Frequency Facilitation at Mossy Fiber–CA3 Synapses of Freely Behaving Rats Contributes to the Induction of Persistent LTD via an Adenosine-A1 Receptor-Regulated Mechanism

Frequency facilitation (FF), comprising a rapid and multiple-fold increase in the magnitude of evoked field potentials, is elicited by low-frequency stimulation (LFS) at mossy fiber–CA3 synapses. Here, we show that in freely behaving rats, FF reliably occurs in response to 1 and 2 Hz but not in response to 0.25-, 0.3-, or 0.5-Hz LFS. Strikingly, prolonged (~600 s) FF was tightly correlated to the induction of long-term depression (LTD) in freely moving animals. Although LFS at 2 Hz elicited unstable FF and unstable LTD, application of LFS at 1 Hz elicited pronounced FF, as well as robust LTD that persisted for over 24 h. This correlation of prolonged FF with LTD was absent at stimulation frequencies that did not induce FF. The adenosine-A1 receptor appears to participate in these effects: Application of adenosine-A1, but not adenosine-A3, receptor antagonists enhanced mossy fiber synaptic transmission and occluded FF. Furthermore, adenosine-A1 receptor antagonism resulted in more stable FF at 1 or 2 Hz and elicited more potent LTD. These data support the fact that FF contributes to the enablement of long-term information storage at mossy fiber–CA3 synapses and that the adenosine-A1 receptor may regulate the thresholds for this process.

Keywords: CA3, frequency facilitation, hippocampus, in vivo, long-term depression, mossy fiber, synaptic plasticity

Introduction
Frequency facilitation (FF) has been widely described at mossy fiber–CA3 synapses in vitro (Salin et al. 1996; Toth et al. 2000; Moore et al. 2003; Nicoll and Schmitz 2005) and more recently has been reported in freely behaving rats (Klausnitzer and Manahan-Vaughan 2008). It occurs when the stimulation frequency is altered from a very low rate (e.g., 0.025 Hz) to a low rate (1 Hz) and typically is only sustained for as long as the stimulation is given. It comprises a unique property of the mossy fiber synapse and is not seen at any other hippocampal synapse (Salin et al. 1996; Dobrunz and Stevens 1999; Toth et al. 2000; Klausnitzer and Manahan-Vaughan 2008). FF may fulfill the role of holding synaptic information “online,” in keeping with the contribution of the CA3 region to the processing of working memory (Kesner 2007).

Hippocampal plasticity exists in a multitude of forms, ranging from very short-lasting forms such as FF, paired-pulse depression, or posttetanetic potentiation (Lambert and Wilson 1994; Dobrunz et al. 1997; Schmitz et al. 2001; Zucker and Regehr 2002) but can also persist for very long periods of time (long-term potentiation, LTP; long-term depression, LTD) (Bear 1996; Kemp and Manahan-Vaughan 2007). Synaptic plasticity is highly frequency dependent (Malenka and Bear 2004; Huang and Kandel 2005, 2007) and has different properties dependent on the hippocampal subregion in which it is induced (Harris and Cotman 1986; Johnston et al. 1992; Malenka and Nicoll 1993; Martin et al. 2000; Vianna et al. 2000; Straube et al. 2003; Lee et al. 2004). Furthermore, different hippocampal subregions respond with distinct types of synaptic plasticity to novel spatial experience (Kemp and Manahan-Vaughan 2008a). LTD in the dentate gyrus and CA1 regions of freely behaving rats is typically induced by 1 Hz low-frequency stimulation (LFS) whereas these forms of synaptic plasticity do not share the same induction mechanisms (Dudek and Bear 1992; Mulkey et al. 1993, 1994; O’Mara et al. 1995; Manahan-Vaughan 1997; Wang et al. 1998; Pöschel and Manahan-Vaughan 2005, 2007). Typical studies of FF involve applying stimuli for 30–150 s (Salin et al. 1996; Dobrunz and Stevens 1999; Toth et al. 2000; Klausnitzer and Manahan-Vaughan 2008). We investigated whether prolonged (~600 s) FF, induced by LFS at mossy fiber–CA3 synapses can also lead to persistent LTD, consistent with a role of FF in the mechanisms underlying persistent synaptic plasticity.

