Medial Perforant Path Inhibition Mediated by mGluR7 Is Reduced After Status Epilepticus

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INTRODUCTION

Metabotropic glutamate receptors (mGluRs) play key roles in synaptic transmission and synaptic plasticity at many synapses throughout the brain (Anwy1 1999; Sheng and Kim 2002), but several lines of evidence also suggest a pathological role for mGluRs in a variety of neurodegenerative disorders, including epilepsy (Moldrich et al. 2003; Wong et al. 1999). Because mGluRs can powerfully modulate both excitatory and inhibitory signaling (Gereau and Conn 1995; Gutierrez 2002), and inhibitory (Se- myanov and Kullmann 2000; Shigemoto et al. 1996) neurotransmission in the brain (Conn and Pin 1997), we asked whether reduced mGluR function might contribute to dysfunctional synaptic transmission early in the process of epileptogenesis caused by pilocarpine (POLO)-induced status epilepticus.

Animals

All experiments were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and approved by Emory University’s Institutional Animal Care and Use Committee. Metabotropic glutamate receptor 8 (−/−) and mGluR4 (−/−) animals were obtained as a gift from R. Duvoisin...
(Duvoisin et al. 1995) and P. J. Conn. Colonies of mGluR4/8 double KO were generated by crossing the mGluR4- and mGluR8-deficient strains. Double KOs did not display overt behavioral differences from WT mice, because animals remained active and well groomed for the duration of our study. The mGluR4/8 double KO mice were fertile, with litter sizes within the normal range, and they grew at a normal rate. Furthermore, both groups of mice responded similarly to PILO, suggesting that seizure susceptibility for the mGluR4/8 KO mice was not markedly enhanced over WT controls. WT 129sv1/svImJ mice were purchased from (Jackson Laboratories, Bar Harbor, ME). Male mice, 45–75 days old, were used for all experiments. Targeted disruption of the mGluR4 and 8 genes in KOs was confirmed via PCR. DNA was collected from tail snips (0.5 cm) and isolated using QuickExtract DNA extraction solution (Epicenter, Madison WI). PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide.

**PILO treatment**

Animals were injected with atropine methyl bromide (4 mg/kg, ip) (Kobayashi et al. 2003) and, 30 min later, with pilocarpine hydrochloride (320–360 mg/kg, ip). Age-matched controls were injected with an equivalent amount of atropine methyl bromide and 0.9% saline solution. SE was allowed to proceed unabated, typically lasting 1.5–3 h in duration. Only mice that exhibited SE for ≥1.5 h were used for electrophysiological experiments. For the first 72 h following SE, recovering animals were monitored two to four times per day and injected with 0.2–0.4 ml of 5% dextrose in lactated Ringer’s solution (ip) as needed, because some of the animals did not eat or drink normally for 1–3 days after SE. As judged by casual observation, at least 2 of 11 animals not used for electrophysiological experiments developed spontaneous seizures after >45 days.

*Electrophysiology*

Animals were deeply anesthetized with halothane and killed by decapitation. Brains were rapidly removed and chilled in an ice-cold, carbonated (i.e., bubbled with 95% O₂-5% CO₂) cutting artificial cerebrospinal fluid (ACSF) containing the following (in mM): 130 NaCl, 3.5 KCl, 1.25 NaHPO₄, 24 NaHCO₃, 4 MgSO₄, 1 CaCl₂, and 10 glucose (osmolarity 300 ± 5 mOsm). Transverse entorhinal-hippocampal slices (450–500 μM thick) were cut horizontally using a Vibrotome and incubated with cutting ACSF for ~30 min at 25°C. Slices were subsequently transferred to a holding chamber in bathing ACSF containing 2 mM each of MgSO₄ and CaCl₂, where they were maintained for ≥30 min at room temperature until experiments were begun. Slices were individually transferred to a submerged chamber for recording and continuously perfused with oxygenated (95% O₂-5% CO₂) bathing medium (3–5 ml/min). All experiments were performed at a temperature of 29.5–31°C.

Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded using glass micropipettes (5–10 MΩ) filled with normal ACSF bathing medium. Slices from the middle hippocampus were used preferentially. Responses were evoked by stimulation (0.1-ms duration, 25–150 μA amplitude) delivered to either the outer molecular layer [lateral perforant path (LPP)] or middle molecular layer [medial perforant path (MPP)] using Teflon-coated, Pt/Ir monopolar microelectrodes (0.9–1 MΩ impedance; Fig. 1A). Stimulus intensity was adjusted to produce 50–75% of the maximal fEPSP amplitude. (3)-3,4-dicarboxyphenylglycine (DCPG), L- (+)-2-amino-4-phosphonobutyric acid (L-AP4), and (2S,2’R,3’R)-2-(2’,3’-dicarboxycyclopropyl)glycine (DCG-IV) were purchased from Tocris Cookson (Elissville, MO) and bath-applied in all experiments for 10 min. Bicuculline (BIC, 30 μM; Tocris) and N-2-amino-5-phosphonovalerat (o-APV, 30–100 μM; Tocris) were co-applied in most experiments to block GABAa- and N-methyl-D-aspartate (NMDA)-mediated neurotransmission, respectively. All drugs were dissolved in 18.2 MΩ water.
as 10–60 mM stocks solutions, and if not used immediately, were stored in frozen aliquots for <1 mo. Electrode placements in the MPP and LPP were corroborated by observing paired-pulse depression or facilitation, respectively, at the 150-ms interpulse interval (Fig. 1B) (McNaughton 1980) and differential pharmacological responses to the application of L-AP4 or DCPG (Macek et al. 1996; Zhai et al. 2002). Responses that did not exhibit consistent paired-pulse depression (MPP recordings) or facilitation (LPP recordings) were not used.

Histology

PILO-treated rats (Buckmaster and Jongen-Reilo 1999; Williams et al. 2002) and mice (Borges et al. 2003; Shibley and Smith 2002) exhibit distinct patterns of neuronal cell loss in the hippocampus after SE, which resembles the pattern of hippocampal cell damage exhibited in patients with temporal lobe epilepsy (TLE) (Bender et al. 2003). To determine if PILO treatment produced similar patterns of neuronal cell loss in the 129sJ mouse strain used in this study, we examined qualitatively the neuronal cell densities throughout the hippocampus and dentate gyrus in control (n = 5 mice) and PILO-treated animals (n = 5 mice). After recording, slices were fixed in 4% paraformaldehyde, paraffin embedded, sectioned (8 μM), and stained with hematoxylin (Biomedia, Foster City, CA) for light microscopy observation. All sections taken from SE-experienced mice had fewer cells in the hilus and throughout stratum pyramidale (hippocampal areas CA3–CA1), whereas dentate granule cells (GCs) were relatively spared from SE-induced cell death (Fig. 1C). Sections made from control mice never displayed these patterns of neuronal cell loss. These data indicate that PILO-induced SE in our mice produced patterns of neuronal cell loss that were characteristic of animal models of and human temporal lobe epilepsy.

Data analysis

The slope of the fEPSP between 20 and 80% of its amplitude was measured by linear regression analysis (Clampfit 9.0, Axon Instruments, Union City, CA). Reductions in the fEPSP slope after application of mGluR agonists (i.e., maximal percent inhibition) were calculated by comparing the mean fEPSP slopes before and after drug application. Baseline measurements were averaged from data 5 min before drug application, whereas agonist-induced inhibition was determined from the average of responses measured 2.5–15 min after drug exposure; this helped to correct for slight variations in wash-in time between low (DCG-IV) and high (L-AP4) concentrations of mGluR agonists. Differences in agonist-induced inhibition between SE-experienced and control groups were compared by ANOVA or two-tailed, unpaired t-test, as appropriate. Time course data were subjected to repeated measures ANOVA (GraphPad Prism 4.0, GraphPad Software, San Diego, CA). Differences were considered significant if P < 0.05.

