-rabbit (Molecular Probes/Invitrogen) and Cy3-conjugated donkey anti-mouse (Jackson Immunoresearch) secondary antibodies. Synthetic isotocin and vasotocin for blocking controls of anti-oxytocin antibody (see Results) were purchased from Peninsula Laboratories.

Tissue preparation

Animal use followed guidelines established by the NIH and the Institutional Animal Care and Use Committee. Larval zebrafish (8 days postfertilization) were anesthetized with MS-222 and fixed by immersion in 4% paraformaldehyde in PBS for 2 h on ice. Immunofluorescence labeling was carried out using whole-mount brain dissected from fixed larvae or 30 μ m cryosections of whole larvae. Images were acquired using an Olympus FV300 laser-scanning confocal microscope, initially processed using Olympus FluoView software, and later exported into Adobe Photoshop 7.0 or NIH ImageJ for final processing. In some cases, images were acquired using conventional epifluorescence microscopy and a cooled CCD camera (Retiga EX, Q-Imaging, Inc.). No staining above background was detectable in sections incubated with secondary antibody alone or with primary antibody preincubated with blocking fusion protein. Contrast and brightness were adjusted using Photoshop 7.0 or ImageJ, but in cases where comparisons between panels were important, the same adjustments were applied to all relevant images.

Electrophysiology

CNGA5 channels were expressed in *Xenopus* oocytes and macroscopic cGMP-activated currents were recorded from excised, inside-out patches as described by Tetreault et al. (2006). Relative Ca^{2+} and Mg^{2+} permeability ratios were determined from reversal potentials measured under bi-ionic conditions with 100 mM Na^{+} in the pipette and either 50 mM Ca^{2+} or Mg^{2+} in the bath. To avoid interference from endogenous Ca^{2+} -dependent Cl^{-} currents in the oocyte membranes, gluconate was used instead of Cl^{-} in the solutions. In order to use a Ag/AgCl electrode in the absence of Cl^{-} ions, a micro-agar salt bridge (Shao and Feldman, 2007) was constructed inside the patch-clamp electrode holder using polyimide tubing. The

electrode solution contained 100 mM Na-gluconate, 2 mM Hepes, and 1 mM EGTA (pH 7.2, adjusted with N-methyl-D-glucamine (NMDG)). Intracellular (bath) solutions were either identical to the electrode solution or contained 50 mM Ca-gluconate or 50 mM Mg-gluconate substituted for the 100 mM Na-gluconate. Block by NMDG was negligible in the voltage range of the reversal potentials. Macroscopic currents were recorded in response to 250-ms voltage pulses ranging from -50 mV to $+50$ mV in 5 mV increments from a holding potential of 0 mV. Leak currents were measured in the absence of cGMP and then subtracted from currents measured in the presence of 1 mM cGMP. Currents were filtered at 2 kHz (low pass Bessel) and sampled at 10 kHz. Currents were measured after voltage-dependent gating and before significant ion depletion effects (Karpen et al., 1988; Zimmerman et al., 1988). Pipette offset was corrected in symmetrical sodium solutions containing 1 mM cGMP. Voltages were corrected for liquid junction potentials, which were measured as described (Baumann et al., 1994). All recordings were at room temperature.

Reversal potentials of the cGMP-activated currents were determined by fitting a straight line to the I-V curve in the range near where the current crossed zero. The reversal potential was taken as the voltage where this line had zero current. Permeability ratios, P_{Ca}/P_{Na} and P_{Mg}/P_{Na} , were calculated from the reversal potentials according to Hille (2001):

$$P_{Ca}/P_{Na} = 1/4 [Na^+]_o / [Ca^{2+}]_i \exp [-2FE_{rev}/RT]$$

where $[Na^+]_o$ and $[Ca^{2+}]_i$ are ionic activities outside and inside the membrane, E_{rev} = reversal potential, F = Faraday's constant, R = gas constant, and T = temperature (293 °K).

