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Upregulation of presynaptic mGluR2, but not mGluR3 in the epileptic medial perforant path

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ABSTRACT

Presynaptic metabotropic glutamate receptors (mGluRs) at glutamatergic synapses play a major role in governing release probability. Previous reports indicated a downregulation of group III mGluRs at the lateral perforant path-granule cell synapse in the chronically epileptic hippocampus. Here, we investigated the mGluR-dependent presynaptic inhibition at the medial perforant path-granule cell synapse in the pilocarpine-treated chronically epileptic rat. The specific group II mGluR agonist (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV, 10 μM) significantly depressed medial perforant path-evoked responses in control slices, but significantly more so in epileptic tissue. This depression was accompanied by a significant increase of the paired-pulse ratio in both animal groups indicating a presynaptic mechanism. Moreover, we also found that this significantly enhanced DCG-IV effect in the medial perforant path recorded in slices from pilocarpine-treated rats was due to a significant increase of mGluR2, but not mGluR3 transcripts in the entorhinal cortex using quantitative real-time reverse transcriptase-PCR. Immunohistochemistry confirmed the increased expression of group II mGluRs in the epileptic medial molecular layer. These results demonstrate that chronic epilepsy not only causes downregulation of mGluRs in the hippocampus, but may also lead to enhanced expression of these receptors – at least in the medial perforant path.

1. Introduction

Presynaptic metabotropic glutamate receptors (mGluRs) play a key role in synaptic transmission throughout the brain and act to modulate release probability. In particular, group II and group III mGluRs are abundantly expressed at presynaptic terminals and generally depress glutamatergic transmission. In the hippocampus, group II mGluRs (mGluRs 2 and 3) are located on medial perforant path synapses terminating on dentate gyrus granule cells, while group III mGluRs (mGluR 4, 7 and 8) are present on lateral perforant path synapses (Bradley et al., 1996; Shigemoto et al., 1997). Since the medial and the lateral perforant paths are functionally distinct, but anatomically adjacent fiber tracts in the dentate gyrus molecular layer, their characteristic mGluR expression is a valuable tool to experimentally distinguish both pathways. The compound L-(+)-2-amino-4-phosphonobutyric acid (L AP4) displays high specificity for group III mGluRs (Watkins and Collingridge, 1994) and, hence, depresses lateral, but not medial perforant path responses (Koerner and Cotman, 1981; Bushell et al., 1995; Johansen et al., 1995; Macek et al., 1996; Dietrich et al., 1997; Zhai et al., 2002). On the other hand, (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclo-propyl)glycine (DCG-IV) is a specific group II mGluR agonist (Kamiya et al., 1996; Yeckel et al., 1999; Kirschstein et al., 2004), and thus inhibits synaptic transmission at medial perforant path synapses (Macek et al., 1996; Kilbride et al., 1998).

Under pathological conditions such as chronic epileptic seizures, a number of alterations in hippocampal mGluR function and expression have been described. For instance, in the lateral perforant path, a reduced sensitivity to L-AP4 indicated the functional downregulation of presynaptic group III mGluRs, probably of the major subunit mGluR8, in different animal models of temporal lobe epilepsy (TLE) as well as in human Ammon's horn sclerosis (Dietrich et al., 1999; Friedl et al., 1999; Klapstein et al., 1999; Kral et al., 2003). However, there are limited data on the role of group II mGluRs in medial perforant path. In the dentate gyrus, the group II mGluR agonist DCG-IV led to a more pronounced depression of the granule cell input to hilar interneurons following pilocarpine-induced

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status epilepticus. This may contribute to granule cell disinhibition, thus rather suggesting an enhanced group II mGluR function at these synapses (Doherty and Dingledine, 2001). Hence, it is an intriguing question whether there is evidence for an increased group II mGluR function at the glutamatergic medial perforant path-granule cell synapse in the epileptic hippocampus. Indeed, we found that the mGluR-dependent presynaptic inhibition is significantly enhanced at this synapse, which was due to an upregulation of mGluR2, but not mGluR3, in the epileptic entorhinal cortex.