Materials and Methods
Electrophysiology
Male Wistar rats (7–8 weeks, Charles River, Germany) were anaesthetized (Pentobarbital 52 mg/kg) and underwent chronic implantation of a bipolar stimulation electrode and a monopolar recording electrode to enable monitoring of evoked potentials at mossy fiber–CA3 synapses.

The recording electrode was placed above the CA3 pyramidal cell layer of the dorsal hippocampus (antero-posterior (AP): -3.2; mediolateral (ML): 2.2) and the stimulation electrode was implanted at coordinates corresponding to the mossy fiber projections (AP: -3.5; ML: 2.0) (based on Derrick and Martinez 1994) (Fig. 2F). Mossy fiber responses were identified based on their appearance, characterized by negative response with an onset of 3–4 ms and a peak latency of 8–10 ms (Derrick et al. 1991).

To enable drug injections, a guide cannula was placed in the ipsilateral cerebral ventricle, as described previously (Manahan-Vaughan 1997). Experiments were commenced 7–10 days after surgery. During all experiments, the animals could move freely in the recording chamber (40 × 40 × 50 cm) and had free access to food and water. To allow the animals to acclimatize they were transferred to the experiment room the day before the experiment took place.

The head stage was connected to an amplifier and stimulator via a flexible cable with a swivel connector. Recordings were analyzed and stored on a computer and the electroencephalography was monitored throughout experiments.

Measurement of Evoked Potentials and Data Analysis
To evoke field excitatory postsynaptic potentials (fEPSPs), a biphasic pulse was given with a half-wave duration of 0.2 ms. For recordings, the stimulation intensity was set to produce an fEPSP, which was 40% of the maximal obtainable. The intensity was found on the basis of an input–output curve (maximal stimulation 900 μA). Each recording consisted of an average of 5 consecutive pulses at 0.025 Hz (test-pulse stimulation). To ensure stability of recordings, all animals were first
tested in a baseline experiment over the same time period as subsequent experiments.

For each time point, 5 consecutively evoked responses at 40-s intervals were averaged. These first 30 min of recording (6 time points) served as baseline, and the data subsequently obtained were expressed as the mean percentage ± the standard error of the mean (SEM) of this average baseline value. Following monitoring of basal synaptic transmission for 30–45 min, drug or vehicle injection was applied. The stability of basal synaptic transmission was then followed for 2.5 h, with further recordings conducted 24 h after injection.

For analysis of differences between groups, a 2-way analysis of variance (ANOVA) was applied. For differences of fEPSPs during FF, the frequency of 0.25, 0.3, 0.5, 1, and 1200 pulses at 2 Hz. The effects of paired-pulse stimulation on fEPSP evoked by tetanic stimulation (100 Hz) of the mossy fiber pathway to CA3. No difference in the profile of LTP was observed when control animals (n = 7). Changes in timescale are indicated by line breaks.

Drugs
The adenosine-A1 receptor antagonist, 1,3-dipropyl-8-(4-acrylate)phenylxanthine (phenylxanthine), was dissolved in a solution containing 5% 1 N sodium hydroxide (NaOH) (with HCl added to correct for pH) and 95% distilled water. The N-methyl-D-aspartate (NMDA) receptor antagonist (-)-2-aminophosphononic acid (AP5; Tocris-Cookson Ltd., Bristol, United Kingdom) was first dissolved in 5 μL of 1 N NaOH, then 0.9% sodium chloride (NaCl) was added to make up a solution of 100 μL volume. The selective adenosine-A1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, Sigma-Aldrich) was dissolved in 10 mM dimethylsulfoxide (DMSO). The adenosine-A3 receptor antagonist 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(2)-dihydropyridine-3,5-dicarboxylate (MR1 191, Sigma-Aldrich) was initially dissolved in 100% DMSO and diluted with 0.9% NaCl to obtain a final concentration of DMSO of 0.1%. The group II metabotropic glutamate receptors (mGluRs) agonist (2S,2R,3,R)-2-(2,3′-dicarboxycyclopropyl)glycine (DCG-IV, Tocris Cookson, Bristol, United Kingdom) was dissolved in isotonic saline (0.9% NaCl) solution. The amount of DCG-IV that was used (20 ng) was chosen because it has no effect on evoked responses in the dentate gyrus (Klausnitzer and Manahan-Vaughan 2008). Phenylxanthine was applied in a 125 μg dose, which had been previously shown to elicit significant increases in mossy fiber transmission in vivo (Klausnitzer and Manahan-Vaughan 2008). Drugs were injected in a 5-μL volume, as an acute, single injection, into the ipsilateral lateral ventricle via the implanted cannula.