A note on nomenclature of zebrafish CNG channels

In keeping with the nomenclature standards of mammalian CNG channels (Bradley et al., 2001), we originally used the name CNGA5 for the brain-specific CNG channel α subunit studied here (Tetreault et al., 2006), because it was the fifth α subunit to be cloned, expressed, and characterized. However, inspection of the relatedness dendrogram for the superfamily of cyclic nucleotide-gated channels (Accession TF318250; www.treefam.org) suggests that zebrafish CNGA5 may be a paralog of CNGA2, which arose from genome duplication in the ray-finned fish lineage. If it can be confirmed that these two putative paralogs lie within duplicated chromosome segments, then the preferred standard nomenclature (zfinfo.org/zf_info/nomen.html) for the two proteins would be Cnga2a and Cnga2b, with the latter being CNGA5. Because this naming convention would conflict with established usage of "a" and "b" suffixes to refer to splice variants of mammalian CNG channels, the alternative zebrafish nomenclature of Cnga2.1 and Cnga2.2 would likely be more appropriate. However, it has not yet been established that CNGA5 is in fact a paralog of CNGA2 in zebrafish, so we will continue to use the term CNGA5 in the results presented here.

RESULTS

Anatomical characterization of CNGA5 immunostaining in zebrafish brain

We used monoclonal antibody L55/54 (see Materials and Methods for details on antibody generation and characterization of specificity) to localize CNGA5 by immunofluorescence staining in dissected whole-mount larval brains or cryosections of larval zebrafish. As shown in Fig. 3, the staining in whole-mount brain was highly discrete, with intense labeling of a small cluster of cell bodies in the diencephalon (arrow, Fig. 3A) and bright puncta that appear to lie along axons ramifying in distinct locations within the midbrain and hindbrain and extending into the spinal cord. Supplemental Video S1 presents a three-dimensional reconstruction from a series of confocal optical sections in the sagittal plane for the brain shown

in Fig. 3A. Figure 3B shows a higher magnification confocal image of labeled puncta in the midbrain, viewed in the horizontal plane about midway between the dorsal and ventral surfaces of the brain. In addition to the immunofluorescence in the brain, a cluster of intensely stained boutons was observed in a subregion of the pituitary, indicated by the asterisk in Fig. 3A. Double labeling with pituitary hormones (see below) confirmed that this staining is indeed within the pituitary.

To establish more closely the location of the CNGA5-positive somata, we turned to coronal cryosections immunostained with L55/54. The labeled cell bodies were located in the dorsal preoptic region (Fig. 4), at the anterior-posterior level of the optic chiasm. This position in the posterior preoptic region is consistent with the intense L55/54 staining in the pituitary, because cell bodies of neurons that project to the pituitary are found at this approximate position (among others) in other fish species (Johnston and Maler, 1992; Corrêa and Zupanc, 2004; Saito et al., 2004). At higher magnification, staining in cell bodies appeared to be in intracellular clusters (inset, Fig. 4), in addition to smaller clusters that seemed to lie along axons exiting in the ventrolateral direction to join the medial and lateral forebrain bundles. This pattern would be consistent with synthesis of CNGA5 protein in preoptic neuroendocrine cells and export along the axon toward sites of projection.

We next examined the projection pattern of the CNGA5-positive processes along the rostral-caudal axis, using coronal cryosections from larval fish. Figure 5A shows an overview from a projection of confocal optical sections through a whole-mount brain in the horizontal plane, with the approximate positions of the coronal sections shown in Fig. 5B–G marked by the corresponding letters. Staining was observed posterior to the cell bodies in the preoptic region (Fig. 5B), suggestive of caudal-projecting axons that diffusely flank the posterior tuberculum (Fig. 5C; PT), with branches descending toward the pituitary (Fig. 5C, white arrows) and ascending to the optic tectum (Figs. 5C,D, white arrowheads). At the level of the bright staining in the pituitary, staining in the midbrain continued to flank midline cell bodies (Fig. 5D), but in the hindbrain, staining was restricted to the ventral part of the brain (Figs. 5E, F), associated with the intermediate reticular formation (Fig. 5E, IMRF) and inferior reticular formation (Fig. 5F, IRF). In the caudal medulla (Fig. 5G), staining moved dorsolaterally, consistent with axons projecting in lateral fiber tracts along the spinal cord.

