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NEUROPEPTIDE Y REGULATES RECURRENT MOSSY FIBER SYNAPTIC TRANSMISSION LESS EFFECTIVELY IN MICE THAN IN RATS: CORRELATION WITH Y2 RECEPTOR PLASTICITY

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Abstract—A unique feature of temporal lobe epilepsy is the formation of recurrent excitatory connections among granule cells of the dentate gyrus as a result of mossy fiber sprouting. This novel circuit contributes to a reduced threshold for granule cell synchronization. In the rat, activity of the recurrent mossy fiber pathway is restrained by the neoexpression and spontaneous release of neuropeptide Y (NPY). NPY inhibits glutamate release tonically through activation of presynaptic Y2 receptors. In the present study, the effects of endogenous and applied NPY were investigated in C57Bl/6 mice that had experienced pilocarpine-induced status epilepticus and subsequently developed a robust recurrent mossy fiber pathway. Whole cell patch clamp recordings made from dentate granule cells in hippocampal slices demonstrated that, as in rats, applied NPY inhibits recurrent mossy fiber synaptic transmission, the Y2 receptor antagonist (S)-N^2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6H)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide (BIIE0246) blocks its action and BIIE0246 enhances synaptic transmission when applied by itself. Y5 receptor agonists had no significant effect. Thus spontaneous release of NPY tonically inhibits synaptic transmission in mice and its effects are mediated by Y2 receptor activation. However, both NPY and BIIE0246 were much less effective in mice than in rats, despite apparently equivalent expression of NPY in the recurrent mossy fibers. Immunohistochemistry indicated greater expression of Y2 receptors in the mossy fiber pathway of normal mice than of normal rats. Pilocarpine-induced status epilepticus markedly reduced the immunoreactivity of mouse mossy fibers, but increased the immunoreactivity of rat mossy fibers. Mossy fiber growth into the inner portion of the dentate molecular layer was associated with increased Y2 receptor immunoreactivity in rat, but not in mouse. These contrasting receptor changes can explain the quantitatively different effects of endogenously released and applied NPY on recurrent mossy fiber transmission in mice and rats.

Key words: neuropeptide Y, mossy fiber, Y2 receptor, hippocampus, epilepsy.

Mossy fibers are axons of the hippocampal dentate granule cells. The mossy fiber pathway normally projects to the pyramidal cells of area CA3 and to inhibitory interneurons of the CA3 area and dentate gyrus, but makes very few recurrent synapses onto granule cells. Thus the pathway drives monosynaptic excitation and feedforward inhibition to CA3 pyramidal cells, but only feedback inhibition to granule cells. A unique feature of temporal lobe epilepsy is the sprouting of mossy fibers within the dentate gyrus, which results in formation of novel monosynaptic recurrent excitatory circuitry (Nadler, 2003). The development of recurrent excitation, coupled with the loss of certain inhibitory neurons (Obenaus et al., 1993; Buckmaster and Jongen-Rêlo, 1999), may enhance the participation of granule cells in seizures. Studies of hippocampal slices support this view (Tauck and Nadler, 1985; Cronin et al., 1992; Patrylo and Dudek, 1998; Hardison et al., 2000; Okazaki and Nadler, 2001; Gabriel et al., 2004; Santhakumar et al., 2005), although the ability of the recurrent mossy fiber pathway to synchronize granule cell discharge depends on stimulus frequency (Feng et al., 2003), $[K^+]_o$ (Patrylo and Dudek, 1998; Hardison et al., 2000) and coreleased transmitters/modulators (Tu et al., 2005). The ability of mossy fibers to drive the CA3 pyramidal cell population is similarly constrained (Weisskopf et al., 1993; Henze et al., 2002).