RESULTS

mGluR7-mediated inhibition in the MPP is diminished 3–9 days after PILO-induced SE

Although mGluR agonists can differentially activate receptors between groups, most compounds do not discriminate for specific receptor subtypes within groups. To study the mGlu7 receptor subtype in mouse hippocampal slices, we measured the actions of the selective group III agonist, L-AP4 (600 μM), in mice bearing deletions of mGluRs 4 and 8, the other group III mGluRs localized to the dentate (Bradley et al. 1996; Shigemoto et al. 1997). In the MPP of SE-naive animals, L-AP4 reversibly reduced the fEPSP slope equally in slices taken from either WT or mGluR4/8 double KO mice (Fig. 2, A–C). In the LPP of untreated animals, L-AP4 (600 μM) suppressed fEPSPs by 40 ± 3% in slices taken from WT mice, however, L-AP4 only slightly inhibited responses in mGluR4/8 double KO animals (5 ± 2%; Fig. 2D). This suggests that mGluR7-mediated presynaptic inhibition is functionally limited to the MPP, but not the LPP.

We also asked whether presynaptic inhibition mediated by mGluR7 might be altered after PILO-induced SE. In WT mice, MPP inhibition produced by 600 μM L-AP4 was significantly reduced 3–9 days after PILO-induced SE, changing from 14 ± 3% in controls to 4 ± 2% after PILO (Fig. 2B, left; P < 0.05, unpaired t-test). To confirm this result, we performed the same set of experiments using mGluR4/8 double KO animals. As with WT mice, 600 μM L-AP4 was significantly less effective in reducing the MPP fEPSP in PILO-treated mGluR4/8 animals (Fig. 2B, right; P < 0.05, unpaired t-test). In the LPP, there were no differences in L-AP4 sensitivity after SE (Fig. 2D). These results suggest that presynaptic inhibition mediated by mGluR7 in the MPP is functionally decreased after PILO-induced SE.

Perforant path inhibition mediated by mGluR2/3 is unchanged 3–9 days after PILO-induced SE

mGluR2/3 has been predominantly localized to the MPP, although it has also been described in low levels in the LPP (Shigemoto et al. 1997). In the MPP of untreated animals, the selective group II agonist, DCG-IV (300 nM), reversibly depressed the slope of the fEPSP by 55 ± 2% in WT and 58 ± 4% in mGluR4/8 double KO animals (Fig. 3, A–C). In the LPP, DCG-IV (300 nM) depressed the slope of the fEPSP by 26 ± 3% in WT animals and 13 ± 5% in mGluR4/8 KO mice.

Group II mGluR function is enhanced in the central amygdala after electrical kindling (Neugebauer et al. 2000) and in the hilus after PILO-induced SE (Doherty and Dingledine 2001). In this study, group II mGluR-mediated inhibition in the MPP was similar in both WT and mGluR4/8 double KO animals 3–9 days after PILO-induced SE (Fig. 3, A–C). In the LPP, the peak level of inhibition exhibited also did not change after SE for either WT or mGluR4/8 double KO mice after PILO (Fig. 3D). This suggests that presynaptic inhibition mediated by mGluR2/3 in the perforant path is not reduced 3–9 days after PILO-induced SE, and accordingly, does not likely contribute to epileptogenesis.

Group III mGluR presynaptic inhibition in the LPP is mediated exclusively by mGluR8 and is not diminished 3–9 days after PILO-induced SE

DCPG appears to be a highly selective mGluR8 activator (Thomas et al. 2001). To evaluate the selectivity of DCPG in mouse hippocampal slices, we measured its actions in the LPP of WT animals and mice bearing deletions of mGluR8. We also applied the well-described group III agonist, L-AP4, in a concentration known to selectively activate mGluR4 and 8, but not mGluR7 (60 μM), in heterologous receptor-expression systems (Conn and Pin 1997) and compared its actions to those responses elicited by the application of DCPG.