CNGA5 immunostaining in zebrafish brain is localized to synaptic terminals

Because some of the punctate structures stained by anti-CNGA5 along putative fiber tracts resembled *en passant* synapses and terminal boutons, we double-labeled larval zebrafish brains with L55/54 and a rabbit polyclonal antibody against the synaptic vesicle protein SV2A, which is commonly used as a marker of presynaptic terminals. At the level of resolution provided by fluorescence microscopy, colocalization of SV2A and CNGA5 immunoreactivity was observed at CNGA5-positive structures in both the brain and pituitary, as illustrated in Figure 6A,B. Rotation of 3D reconstructions (Supplemental Video S2) from Z-axis stacks of confocal images confirmed that SV2A staining was contained within the CNGA5-positive structures, consistent with clusters of synaptic vesicles within synaptic terminals. The colocalization of the vesicle marker with CNGA5 immunostaining suggests that CNGA5 channels are found at synaptic terminals. Of course, since SV2A marks synaptic terminals of all types, numerous SV2-positive puncta do not coincide with CNGA5 immunostaining, which is specific for a small subset of putative synaptic terminals.

CNGA5-positive terminals are not associated with anterior pituitary cells

The location of the brightly CNGA5-positive terminals near the pars intermedia of the pituitary suggested that they might be the terminals of hypothalamic neurons that secrete releasing factors controlling the release of one or more anterior pituitary hormones from specific classes

of pituitary cells. Fish lack a portal system like that of mammals and instead have direct innervation of anterior pituitary cells by the hypothalamic neurons controlling their secretory activity (Weltzien et al., 2004). This innervation arrangement allowed us to use double-label immunofluorescence staining with the anti-CNGA5 monoclonal antibody and rabbit antisera directed against hormones of the anterior pituitary to determine if CNGA5-positive terminals contact specific hormone-secreting cell types. In fish, cells secreting each of the adenohypophysial hormones form clusters in particular anterior-posterior locations (Weltzien et al., 2004; Kasper et al., 2006). In the region of the pituitary where the CNGA5-positive terminals are located, cells secreting α -MSH, ACTH, or somatolactin are expected to be found, based on the pattern in other fish species. (Somatolactin is a fish-specific pituitary hormone, related to growth hormone and prolactin; Kaneko, 1996.) In keeping with this expectation, discrete cell clusters expressing these hormones were indeed observed near the CNGA5 immunoreactivity in the zebrafish pituitary, as shown in Fig. 6C–E. However, although they were located nearby, the terminals marked with CNGA5 staining did not directly contact melanotrope, corticotrope, or somatolactotrope. Therefore, CNGA5 channels are not found in the terminals of the regulatory neurons that secrete releasing factors for any of these three types of anterior pituitary cells. We next widened the search, using antisera against prolactin, luteinizing hormone, follicle stimulating hormone, and growth hormone to localize other subsets of anterior pituitary cells. All of these antisera labeled clusters of cells in locations similar to those observed in the pituitary of other teleosts (Weltzien et al., 2004; Kasper et al., 2006). However, the CNGA5-positive terminals did not contact any of these cell types (data not shown). Therefore, we conclude that the cells whose synaptic terminals express CNGA5 in the pituitary are unlikely to be involved in regulation of the adenohypophysis.