2. Materials and methods

2.1. Pilocarpine-induced status epilepticus and slice preparation

A prolonged status epilepticus was induced by pilocarpine injection (340 mg/kg s.c.) in male 30 day-old Wistar rats (100-130 g; Charles River Laboratories, Sulzfeld, Germany). Methyl scopolamine (1 mg/kg s.c.) was applied 30 min prior to the pilocarpine treatment to reduce peripheral cholinergic effects. When status epilepticus did not develop within 60 min, the rats received a second pilocarpine injection (170 mg/kg s.c.). Status epilepticus was terminated after 40 min by diazepam injection (4 mg/kg s.c.) which was repeated when necessary to stop the seizures. Control rats were also treated with methyl scopolamine and diazepam, but received saline instead of pilocarpine. Animals that did not develop seizures despite of pilocarpine treatment were used to control for pilocarpine-mediated effects that were independent of status epilepticus. Rats were housed in a fixed 12-hours light-and-dark environment (lights on from 06:00 h to 18:00 h), and received food and water *ad libitum*. All procedures with experimental animals were approved by the Animal Care and Use Committee. Analyses were performed with 3- to 4-month-old rats (i.e. >2 months survival time) and after the pilocarpine-treated rats had experienced chronic recurrent limbic seizures.

After deep anesthesia with diethyl ether (Mallinckrodt Baker, Deventer, Netherlands), rats were decapitated and the brain was rapidly removed and submerged into oxygenated ice-cold dissection solution containing (in mM) 125 NaCl, 26 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 0.2 $CaCl_2$, 5 $MgCl_2$ and 13 D-glucose (gassed with 95% O_2, 5% CO_2; pH 7.4). Horizontal brain slices (400 μm) of the hippocampus were prepared using a vibratome (Integraslice 7550MM, Campden Instruments, Loughborough, UK), and then transferred into a holding chamber with artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 26 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgCl_2$ and 13 D-glucose (gassed with 95% O_2, 5% CO_2; pH 7.4).

2.2. Electrophysiological recording and data analysis

For electrophysiological recordings, slices were transferred into an interface chamber (BSC-HT, Harvard Apparatus, Holliston, USA) maintained at 32 °C (TC-10, npi electronic GmbH, Tamm, Germany) and superfused with ACSF (2-3 ml/min). Field excitatory postsynaptic potentials (fEPSPs) were recorded using borosilicate glass pipettes (GB150-8P, Science Products, Hofheim am Taunus, Germany) with a tip resistance of 2–3 MΩ (pulled with PIP5 from HEKA Elektronik, Lambrecht, Germany) filled with ACSF. Bipolar stimulating electrodes were made from Teflon-insulated platinum wires (PT-2T, Science Products), and double-pulse stimulation (interstimulus interval 40 ms) was delivered using a Master-8 stimulator (A.M.P.I., Jerusalem, Israel) and a stimulus isolator (A365, World Precision Instruments, Sarasota, USA) at a baseline rate of 0.033 Hz. The baseline stimulation strength was adjusted to 30-40% of the maximal fEPSP amplitude and typically in the range of 50–100 μA. The evoked potentials were amplified and filtered at 1 kHz (EXT-10-2F, npi), digitized (Micro1401, CED, Cambridge Electronic Design, Cambridge, UK) and stored for offline analysis (Signal 2.16, CED).

To record from the medial perforant path-granule cell synapse, both the stimulating and the recording electrodes were placed into the middle molecular layer (mML) of the dentate gyrus. These synapses typically display paired-pulse depression upon double-pulse stimulation (Dietrich et al., 1997; Kilbride et al., 1998). The influence of group II mGluRs was tested by bath application of (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV, Tocris, Bristol, UK), and (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495, Tocris). All other chemicals were purchased from Sigma (Taufkirchen, Germany). Paired-pulse ratio (PPR) was calculated as the ratio of the 2nd pulse-evoked fEPSP to the 1st pulse-evoked fEPSP, and then normalized to baseline values.

2.3. Real-time reverse transcriptase PCR

Total RNA was isolated from pooled entorhinal cortex and dentate gyrus, respectively, using TRIZOL reagent. Total RNA was reverse-transcribed using Moloney murine leukaemia virus reverse transcriptase (RT, 200 U/μl) and RNasin Plus RNase inhibitor (40 U/μl, both Promega Corporation, Madison, WI, USA) in the presence of random hexamers (3 μg/μl) and dNTP Mix (10 mM each, Invitrogen, Carlsbad, CA, USA). Real-time RT-PCRs were performed using a reaction mastermix consisting of 10× buffer, Mg^{2+} (Cf = 4 mM), deoxyribonucleotide triphosphates (Cf = 200 μM), Platinum taq polymerase (0.6 U/20 μl reaction, Invitrogen) and SYBR Green (concentration as recommended by the manufacturer, Qiagen Inc., Valencia, CA, USA). The mastermix was aliquoted, cDNA and primers (Cf = 20 μmol) were added. The amplicons for mGluR2 and mGluR3 were 250 bp and 261 bp, respectively (primers for mGluR2: TTTAGGTCAGAAGCCAGAGT, CAGTAACCATCCCTATCC; for mGluR3: TATTCTCAGTCCTCTGCAAG, TTGTAGCACATCACTACATACC). As the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used (primers: TGTGTCCGTCGTGGATCTGA, TTGCTGTTGAAGTCGCAGGAG, all primers from TIB Molbiol, Berlin, Germany).