Immunohistochemically, mGluR8 appears to be located exclusively in the LPP, expressed within the outer one-third of the molecular layer (Shigemoto et al. 1997; Zhai et al. 2002).
In slices from untreated WT animals, the dose-response curve for DCPG yielded a median effective concentration (EC50) in the LPP of 900 nM (Fig. 4A). To look for reductions in inhibition, a maximal concentration of DCPG (3 μM) was applied. At 3 μM, DCPG reversibly inhibited evoked fEPSPs in the LPP of SE-naïve animals by 26 ± 3%, but was without effect (0 ± 2%) in slices taken from untreated mGluR8 KO mice (Fig. 4, A–D). L-AP4, by comparison, produced a peak inhibition of 19 ± 2% in the LPP, and did not affect (3 ± 2%) slices taken from mGluR8 KO animals (Fig. 4D). In the MPP, DCPG slightly inhibited WT mice (6 ± 1%) and was without effect in mGluR8 KO animals (0 ± 1%; Fig. 4, E and F). This finding suggests that mGluR8 alone mediates presynaptic inhibition in the LPP.

Previous studies have shown that mGluR8 function in the perforant path is chronically reduced after the development of epilepsy (Dietrich et al. 1999; Kral et al. 2003; Tang et al. 2001), suggesting a reduction of presynaptic inhibition of glutamate release may contribute to maintained hyperexcitability within the dentate. To determine if mGluR8-mediated presynaptic inhibition is also reduced before spontaneous seizures begin (i.e., during epileptogenesis), the sensitivity to DCPG within the LPP was measured during the latent period in mice. Three to 9 days after SE in PILO-treated WT mice, DCPG-mediated inhibition was unchanged in the LPP (Fig. 4B–D). The peak level of inhibition was 23 ± 2% for PILO-treated animals versus 26 ± 3% for WT controls. Similarly, DCPG-induced inhibition in the MPP was not altered after PILO (6 ± 1% inhibition in control vs. 8 ± 2% after SE; Fig. 4, E and F). These results indicate mGluR8-mediated presynaptic inhibition is not reduced 3–9 days after PILO-induced SE and suggest that the late reduction in mGluR8 function observed in spontaneously seizing animals (Dietrich et al. 1999; Kral et al. 2003; Tang et al. 2001) might be a consequence of spontaneous recurrent seizures.

**DISCUSSION**

The main conclusions of this study are the following. First, in the LPP of untreated animals, presynaptic inhibition is mediated by mGluR2/3 and 8, but not mGluR4 or 7. Second, in
the MPP of untreated animals, synaptic transmission is inhibited by activation of mGluR2/3 and 7, but not mGluR4 or 8. Third, after PILO-induced SE, mGluR7-mediated inhibition is significantly diminished in the MPP, whereas mGluR8 (LPP) and mGluR2/3 (MPP and LPP)-mediated inhibition are not altered. These findings indicate that presynaptic group II/III mGluR-mediated inhibition within the perforant path of the dentate is remarkably layer-specific and is consistent with the notion that a reduction of mGluR7 function may contribute to epileptogenesis.

Several observations indicate that mGluR7 is the only group III mGluR to mediate presynaptic inhibition within the MPP. First, the high concentration of L-AP4 (600 μM) required to inhibit fEPSPs (Fig. 2, A–C) suggests that the mGlu7 receptor subtype mediated this response (Conn and Pin 1997; Wu et al. 1998). Second, the same concentration of L-AP4 produced a level of inhibition in mGluR4/8 double KO mice that was equivalent to WT mice (Fig. 2, A–C). Third, because a lower concentration of L-AP4 (60 μM) is likely to activate only mGluR4 and mGluR8, the lack of an effect of L-AP4 in untreated WT mice (Fig. 4F) further indicates that only mGluR7 inhibits presynaptic glutamate release in the MPP.

mGluR7-mediated inhibition in the MPP was reduced 3–9 days after SE (Fig. 2, A–C). Because there is extensive cell loss in the entorhinal cortex in animal models (Kobayashi et al. 2003) or human epilepsy (Duvoisin et al. 1995), it might be expected that the loss of mGluR7-bearing afferent projections might account for the diminished L-AP4-mediated inhibition within the MPP we observed. Indeed, mGluR7b-bearing hilar mossy cells, which project to the inner molecular layer of the dentate gyrus, have been shown to be preferentially lost in epileptic patients with Ammon’s Horn Sclerosis (Blumcke et al. 2000). However, it is the neurons found in medial portion of layer II that comprise the afferents of the MPP (Witter et al. 2000), and the majority of cell loss in the entorhinal cortex occurs in layer III after PILO (Kobayashi et al. 2003). Thus it seems improbable that a selective loss in mGluR7-bearing neurons explains these data. Rather, this finding suggests that a down-regulation or ineffectual downstream messenger coupling is more likely to account for the observed reduction in mGluR7-mediated presynaptic inhibition.