CNGA5 is expressed in isotocin-secreting neuroendocrine cells

In addition to the anterior-pituitary hormones, the mammalian pituitary secretes the nonapeptide hormones vasopressin (CYFQNCPRG) and oxytocin (CYIQNCPLG), which are released directly into the blood supply of the neurohypophysis from synaptic terminals of neuroendocrine cells whose somata are located in the hypothalamus. In fish, the equivalent neurohypophysial hormones are vasotocin (CYIQNCPRG) and isotocin (CYISNCPIG). Instead of the distinct anatomical separation of anterior and posterior pituitary found in mammals, the neurohypophysis interdigitates with the adenohypophysis in teleosts. This anatomical arrangement raises the possibility that the cluster of CNGA5-positive terminals within the zebrafish pituitary could be located in the equivalent of the mammalian posterior pituitary, which would explain why CNGA5 immunoreactivity was not associated with any of the adenohypophysial cell types. To test this possibility, we double-labeled sections of zebrafish brain and pituitary with anti-CNGA5 and a rabbit polyclonal antibody against mammalian oxytocin. Consistent with previous results in trout for neurons that contain vasotocin or isotocin (Saito et al., 2004), neurons labeled with anti-oxytocin projected widely in the central nervous system, as well as to the neurohypophysis, as shown in Fig. 7A,B. Extensive colocalization of the anti-CNGA5 and anti-oxytocin immunoreactivity was observed in the pituitary endings (Fig. 7A,B), demonstrating that the CNGA5-positive terminals in the pituitary are indeed vaso/isotocin-secreting terminals of the zebrafish neurohypophysis. In the brain, strong colocalization of CNGA5 and oxytocin immunostaining could also be observed (e.g., inset, Fig. 7B), but not all CNGA5-positive puncta in the brain were associated with detectable anti-oxytocin staining. This leaves open the possibility that CNGA5 might be associated with additional brain systems besides those releasing neurohypophysial hormones. In the pituitary, however, every CNGA5-positive cluster was associated with anti-oxytocin staining, suggesting that vaso/isotocin terminals in the neurohypophysis are the primary site of CNGA5 expression in the pituitary.

The anti-oxytocin antibody we used in these experiments could potentially interact with either or both of the fish hormones vasotocin and isotocin. To establish whether the staining we observed represents isotocin or vasotocin, we incubated the antibody with a molar excess of synthetic isotocin or vasotocin and then performed double immunofluorescence experiments with anti-CNGA5. As shown in Fig. 8, staining with the anti-oxytocin antibody was eliminated by pre-incubation with isotocin but not vasotocin, indicating that CNGA5 immunostaining in the pituitary is restricted to neuroendocrine terminals that release the hormone isotocin.

In the preoptic region of the hypothalamus, where the cell bodies of isotocin-secreting neuroendocrine cells are located, anti-oxytocin immunostaining also colocalized with CNGA5 immunoreactivity in neuronal cell bodies, shown in Fig. 7C–E. The position of the oxytocin-positive cells within the diencephalon agrees with the location of isotocin neurons in zebrafish brain revealed by *in situ* hybridization (Unger and Glasgow, 2003; compare their Fig. 3H with our Fig. 4 and Fig. 5B). Therefore, the CNGA5-positive cell bodies shown earlier (Fig. 4) appear to give rise to the isotocinergic system of the neurohypophysis and the brain. In 8-day larval fish, the cell bodies of isotocin-positive preoptic neurons were small: the surface area of a polygon drawn around the perimeter of the soma averaged $39 \pm 3 \mu\text{m}^2$ (mean $\pm$ s.e.m.; $N=13$) corresponding to a diameter of $\sim 7 \mu\text{m}$. However, the isotocin neurons were significantly larger than diencephalic cells that were immunoreactive for tyrosine hydroxylase ($24 \pm 1 \mu\text{m}^2$, $N=14$, $p=0.0002$), consistent with the position of isotocin neurons in the dorsal, magnocellular portion of the preoptic region (Fig. 4; Unger and Glasgow, 2003).