Glutamate pathways, including the mossy fibers, do not normally express neuropeptide Y (NPY). In the pilocarpine model of temporal lobe epilepsy, however, NPY immunoreactivity appears de novo in the mossy fiber pathway, including the recurrent projection (Lurton and Cavalheiro, 1997; Scharfman et al., 2000; Tu et al., 2005). Spontaneous release of NPY from recurrent mossy fiber terminals in rats reduces the probability of glutamate release from those terminals by activating presynaptic Y2 receptors. Thus endogenous NPY impedes the ability of recurrent mossy fibers to synchronize granule cell dis-

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Abbreviations: AANPY, [ala^{31},aib^{32}]-neuropeptide Y; ACSF, artificial cerebrospinal fluid; BIIE0246, (S)-N^2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6H)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide; D-AP5, D-2-amino-5-phosphonopropanoate; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; NBQX, 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[F]quinoxaline; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.1% (v/v) Triton X-100; QX-314, N-ethyllidocaine; S.E.M., standard error of the mean.

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charge (Tu et al., 2005). The normal and recurrent mossy fiber pathways of pilocarpine-treated epileptic mice also express NPY immunoreactivity (Borges et al., 2003). Mice with genetically-determined alterations of NPY mechanisms may provide further insight into the role of NPY in mossy fiber function and in epileptogenesis generally. Because these mutant strains are usually generated on a C57Bl/6 background, our initial studies tested the effects of NPY receptor ligands on the recurrent mossy fiber pathway of pilocarpine-treated C57Bl/6 mice. Surprisingly, we found that NPY regulates synaptic transmission in this pathway much less effectively in mice than in rats.

EXPERIMENTAL PROCEDURES

Materials

NPY (porcine), D-gluconic acid lactone, cesium hydroxide (99.9%; 50% by weight), Hepes, EGTA, creatine phosphate, creatine phosphokinase, pilocarpine hydrochloride, (−)scopolamine methyl bromide, terbutaline hemisulfate and diaminobenzidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA); D-2-amino-5-phosphonopropanoate (D-AP5) and 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[F]quinoxaline (NBQX) from Tocris Cookson (Ellisville, MO, USA); D-trp^{32}NPY and [ala^{31},aib^{32}]-neuropeptide Y (AANPY) from Phoenix Pharmaceuticals (Belmont, CA, USA); bicuculline methiodide from Research Biochemicals (Natick, MA, USA); N-ethyllidocaine (QX-314) from Alomone Laboratories (Jerusalem, Israel); rabbit anti-Y2 receptor antibody from Neuromics (Bloomington, MN, USA); normal goat serum from Peninsula Laboratories (San Carlos, CA, USA); and biotinylated goat anti-rabbit IgG and Vectastain Elite ABC kit from Vector Laboratories (Burlingame, CA, USA). (S)-N^2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6H)-oxodibenz[b,e]azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl]acetyl]-N-[2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide (BIIE0246) was a gift from Boehringer-Ingelheim (Biberach an der Riss, Germany).

Induction of recurrent mossy fiber growth

Male C57Bl/6 mice (8–12 weeks of age; Harlan Sprague-Dawley, Madison, WI, USA) received a single injection of pilocarpine hydrochloride (265 mg/kg, i.p.) 30 min after an injection of scopolamine methyl bromide and terbutaline hemisulfate (2 mg/kg, i.p., each). More than 90% of mice treated in this way developed status epilepticus, defined as a continuous limbic motor seizure of stage 2 or higher (Racine, 1972). The seizures lasted for at least 5 h and were allowed to self-terminate. A fan mounted atop the cage blew a stream of cool air on the animal, to minimize seizure-induced hyperthermia (Sloviter et al., 2003). Age-matched untreated mice were used as controls. Germline Y2 receptor knockout (Y2$^{-/-}$) mice (Sainsbury et al., 2002) were propagated by breeding homozygotes.

Male Sprague–Dawley rats (175–200 g; Zivic Laboratories, Pittsburgh, PA, USA) were treated similarly, except that the dose of pilocarpine hydrochloride was 340–380 mg/kg, i.p. Controls were age-matched and untreated.

Animal protocols were approved in advance by the Duke University Animal Care and Use Committee according to guidelines of the National Institutes of Health. Every effort was made to minimize the number of animals used, as well as their pain and suffering.