Real-time RT-PCR was done using the ep mastercycler (software realplex 1.5, Eppendorf, Hamburg, Germany) with cycling parameters 95 °C for 2 min once, followed by 95 °C for 30 s and 60 °C for 45 s, with normalized fluorescence read at 60 °C (530 nm) for 40 cycles. Single product amplification was confirmed by melting curve. Both mGluR2−mRNA and mGluR3−mRNA expression levels in dentate gyrus and entorhinal cortex were determined by comparison with GAPDH-mRNA as the mean of $2^{\Delta\Delta Ct} \pm$ SEM.

2.4. Immunohistochemistry

For immunohistochemistry, pilocarpine-treated ($n = 5$) and control rats ($n = 4$) were deeply anesthetized with S-ketamine and xylazine (100 mg/kg S-ketamine and 15 mg/kg xylazine, i.p.), then transcardially perfused with phosphate-buffered saline (PBS) followed by ice-cold 4% paraformaldehyde (PFA). Brains were removed, post-fixed in 4% PFA (overnight) and transferred to 30% sucrose solution (24–48 h). Brains were then sectioned horizontally into 30 μm slices on a Leica microtome and collected in phosphate-buffered saline (PBS). Section sequence was preserved and every tenth section was further used for antibody treatment, with sections from pilocarpine-treated and control brains always treated in parallel using the same solutions. Selected sections were first blocked with 3% normal goat serum in PBS plus 0.03% Triton X-100 for 30 min, then incubated overnight with primary antibody (rabbit polyclonal anti-mGluR2/3, 1:1,500; Millipore, Billerica, MA, USA). The following day, slices were rinsed (2× 15 min in PBS) and incubated for 2 h either with Cy3-conjugated goat anti-rabbit IgG antibody (1:800; Invitrogen, Darmstadt, Germany) for immunofluorescence analysis, or with biotin-conjugated goat anti-rabbit IgG antibody (1:250; Vector Laboratories, Burlingame, CA) for light microscopy. For immunofluorescence, slices were then counterstained with 4′,6′-diamidin-2-phenyindol (DAPI), mounted on glass slides and embedded with fluorescence-protecting mounting medium (Dako, Hamburg, Germany). For light microscopy, sections were subjected to biotin-avidin-peroxidase-complex solution (ABC-Kit, Vector Laboratories) and antibody binding was visualized by incubating sections in a solution containing 0.04% 3, 3′ diaminobenzidine, 0.01% H_2O_2 and 0.01% $NiCl_2$. Sections were dehydrated in a graded ethanol series and coverslipped with Entellan (Merck, Darmstadt, Germany).

For analysis, four sections containing hippocampus (approximate positions: −7.6, −7.34, −7.1, −6.82 mm from Bregma, according to Paxinos and Watson, 1998) were selected from the Cy3-immunostained series. Using a Zeiss LSM 510 Meta confocal microscope (Leica, Wetzlar, Germany), pictures were taken from the dentate gyrus (DG) molecular layer at 200× magnification at a defined position (the position where a virtual line extending the CA3 pyramidal cell layer was crossing the granule cell layer; approximately corresponding to the crest of DG). Two squares of $44 \times 44\ \mu m^2$ were imposed on the middle (mML) or inner molecular layer (iML), respectively (see Fig. 3C–D), and staining intensity was determined within these squares using ImageJ (Abramoff et al., 2004). Data from the four sections were averaged to obtain one representative value ($n = 1$) per animal. Data are presented as mGluR2/3 immunoreactivity in mML relative to iML. For light microscopy, immunostained sections were viewed and photographed with a Zeiss Axioskop 2 microscope (Zeiss, Oberkochen, Germany).

2.5. Statistical analysis

All data are expressed as means ± SEM. Statistical comparisons were performed using Student's two-tailed *t*-test unless otherwise specified. Significant differences are indicated by asterisks ($^{*}p < 0.05$; $^{**}p < 0.01$) for comparisons between control and pilocarpine-treated animals (unpaired test), and by diamonds ($^{\diamond}p < 0.05$; $^{\diamond\diamond}p < 0.01$) for comparisons between baseline and drug effect (paired test).

3. Results

3.1. Enhanced group II mGluR function in the epileptic medial perforant path

Metabotropic glutamate receptor (mGluR) signaling through group II and III receptors conveys an important feedback inhibition to excitatory glutamatergic synapses in the dentate gyrus molecular layer. Since group III mGluRs are downregulated in the lateral perforant path of the chronically epileptic hippocampus (Dietrich