CNGA5 channels are highly permeable to Ca^{2+}

The presynaptic location of CNGA5 channels suggests they may provide a cyclic nucleotide-sensitive pathway for calcium entry and thus for modulation of isotocin release. Other known CNG channels have appreciable permeability to Ca^{2+} , and calcium influx through the channels is important in sensory adaptation (Fain et al., 2001) and in activation of chloride channels (Matthews and Reisert, 2003). However, CNG channel subtypes differ considerably in calcium permeability (P_{Ca}), with olfactory channels having substantially higher permeability than rod photoreceptor channels (Frings et al., 1995). To determine whether channels formed by CNGA5 are more similar in P_{Ca} to olfactory or rod channels, we measured reversal potentials of current through heterologously expressed channels under bi-ionic conditions in inside-out patches, as shown in Fig. 9. The average reversal potential in 7 patches was $-19.8 \pm 3.4 \text{ mV}$ for calcium and $-20.1 \pm 4.7 \text{ mV}$ for magnesium ($\pm$ SD), with 50 mM divalent ion in the bath and 100 mM sodium in the pipette. Permeability ratios for Ca^{2+} and Mg^{2+} relative to Na^+ ($P_{\text{Ca}}/P_{\text{Na}}$ and $P_{\text{Mg}}/P_{\text{Na}}$, respectively) were then calculated from the reversal potentials as described (Hille, 2001). To provide comparison with other CNG channels, we also measured divalent cation permeability ratios for bovine CNGA1 (rod photoreceptor) and rat CNGA2 (olfactory receptor) channels. The results are summarized in Table 2. Under our experimental conditions, $P_{\text{Ca}}/P_{\text{Na}}$ and $P_{\text{Mg}}/P_{\text{Na}}$ for mammalian CNGA1 and CNGA2 were similar to the calcium and magnesium permeability ratios reported previously for rod and olfactory channels (Frings et al., 1995). The relative P_{Ca} of zebrafish CNGA5 channels was comparable to the highly calcium-permeable olfactory CNG channel and substantially higher than the mammalian rod channel. This indicates that CNGA5 channels more closely resemble olfactory CNG channels, in which a large portion of inward current is carried by calcium ions under physiological conditions (Frings et al., 1995).

DISCUSSION

We have demonstrated here that the novel CNG channel alpha subunit, CNGA5, is enriched in synaptic terminals of isotocinergic neurons in zebrafish brain and neurohypophysis. Isotocin is a nonapeptide hormone (CYISNCPIG), which is equivalent to mammalian oxytocin

(CYIQNCPLG). In mammals, the classical effects of oxytocin are to stimulate uterine smooth muscle during parturition and to elicit milk ejection during lactation, but oxytocin also has a number of additional behavioral roles. Oxytocin-releasing neurons have widespread connections in the mammalian brain, and oxytocin release in the CNS has been implicated in a number of social behaviors related to pair bonding, mating, and parenting (Gimpl and Fahrenholz, 2001; Wang and Aragona, 2004; Hammock and Young, 2006). As with oxytocinergic neurons of mammals, isotocinergic neurons project widely in the brain of trout (Saito et al., 2004) and zebrafish (present study). In addition, isotocin and vasotocin influence social and reproductive behaviors in fish (Goodson and Bass, 2000, 2001; Ohya and Hayashi, 2006), much like the equivalent hormones in mammals. Therefore, the posterior pituitary hormones seem to play similar functional roles in the central nervous system of both fish and mammals.

The immunocytochemical localization of CNGA5 in synaptic terminals and the high calcium permeability of channels formed by CNGA5 indicate that cyclic nucleotides may modulate release of isotocin through activation of CNGA5 channels. Both cGMP and cAMP are full agonists for CNGA5 (Tetreault et al., 2006), but the channel is substantially more sensitive to cGMP ($K_{1/2} = 7 \mu\text{M}$) than cAMP ($K_{1/2} = 280 \mu\text{M}$). This suggests that the relevant agonist is likely to be cGMP, although cAMP could activate the channel in microdomains containing a cAMP signaling complex. Nitric oxide (NO) is known to modulate the activity of oxytocinergic neurons in mammals (Gimpl and Fahrenholz, 2001), and one possible scenario is that activation of soluble guanylyl cyclase by NO leads to production of cGMP and opening of CNGA5 channels. Alternatively, NO may directly activate CNGA5 channels by S-nitrosylation, as occurs in rat olfactory CNG channels (Broillet and Firestein, 1997). The cysteine residue required for activation by S-nitrosylation (Cys-460 of rat CNGA2; Broillet, 2000) is located within a 100% conserved region of CNGA5 in the linker connecting the cyclic nucleotide binding domain to the sixth transmembrane segment, which suggests that CNGA5 might also respond directly to NO.

