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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 CNS, 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 Ca^{2+}, 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.

Key words: oxytocin, cyclic nucleotides, calcium entry, neuroendocrine cells, synaptic plasticity, neurohypophysis.

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 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

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Abbreviations: ACTH, adrenocorticotropic 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; 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; 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 2 A; α-MSH, alpha melanocyte stimulating hormone; T, thalamus; TeO, tectum opticum.

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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 His_6 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. Fig. 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, USA.

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.

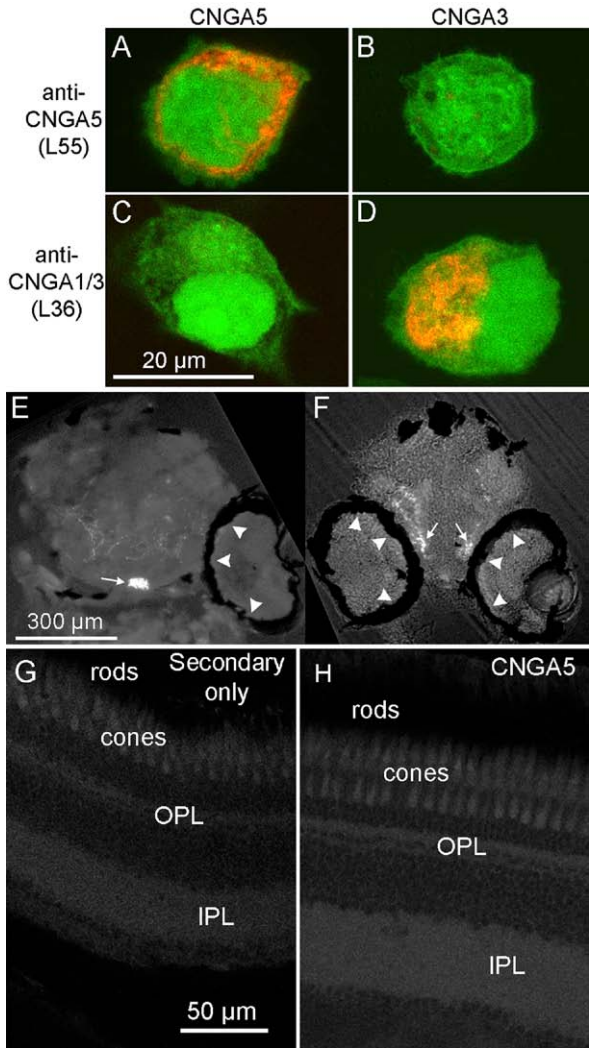

Fig. 1. Specificity of anti-CNGA5 monoclonal antibody L55/54 for CNGA5 channels. (A–D) COS1 cells were co-transfected with cDNAs for EGFP and for full-length zebrafish CNGA5 (A, C) or for full-length goldfish CNGA3 (B, D). L55/54 stained GFP-positive COS1 cells that express CNGA5 (A) but not CNGA3 (B). 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.