Verification of Mossy Fiber-CA3 Recordings
The mossy fiber synapse is very sensitive to agonism of group II mGluRs by DCG-IV that selectively inhibits mossy fiber but not associational-commissural EPSPs (Kamiya et al. 1993; Yeckel et al. 1999; Goussakov et al. 2000). Animals were excluded from the mossy fiber-CA3 study when the fEPSP responses evoked in the stratum lucidum failed to show strong sensitivity (i.e., a reduction of test pulse-evoked responses by 60% or greater) to DCG-IV, 1 h after DCG-IV application. Postmortem histological analysis of the electrode localizations was also conducted (Bock 1989; Manahan-Vaughan et al. 1998).

Results

LTP Elicited at Mossy Fiber Synapses Is Not Sensitive to NMDA Receptor Blockade
An important issue to address in conducting experiments in the freely behaving animal is the verification that evoked
potentials derive predominantly from the activation of mossy fiber synapses. One strategy that we employed was to assess the sensitivity of the synapses to DCG-IV (see Materials and Methods). A second strategy was to assess if LTP is sensitive to treatment with NMDA receptor antagonists. Previous reports indicate that LTP at commissural associational, but not mossy fiber, synapses can be prevented by application of an NMDA receptor antagonist prior to or during the tetanus (Harris and Cotman 1986; Zalutsky and Nicoll 1990). In control animals, we induced reliable LTP by applying high-frequency tetanization (100 Hz). We then treated animals with a concentration of AP5 that we had previously shown to be effective in blocking LTP at CA1 (Manahan-Vaughan 1997) and dentate gyrus (Manahan-Vaughan et al. 1998) synapses in freely behaving rats. No significant effect on LTP was observed (ANOVA: $F_{(1,47)} = 0.40; P = 0.53, n = 7$) (Fig. 1B). This supports the likelihood that we recorded predominantly from mossy fiber synapses in our study.

Application of LFS Reveals That FF Is Highly Frequency Dependent

Application of LFS (600 pulses) at a range of frequencies revealed that the successful induction of FF in vivo is tightly dependent on the LFS frequency implemented (Fig. 2). No significant differences were observed between fEPSPs elicited with test-pulse stimulation and fEPSPs elicited with LFS at 0.25 Hz (ANOVA, $F_{(1,35)} = 6.13; P = 0.42, n = 6$), at 0.3 Hz (ANOVA, $F_{(1,31)} = 12.59; P = 0.08, n = 7$), and 0.5 Hz (ANOVA, $F_{(1,36)} = 24.47; P = 0.16, n = 6$, Fig. 2). LFS at 1 Hz elicited robust FF that attained a maximal level of $190 \pm 18.2\%$, declined slightly during LFS, but remained significant from pre-LFS values throughout the 600-s stimulation period (ANOVA, $F_{(1,11)} = 395.67; P < 0.0001, n = 16$, Fig. 2D). LFS at 2 Hz (1200 pulses) elicited facilitation that was highly variable but still differed significantly from fEPSP responses elicited by test-pulse stimulation (0.025 Hz; ANOVA, $F_{(1,49)} = 448.74; P < 0.0001, n = 9$) (Fig. 2E). The synaptic depression elicited by 2-Hz LFS was significantly less and more unstable than that elicited by 1-Hz LFS (Fig. 3D,E) (ANOVA, $F_{(1,14)} = 14.73; P < 0.001$). However, in both cases (600 pulses compared with 1200 pulses), persistent LTD occurred.

**Paired Pulse Facilitation (PPF) of Mossy Fiber Responses Occurs Before but not After Induction of LTD**

PPF has been described at mossy fiber synapses in the hippocampal slice preparation (Salin et al. 1996; Henze et al. 2000). To assess whether PPF is present at mossy fibers of freely moving rats, we tested the effects of a range of ISIs. We found that ISIs of either 20, 40, 100, 300, 500, or 1000 ms elicited no significant effects on evoked responses (Fig. 3A, all $n = 3$). An ISI of 50 ms, elicited a significant enhancement of the second compared with the first evoked fEPSP, however ($t$-test: $P = 0.048, n = 3$, Fig. 3B,C) in line with previous in vitro reports (Kamiya and Ozawa 1998). We then applied paired-pulse stimulation at a 50-ms ISI after LFS (1 Hz, 600 pulses) to induce LTD, to evaluate the presynaptic involvement in synaptic plasticity at mossy fiber-CA3 synapses. After LFS, application of paired-pulse stimulation with a 50-ms ISI had no effect on the second fEPSP ($t$-test: $P = 0.65$, Fig. 3B,C).