In either case, however, it is not yet clear what functional effect activation of CNGA5 channels might have on isotocin release, because opening of presynaptic CNGA5 channels could either facilitate or depress release, depending on the downstream targets. For example, calcium influx through the open channels might enhance release by directly activating exocytosis, or it might inhibit release by activating calcium-dependent potassium channels. Independent of direct calcium influx through CNGA5 channels, depolarization resulting from channel opening could also modulate isotocin secretion positively or negatively, by promoting either activation or inactivation of voltage-gated channels.

CNG channels of mammalian brain have wide-ranging expression in a variety of cell types and tissues beyond the CNS, but the CNGA5 channel described here is specific to the zebrafish brain and pituitary, and especially to the isotocinergic system. This high specificity, combined with tools for genetic manipulation available in zebrafish, will facilitate studies to establish the functional role of CNG channels in transmitter release in a discrete neural system in the CNS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ACTH, adrenocorticotrophic hormone
 CNG channels, cyclic nucleotide-gated channels
 CNGA1, cyclic nucleotide-gated channel alpha subunit 1

CNGA2, cyclic nucleotide-gated channel alpha subunit 2
 CNGA3, cyclic nucleotide-gated channel alpha subunit 3
 CNGA5, cyclic nucleotide-gated channel alpha subunit 5
 CNS, central nervous system
 C-terminal, carboxy terminal
 FSH, follicle stimulating hormone
 GH, growth hormone
 GST, glutathione S-transferase
 Hy, hypophysis
 IMRF, intermediate reticular formation
 IRF, inferior reticular formation
 LH, luteinizing hormone
 α -MSH, alpha melanocyte stimulating hormone
 MO, medulla oblongata
 NMDG, N-methyl-D-glucamine
 oc, optic chiasm
 PBS, phosphate buffered saline
 Po, preoptic region
 PRL, prolactin
 PT, posterior tuberculum
 SL, somatolactin
 SV2A, synaptic vesicle protein 2A
 T, thalamus
 TeO, tectum opticum

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Fig. 1. Specificity of anti-CNGA5 monoclonal antibody L55/54 for CNGA5 channels. (A–D) COS1 cells were co-transfected with cDNAs for EGFP and for full-length zebrafish CNGA5 (A,C) or for full-length goldfish CNGA3 (B,D). L55/54 stained GFP-positive COS1 cells that express CNGA5 (A) but not CNGA3 (B). Antibody L36/12, which was raised against goldfish CNGA3 and detects both CNGA1 and CNGA3 in multiple species, showed the reverse pattern, labeling GFP-positive cells that express CNGA3 (D) but not CNGA5-expressing cells (C). (E) In an oblique section of 8-day larval zebrafish that allowed both the pituitary and one eye to be viewed in a single section, L55/54 intensely stained the cluster of terminals of isotocinergic cells in the pituitary (arrow), but produced no staining in the photoreceptor layer of the retina

(arrowheads). (F) In a coronal section through both eyes, L55/54 labeled neuronal processes and somata in the brain (arrows), but not the photoreceptor layer of the retina (arrowheads). Images in E and F were constructed by superimposing both fluorescence and bright-field views, to better reveal the overall morphology and the non-stained structures within the eye. (G,H) Lack of staining with anti-CNGA5 in adult zebrafish retina. L55/54 antibody against CNGA5 (H) produced no staining above that observed with the secondary antibody alone (G). In both G and H, contrast was enhanced to allow the dim background staining of retinal layers to be discernible. Images in G and H are single confocal optical sections of immunostained cryosections. OPL: outer plexiform layer; IPL: inner plexiform layer. Thus, antibody L55/54 does not cross react with CNG channels of photoreceptor cells in larval or adult zebrafish.

