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HETEROLOGOUS EXPRESSION OF THE INVERTEBRATE FMRFamide–GATED SODIUM CHANNEL AS A MECHANISM TO SELECTIVELY ACTIVATE MAMMALIAN NEURONS

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Abstract—Considerable effort has been directed toward the development of methods to selectively activate specific subtypes of neurons. Focus has been placed on the heterologous expression of proteins that are capable of exciting neurons in which they are expressed. Here we describe the heterologous expression of the invertebrate FMRFamide (H-phenylalanine-methionine-arginine-phenylalanine-NH_2) –gated sodium channel from *Helix aspersa* (*Ha*FaNaC) in hippocampal slice cultures. *Ha*FaNaC was co-expressed with a fluorescent protein (green fluorescent protein (GFP), red fluorescent protein from *Discosoma* sp (dsRed) or mutated form of red fluorescent protein from *Discosoma* sp (tdTomato)) in CA3 pyramidal neurons of rat hippocampal slice cultures using single cell electroporation. Pressure application of the agonist FMRFamide to *Ha*FaNaC-expressing neuronal somata produced large prolonged depolarizations and bursts of action potentials (APs). FMRFamide responses were inhibited by amiloride (100 μM). In contrast, pressure application of FMRFamide to the axons of neurons expressing *Ha*FaNaC produced no response. Fusion of GFP to the N-terminus of *Ha*FaNaC showed that GFP-*Ha*FaNaC was absent from axons. Bath application of FMRFamide produced persistent AP firing in *Ha*FaNaC-expressing neurons. This FMRFamide-induced increase in the frequency of APs was dose-dependent. The concentrations of FMRFamide required to activate *Ha*FaNaC-expressing neurons were below that required to activate the homologous acid sensing ion channel normally found in mammalian neurons. Furthermore, the mammalian neuropeptides neuropeptide FF and RFamide-related peptide-1, which have amidated RF C-termini, did not affect *Ha*FaNaC-expressing neurons. Antagonists of NPFF receptors (BIBP3226) also had no effect on *Ha*FaNaC. Therefore, we suggest that heterologous-expression of *Ha*FaNaC in mammalian neurons could be a useful method to selectively and persistently excite specific subtypes of neurons in intact nervous tissue. Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: AP, action potential; ASIC, acid sensing ion channel; dsRed, red fluorescent protein from *Discosoma* sp; FaNaC, H-phenylalanine-methionine-arginine-phenylalanine-NH_2–gated sodium channel; FMRFamide, H-phenylalanine-methionine-arginine-phenylalanine-NH_2; GFP, green fluorescent protein; GFP*Ha*FaNaC, H-phenylalanine-methionine-arginine-phenylalanine-NH_2–gated sodium channel from *Helix aspersa* with an N-terminal green fluorescent protein fusion; *Ha*FaNaC, H-phenylalanine-methionine-arginine-phenylalanine-NH_2–gated sodium channel from *Helix aspersa*; P, postnatal day; PCR, polymerase chain reaction; QRFP, pyroglutamylated RFamide peptide; RFRP, RFamide-related peptide; SynGFP, synaptophysin green fluorescent protein fusion protein; tdTomato, mutated form of red fluorescent protein from *Discosoma* sp.

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Determining the role that specific subtypes of neurons play in neuronal network function is essential for understanding how the nervous system operates and how dysfunction of specific subtypes of neurons contributes to psychiatric and neurological diseases. One method to study the role of specific neuronal subtypes in neural network function is by activating a specific set of neurons in a neural network with either direct electrical stimulation of nervous tissue or chemical stimulation through the application of an exogenous excitatory molecule (e.g. exogenous application of the ubiquitous excitatory neurotransmitter glutamate). However, electrical stimulation and excitatory chemical application activate all axons and neuronal processes in the exposed tissue and therefore neither stimulation technique can selectively activate a specific subtype of neuron in intact nervous tissue.