Preparation and incubation of hippocampal slices

Experiments on mice were interleaved with some of the previously reported experiments on rats (Tu et al., 2005), so that the results could be compared across species. Studies on mice and rats were performed with the same recording system and utilized identical experimental protocols.

Mice were decapitated under ether anesthesia 10–20 weeks after pilocarpine administration. Transverse 400 μm-thick slices of the caudal hippocampus were prepared with a vibratome, incubated in a high-Mg^{2+} artificial cerebrospinal fluid [high-Mg^{2+} ACSF, which contained (in mM) 122 NaCl, 25 $NaHCO_3$, 3.1 KCl, 1.8 $CaCl_2$, 12 $MgSO_4$, 0.4 KH_2PO_4 and 10 D-glucose, pH 7.4] and oxygenated at 34 °C for 45–60 min with 95% O_2/5% CO_2. The slices were then maintained at room temperature (22–24 °C) until experimentation began.

Whole cell patch clamp recording

A slice was transferred to a submersion-type recording chamber mounted on a microscope stage, submerged in standard ACSF (1.2 mM $MgSO_4$) at room temperature and superfused at 2–3 ml/min. Whole cell patch clamp recordings were obtained from dentate granule cells with the assistance of infrared-differential interference contrast optics. Patch electrodes pulled from borosilicate glass (1.5 mm outer diameter; Sutter Instruments, Novato, CA, USA) had a tip resistance of 6–7.5 MΩ. The tip was filled with solution that contained (in mM) 140 cesium gluconate, 15 Hepes, 3.1 $MgCl_2$, 1 $CaCl_2$ and 11 EGTA, pH 7.2 and 276 mOsm. The electrode was then backfilled with an internal solution that contained (in mM) 120 cesium gluconate, 10 Hepes, 2 MgATP, 1 EGTA, 5 creatine phosphate, 20 U/ml creatine phosphokinase and 10 QX-314, pH 7.2 and 276 mOsm. Whole cell access was achieved in current clamp mode; only cells with $V_m > -70$ mV on break-in (after correction for a 10-mV liquid junction potential) were accepted for study. R_N was determined immediately after break-in from the current deflection produced by a 200 ms, 5 mV hyperpolarization from a holding potential of −80 mV.

Recordings were made with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) beginning ~20 min after break-in. Series resistances (<20 MΩ) were compensated 70–75%. Recordings were rejected if the series resistance varied by >20%. Constant-current rectangular electrical stimuli (0.1 ms duration) were delivered through a monopolar nichrome electrode (25 μm-diameter) insulated to the tip with polymerized polyvinyl resin. Signals were filtered at 2 kHz, sampled at 10 kHz and stored for analysis offline with pClamp8 software (Axon Instruments). NPY receptor ligands were applied at a concentration that was expected to produce a maximal response. Their effects were evaluated between 5 and 8 min after the compound was added to the superfusion medium. By this time, the effect of the compound was maximal.

The NMDA receptor-mediated component of the compound recurrent mossy fiber EPSC was isolated pharmacologically by addition to the superfusion medium of 30 μM bicuculline, to block $GABA_A$ receptors, and 10 μM NBQX, to block AMPA and kainate receptors. Recordings were made at a holding potential of −20 or −30 mV. The mossy fibers were stimulated under each condition with a train of 10 pulses at a frequency of 0.1 Hz and intensity of 100–500 μA. The effects of test compounds were computed from the average of 10 evoked responses.

Results are expressed as means±standard error of the mean (S.E.M.) and the effects of NPY receptor ligands were assessed by paired *t*-test.

Immunohistochemistry

Each of the five experiments included brain sections from five animals: one mouse and one rat that had experienced pilocarpine-induced status epilepticus 10–20 weeks earlier, one control mouse and rat, and one Y2$^{-/-}$ mouse. Animals were deeply anesthetized with pentobarbital and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% (w/v) phosphate-