Fig. 2. Preincubation with antigenic protein eliminates immunostaining with L55/54 in zebrafish brain and pituitary. The intensity scale of the images is inverted, so that bright objects appear dark. (A) Control immunostaining showing the typical pattern of CNGA5 immunoreactivity in a sagittal section through the midbrain and pituitary. (B) When preincubated with the antigenic protein, L55/54 no longer produces detectable staining above background in a comparable sagittal section. (C) Horizontal section through the hindbrain, showing the parallel descending tracts of CNGA5 immunoreactivity on the two sides of the brain. (D) Preincubation with the antigenic protein abolished immunostaining in a comparable section through the hindbrain.

Fig. 3.

Overview of CNGA5 immunoreactivity in zebrafish brain and pituitary. Immunofluorescence L55/54 staining followed by confocal microscopy. Except in the inset in A, the intensity scale is inverted, so that bright objects appear dark. (A) Sagittal view of a whole-mount brain from an 8-day-old larval fish. The image is a Z-axis projection of confocal optical sections spanning 150 μm , starting near the midline. The arrow indicates cell bodies of CNGA5-immunoreactive neurons, and the asterisk marks a cluster of bright immunostaining in the pituitary. Video S1 shows a 3D reconstruction of the same Z-axis series of optical sections. The inset shows a single optical section through the pituitary at higher magnification to show the staining in terminal boutons. (B) View in the horizontal plane of CNGA5 immunostaining in the midbrain

of a whole-mount brain from an 8-day-old larval fish. CNGA5 immunoreactivity is restricted to discrete puncta that appear to lie along fibers forming a bilateral plexus. The arrow indicating the rostral direction is located at the midline.

Fig. 4. Somata of CNGA5 immunoreactive cells are located in the preoptic region of the hypothalamus. (A) Coronal section of an 8-day zebrafish larva at the level of the optic chiasm, immunostained with antibody L55/54 against CNGA5 and viewed using epifluorescence illumination. The intensity scale is inverted, so that bright objects appear black. The inset shows a higher magnification view of the cluster of cells on the right. (B) The same field of view as A, imaged using brightfield, oblique illumination. The arrowheads and the label for the preoptic region (Po) are at the same positions within the field of view in A and B.

Fig. 5.

The course of CNGA5-positive projections along the rostral-caudal axis of 8-day larval zebrafish brain. (A) Overview of CNGA5 staining in a whole mount brain in the horizontal plane, formed from a montage of 14 projections of confocal optical sections from the dorsal to the ventral surface of the brain. Letters B–G indicated approximate rostral-caudal positions of the coronal sections shown in panels B–G. The cell bodies in the preoptic region are at the left, and the spinal cord is at the far right. (B–G) Coronal sections of an 8-day zebrafish larva immunostained with anti-CNGA5 (bright spots). Brightfield images are superimposed to provide positional reference for the immunostaining relative to relevant brain regions. Arrows in C indicate fibers descending toward the neurohypophysis. Arrowheads in C and D indicate

punctate staining within the optic tectum. Abbreviations: Hy, hypophysis; IMRF, intermediate reticular formation; IRF, inferior reticular formation; MO, medulla oblongata; oc, optic chiasm; Po, preoptic region; PT, posterior tuberculum; T, thalamus; TeO, tectum opticum.

Fig. 6. CNGA5 immunoreactivity is located in synaptic terminals in the brain and pituitary. Images are confocal optical sections from 30 μm cryosections of 8-day-old larval zebrafish. (A,B) Sections were double-labeled with anti-CNGA5 (red) and a marker of synaptic vesicles, anti-SV2A (green), in the pituitary (A) and the brain (B). (C–E) CNGA5-positive terminals in the pituitary do not make contact with melanotropes (C), corticotropes (D), or somatolactotropes (E). Each panel in C–E is a stereo pair of 3D-reconstructions from a series of Z-axis confocal sections, with anti-CNGA5 in red and the indicated hormone antibodies in green. White dots are registration marks to assist in stereo viewing.