In order to selectively activate specific subtypes of neurons, attempts have been made to heterologously express proteins that, when activated, are capable of depolarizing and exciting only those neurons in which they are expressed (Zemelman et al., 2002, 2003; Banghart et al., 2004; Lima and Miesenbock, 2005; Boyden et al., 2005; Li et al., 2005; Nagel et al., 2005; Volgraf et al., 2006; Bi et al., 2006; Ishizuka et al., 2006; Schroll et al., 2006; Chambers et al., 2006; Arenkiel et al., 2007, 2008; Han and Boyden, 2007; Petreanu et al., 2007; Zhang and Oertner, 2007; Zhang et al., 2007; Adamantidis et al., 2007; Aravanis et al., 2007). In order to be effective, the heterologously expressed activator protein cannot be endogenously expressed in the neural system under investigation. The mechanism of activation of the protein should not affect any endogenous molecules in the system under study, and molecules present in the system under study should not affect the activator protein. In the case of the mammalian nervous system, light- or ligand-gated ion channels from nonmammalian species have provided a source for such proteins (Zemelman et al., 2002; Nagel et al., 2003; Banghart et al., 2004; Kramer et al., 2005; Boyden et al., 2005; Li et al., 2005; Herlitze and Landmesser, 2007; Parrish et al., 2006; Chambers et al., 2006). Therefore, in this study we have investigated the potential utility of heterologously expressing the invertebrate FMRFamide (H-phenylalanine-methionine-arginine-phenylalanine-NH_2) –gated sodium channel (FaNaC) (Lingueglia et al., 1995; Jeziorski et al., 2000; Perry et al., 2001; Furukawa et al., 2006) in mammalian neurons for their selective activation.

FaNaC was initially cloned from *Helix aspersa* (Lingueglia et al., 1995), but others have also been found in *Helisoma trivolis*, *Lymnaea stagnalis*, and *Aplysia kurodai* (Lingueglia et al., 1995; Jeziorski et al., 2000; Perry et al., 2001; Furukawa et al., 2006). FaNaCs are activated by the amidated peptide FMRFamide (Price and Greenberg, 1977) and are primarily permeated by sodium ions (Lingueglia et al., 1995). Importantly, when heterologously expressed in cell lines or oocytes, activation of FaNaC shows little desensitization (Lingueglia et al., 1995; Green and Cottrell, 1999, 2002; Jeziorski et al., 2000). Of the different species, FMRFamide-gated sodium channel from *Helix aspersa* (*Ha*FaNaC) has a higher affinity for FMRFamide and produces larger currents than FaNaC from other species (Lingueglia et al., 1995; Jeziorski et al., 2000; Perry et al., 2001; Furukawa et al., 2006). Thus, *Ha*FaNaC may be the best candidate of the FaNaC proteins for gene-targeted activation of mammalian neurons. However, there may be some limitations to the use of *Ha*FaNaC as a gene-targeted method for the activation of neurons in intact mammalian nervous tissue. Although FMRFamide is not found in mammals, other longer peptides with amidated RF C-termini (RFamide peptides) have been found in the mammalian CNS (Yang et al., 1985; Perry et al., 1997; Vilim et al., 1999; Hinuma et al., 2000; for review see Fukusumi et al., 2006). Fortunately, RFamide peptides with an extended N-terminus do not appear to activate *Ha*FaNaC (Lingueglia et al., 1995; Cottrell, 1997). In contrast, the mammalian NPFF receptors are activated by FMRFamide (Tang et al., 1984; Yang et al., 1985; Raffa, 1988, 1989; Brussaard et al., 1989; Roumy and Zajac, 1998; Bonini et al., 2000; Hinuma et al., 2000; Liu et al., 2001). Furthermore, acid sensing ion channels (ASIC), which are homologous to *Ha*FaNaC, are found in the mammalian CNS and are modulated by FMRFamide (Lingueglia et al., 1995, 2006; Askwith et al., 2000; Xie et al., 2003). Therefore, although *Ha*FaNaC may be capable of activating mammalian neurons in which it is heterologously expressed, activation of *Ha*FaNaC by FMRFamide may have secondary effects on endogenous receptors and ion channels present in the mammalian CNS.

In this study, we show that heterologous expression of *Ha*FaNaC in hippocampal CA3 pyramidal neurons permitted those neurons to be potently and persistently activated by application of FMRFamide. Furthermore, these neurons were activated at FMRFamide concentrations below that known to modulate ASICs. Lastly, an antagonist of NPFF receptors had no effect on *Ha*FaNaC activation in CA3 pyramidal neurons. Therefore, the heterologous expression of *Ha*FaNaC in mammalian neurons may provide a method for the selective activation of neurons of choice in the mammalian CNS.