Activation of mGluR2/3 by DCG-IV (300 nM) in the MPP diminished the slope of the fEPSP of both WT and mGluR4/8 double KO animals equally (Fig. 3, A and B), similar to levels described previously for rats (Kew et al. 2001; Macek et al. 1996) and mice (Kew et al. 2002). DCG-IV also inhibited evoked responses in the LPP (Fig. 3D), where the level of inhibition in the LPP (25–30%) was approximately one-half that observed in the MPP (55–60%). These results are consistent with immunohistochemical (Shigemoto et al. 1997) and electrophysiological (Lovinger and McCool 1995; Macek et al. 1996) data describing a role for mGluR2/3 in the LPP and indicate a potent regulatory role for mGluR2/3 across multiple synapses (i.e., both LPP-to-GC and MPP-to-GC) within the dentate molecular layer.

Previous work has shown that DCG-IV (either 1 μM or 200 nM) produced the same level of inhibition at the perforant path-to-GC synapse in sclerotic hippocampal tissue resected from epileptic patients as it did in nonsclerotic control tissue (Dietrich et al. 2002). In rats, mGluR2-mediated synaptic
plasticity was not altered at perforant path inputs to hilar-border interneurons after PILO-induced SE (Doherty and Dingledine 2001). In this study, inhibition mediated by mGluR2/3 was unchanged 3 days after PILO-induced SE (Doherty and Dingledine 2001). In this study, inhibition mediated by mGluR2/3 receptor subtypes in the perforant path (Zhai et al. 2002) and suggest that this mGluR8-mediated inhibition in the LPP do not contribute to the early development of epilepsy.

The selective inhibitory effects of DCPG within the LPP (Fig. 4, A–D) confirm a role for mGluR8-mediated inhibition in the perforant path (Zhai et al. 2002) and suggest that this presynaptic inhibition is confined to the LPP. It is unlikely that inhibition in the LPP is mediated by mGluR4, because 60 μM L-AP4 produced the same level of inhibition as did 3 μM DCPG in WT mice and did not produce any inhibition in mGluR8 KO mice (Fig. 4D).

FIG. 4. Synaptic inhibition by (S)-3,4-dicarboxyphenylglycine (DCPG, 3 μM), a highly selective mGluR8 agonist, in the (A–D) LPP and (E and F) MPP of the dentate gyrus after PILO-induced status epilepticus. A: dose-response curve for DCPG in the LPP. DCPG inhibits responses evoked in the LPP of WT (non-PILO-treated) slices, but not in slices taken from mGluR8 KO animals. B: representative fEPSPs showing DCPG (3 μM) inhibited responses in the LPP of WT control and PILO-treated animals equally, but did not inhibit LPP responses in slices taken from mGluR8 KO mice. Calibration bars = 2 ms, 0.3 mV. C: time course for DCPG-induced effects in the LPP for WT controls (n = 10 slices from 7 mice), PILO-treated (n = 9 slices, 5 mice), or mGluR8 KO (n = 8 slices, 6 mice). Symbols represent means ± SE. Horizontal bars indicate duration of drug application. D: summary data showing peak levels of inhibition in the LPP after the application of DCPG compared with the selective group III agonist, L-AP4 (60 μM; a concentration to activate mGluR4 and mGluR8, but not mGluR7; see RESULTS). Numbers of animals summarized here for 3 μM DCPG in the LPP were as described in C. Numbers of animals used in experiments involving 60 μM L-AP4 were as follows: WT controls, n = 5 slices, 5 mice; mGluR8 KO, n = 4 slices, 3 mice; mGluR8 KO, n = 4 slices, 4 mice. Bars represent means ± SE. E: time course for these effects in the MPP for WT controls (n = 11 slices, 7 mice), PILO-treated (n = 8 slices, 5 mice), or mGluR8 KO (n = 8 slices, 6 mice). F: summary of peak levels of inhibition in the MPP after either DCPG (3 μM) or L-AP4 (60 μM). Numbers of animals summarized here for 3 μM DCPG in the MPP were as described in C. Numbers of animals used in experiments involving 60 μM L-AP4 were as follows: WT controls, n = 5 slices, 5 mice; mGluR8 KO, n = 4 slices, 4 mice. Bars represent means ± SE.
The development of epilepsy, its role is not apparent after SE. Thus, because we measured mGluR8-mediated inhibition in the LPP 3–9 days after PILO, it is possible that a functional reduction had yet to occur. Our data, along with the aforementioned studies, indicate that if mGluR8 is involved in the development of epilepsy, its role is not apparent 3–9 days after PILO-induced SE. However, a chronic reduction in mGluR8-mediated inhibition (Dietrich et al. 1999; Kral et al. 2003) may be important for maintenance of the epileptic phenotype.