Fig. 7.

CNGA5 immunostaining co-localizes with immunoreactivity for isotocin in the pituitary and brain. Images are confocal optical sections from cryosections of 8-day-old larval zebrafish, double labeled with anti-CNGA5 (red) and an antibody against mammalian oxytocin that also recognizes zebrafish isotocin (green). Areas of overlap appear yellow. (A) Overview of staining in the brain and pituitary. (B) Higher magnification views of the co-localized staining in the neurohypophysis (main panel) and in the brain (inset). (C–E) CNGA5 immunostaining is located in cell bodies of isotocin-secreting neuroendocrine cells in the hypothalamus. (C) Anti-isotocin staining in green. (D) Superimposed staining for CNGA5 (red) and isotocin (green). (E) Anti-CNGA5 staining in red.

Fig. 8. Immunostaining in the pituitary with antibody against mammalian oxytocin is blocked by incubating the antibody with isotocin but not vasotocin. (A,B) CNGA5-positive terminals in the pituitary (A) are no longer double labeled with anti-oxytocin (B) when the oxytocin antibody was preincubated with a molar excess of synthetic isotocin. (C,D) Preincubation of anti-oxytocin with a molar excess of vasotocin (D) does not block double labeling of CNGA5-positive terminals (C) in the pituitary.

Fig. 9.

Current-voltage curves for cGMP-activated current in an excised, inside-out patch from a *Xenopus* oocyte expressing CNGA5 channels. Curves are from the same patch. The solution inside the recording pipette contained 100 mM Na^+ , and the bath solution facing the inside of the patch was switched between 50 mM Ca^{2+} (circles) and 50 mM Mg^{2+} (triangles). The reversal potential with 100 mM Na^+ in the bath solution was 0 mV (not shown). The current in each condition is the difference between leak current in the absence of cGMP and the current in the presence of 1 mM cGMP in the bath solution.

Table 1

Rabbit polyclonal antibodies used in immunofluorescence studies in conjunction with mouse monoclonal antibody L55/54 (anti-CNGA5).

Antibody	Source	Dilution	Catalog #
synaptic vesicle protein 2A (SV2A); human	Synaptic Systems	1:200	119 002
alpha-melanocyte stimulating hormone (α MSH); human	Phoenix Pharmaceuticals	1:400	H-043-01
prolactin (PRL); salmon	Dr. Akivoshi Takahashi, Kitasato University, Japan	1:4000	Lot 8206
luteinizing hormone (LH); salmon	Dr. Akivoshi Takahashi	1:4000	Lot 8506
follicle stimulating hormone (FSH); salmon	Dr. Akivoshi Takahashi	1:1000	Lot 8510
somatolactin (SL); salmon	Dr. Akivoshi Takahashi	1:4000	Lot 8906
growth hormone (GH); salmon	Dr. Akivoshi Takahashi	1:4000	Lot 8501
oxytocin; human	Biogenesis	1:1000	7090-0009
ACTH; human	Dr. A.F. Parlow, National Hormone and Pituitary Program, NIDDK	1:1000	AFP-39032082

Table 2

Relative Ca^{2+} and Mg^{2+} permeability of channels formed by zebrafish CNGA5, bovine CNGA1, and rat CNGA2 in inside-out patches from *Xenopus* oocytes.

	zfCNGA5	bCNGA1	rCNGA2
$P_{\text{Ca}}/P_{\text{Na}}$	5.3 ± 1.5 (7)	1.5 (2)	5.5 (1)
$P_{\text{Mg}}/P_{\text{Na}}$	5.6 ± 2.2 (7)	1.9 (2)	6.1 (1)

mean $\pm$ SD, with number of experiments in parentheses