EXPERIMENTAL PROCEDURES

cDNA constructs

*Ha*FaNaC constructs were contracted out to sequencing companies for subcloning into the mammalian expression vector pCMVTnT (Promega, Madison, WI, USA). *Ha*FaNaC cDNA (EMBL accession number: X92113) was donated by Drs. Eric Lingueglia and Michel Lazdunski (CNRS-Université de Nice-Sophia, Antipolis, France). To generate pCMVTnT/*Ha*FaNaC, the entire coding sequence of *Ha*FaNaC was polymerase chain reaction (PCR) amplified and ligated between *Xba*I and *Sal*I sites (Epoch Biolabs, Sugar Land, TX, USA). FMRFamide-gated sodium channel from *Helix aspersa* with an N-terminal green fluorescent protein fusion (GFP*Ha*FaNaC) construct was created by Eton Bioscience Inc. (San Diego, CA, USA) using overlap extension PCR. Green fluorescent protein (GFP) was PCR amplified out of pmaxGFP (Amaxa, Inc., Gaithersburg, MD, USA) and fused to the N-terminus of the *Ha*FaNaC full length coding sequence and ligated between *Eco*R I and *Xba*I sites. Kozak consensus sequences were added immediately upstream of the 5′ ATG start codon of each construct to enhance translation efficiency (Kozak, 1987).

The mutated form of red fluorescent protein from *Discosoma* sp (tdTomato) (Shaner et al., 2004, 2005) (donated by R. Tsien, University of California, San Diego, CA, USA) was ligated out of the bacterial vector pRSET-B and subcloned into *Bam*H I and *Eco*R I sites in pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA). pCMV–red fluorescent protein from *Discosoma* sp (dsRed) Express vector (Clontech, Mountain View, CA, USA) and pmaxGFP (Amaxa Inc.) were also used for visualization. pEGFP/synaptophysin (synaptophysin green fluorescent protein fusion protein, SynGFP) was kindly provided by Ed Ruthazer (McGill University, Montreal). Prior to electroporation into cells, plasmids were purified using Qiagen EndoFree kits (Hilden, Germany).

Hippocampal slice cultures

Use of animals in this study was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and adhered to U.S. federal guidelines of the Animal Welfare Act and Animals Welfare Regulations and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The number of animals used in this study was limited to those necessary to show statistical differences and animals were never exposed to pain or suffering. Postnatal day (P) 7 or P8 Sprague–Dawley rat pups (Zivic Laboratories) were deeply anesthetized with isoflurane, decapitated, and their hippocampus dissected aseptically under an Olympus SZ61 dissection microscope. Organotypic hippocampal slice cultures were prepared using the method developed by Stoppini et al. (1991) with the following modifications: 300 μm transverse slices were cut using a Stoelting Tissue Chopper, and two to three slices were transferred to organotypic Millicell-CM inserts (Millipore, Bedford, MA, USA) in 60 mm Petri dishes containing 2 ml of media (50% minimum essential medium, 25% horse serum and 25% Hanks' balanced salt solution, 36 mM glucose, 25 mM Hepes, 1% penn/strep, pH 7.2). After 1 day in culture, culture medium was replaced with fresh medium containing no antibiotic. Culture medium was replaced thereafter biweekly. Cultures were allowed to grow for 5–7 days prior to single cell electroporation.

Single cell electroporation

Organotypic hippocampal slices were placed on the fixed stage of an Olympus BX51WI microscope equipped with DIC optics. The image of CA3 pyramidal neurons was collected through a 60× (0.9 N.A.) water immersion objective lens, captured with a DAGE-MTI IR1000 CCD camera and displayed on a monochrome video monitor (Vitek VTM-14A, Audio Video Supply, San Diego, CA, USA). Glass pipettes (borosilicate glass (8250, 1.65/1.0 mm)) were pulled using a Narishige PP830 pipette puller (East Meadow, NY, USA). Tips were backfilled with 2 μl of solution containing 49.5 ng *Ha*FaNaC cDNA and 16.5 ng fluorescent marker cDNA in sterile-filtered saline (in mM): 125 NaCl, 3.0 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, and 25 glucose). Pipette tips were visually guided in close apposition to an individual neuron's