Implications of diminished mGluR7-mediated inhibition in epilepsy

The diminution of mGluR7-mediated inhibition in the MPP noted here after SE (Fig. 2) is consistent with a possible role for aberrant excitation within the dentate. On the other hand, the magnitude of mGluR7-mediated inhibition in the MPP is not large, even in untreated animals (Fig. 2, B and C). However, how might this reduction in mGluR7 contribute to the development of hippocampal hyperexcitability and/or spontaneous seizures? Several lines of evidence suggest that changes in mGluR7 are likely to play an integral part in regulating the balance between excitation and inhibition. First, group III agonist L-SOP into the inferior colliculus produced a short (10 min) proconvulsant excitation in sound-sensitive genetically epilepsy-prone rats, followed by a prolonged phase of enhanced protection from subsequent sound-induced seizures that lasted for 2–4 days (Yip et al. 2001). The authors reported an up-regulation in both mRNA and protein levels of mGluR7 in the inferior colliculus that they hypothesized was responsible for the prolonged anticonvulsant effect. Finally, it is important to note that only mice lacking mGluR7 display an increased susceptibility to seizures, whereas other mGluR1, 2, 4, 5, and 8 KO mice do not (Linden et al. 2002; Sansig et al. 2001; Snead et al. 2000). Sansig et al. (2001) attributed the enhanced seizure susceptibility exhibited by mGluR7-deficient mice in part to “very small excitability changes” in hippocampal area CA1 and deficits in recovery from activity-dependent facilitation in neocortex.

Our working hypothesis to describe how the observed changes in mGluR7 function might contribute to dentate hyperexcitability is depicted in Fig. 5. In the latent period after SE, the observed reduction in mGluR7-mediated presynaptic inhibition in the MPP (Fig. 2) would be expected to enhance, perhaps in a subtle manner, the response of GCs to input trains from entorhinal cortex. At the same time, increased mGluR2/3-mediated presynaptic inhibition of GC inputs to hilar-border interneurons (Doherty and Dingledine 2001) would be expected to partially disinhibit GCs, further increasing their firing.

![Diagram showing how the present mGluR-mediated changes might contribute to increased neuronal transfer through the dentate and epileptogenesis.](http://jn.physiology.org/doi/abs/10.1152/jn.01086.2003)
probability during a train of inputs. The combination of enhanced excitatory input and reduced feedback inhibitory control of GCs—both achieved by alterations in mGluR-mediated presynaptic regulation of glutamate release—are proposed to act synergistically to increase the propagation of neuronal signals through the dentate into CA3 during the epileptogenic period. This hypothesis is consistent with findings of enhanced MPP input to, and reduced inhibition of, dentate GCs 3–7 days after PILO-induced SE (Kobayashi et al. 2003). Investigations of enhanced dentate throughput are underway to further test this hypothesis.

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