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**Figure 2.** FF is highly frequency dependent. LFS at 0.25 (A, $n = 6$), 0.3 (B, $n = 7$), or 0.5 Hz (C, $n = 6$) has no significant effect on the amplitude of synaptic responses evoked at mossy fiber-CA3 synapses in the intact freely behaving rat. LFS at 1 Hz (D) or 2 Hz (E, $n = 9$) significantly increases the amplitude of synaptic responses evoked at mossy fiber-CA3 synapses in the intact freely behaving rat ($n = 16$). LFS at 2 Hz elicits an FF that is more variable than that seen with 1-Hz LFS. Analogs represent responses evoked with 0.5 Hz (upper traces) or 1 Hz (lower traces) pre-LFS (i), at the 30th (ii), and 300th (iii) pulse. Vertical scale bar: 4 mV; horizontal scale bar: 8 ms. Hippocampal slices showing the position of the stimulating (upper picture) and recording (lower picture) electrodes in the CA3 region and the mossy fiber pathway, respectively (F, black arrows).
LFS at 1 Hz (600 pulses) was characterized by an initial FF of maximally 190 ± 18.2% that endured for approximately 300 s. After the initial 5 min, the evoked potentials declined to an average of 133 ± 7%. Five minutes after the termination of LFS, a significant synaptic depression was observed with a maximal depression of the fEPSP of 64 ± 4.4% (compared with baseline values) that lasted for over 24 h (Fig. 4D). Reducing the number of pulses to 30 results in FF that has no significant effects on basal synaptic transmission (Klausnitzer and Manahan-Vaughan 2008), suggesting that the duration of FF is a decisive factor in determining whether LTD occurs.

A 2-Hz LFS induced a significant but unstable FF compared with that seen with 1-Hz LFS. Five minutes after termination of 2-Hz LFS, an fEPSP depression to 80 ± 10% (n = 9) compared with baseline values was noted that lasted for over 24 h (Fig. 4E). This response was significantly different compared with values obtained during application of test pulses (ANOVA, F(1,64) = 48.34; P < 0.0001) but was also significantly less than LTD elicited with 1-Hz LFS (P < 0.01).

**LTD Induced by LFS of Mossy Fiber Synapses In Vivo Is Reversed by Tetanic Stimulation**

To exclude that LTD induced as a consequence of LFS of mossy fibers is not a product of synaptic run-down, or other factors unrelated to plasticity, we assessed whether responses could be reversed by HFS at 100 Hz (n = 6). HFS was given 2 h after induction of LTD when responses had reached a steady plateau. A full reversal of LTD with a tendency toward synaptic potentiation became evident (Fig. 5). The potentiation effects endure for as long as recordings were conducted (>25 h after application of LFS. Effects were highly significant (ANOVA, F(1,78) = 70.81; P < 0.0001).

**Antagonism of Adenosine-A1 Receptors Enhances Mossy Fiber Transmission and Prevents FF Induced by Low-Frequency Stimulation**

Application of the adenosine-A1 receptor antagonist phenylxanthine (12.5 μg) significantly increased synaptic responses at mossy fiber–CA3 synapses compared with vehicle-injected controls (ANOVA, F(1,84) = 29.86; P < 0.0001, n = 7 and ANOVA, F(1,95) = 24.24; P < 0.0001, n = 8. Fig. 6A,B, respectively). This is consistent with previous reports using the same concentration in vivo (Klausnitzer and Manahan-Vaughan 2008). FF during stimulation at 1 Hz at mossy fiber–CA3 synapses was significantly occluded (ANOVA, F(1,16) = 130.17; P < 0.0001, n = 7) (Fig. 6C). Maximal FF reached similar levels to that seen under control conditions, but in contrast to controls, FF did not decline and was sustained at these high levels for the duration of LFS. LTD elicited by 1-Hz LFS in the presence of phenylxanthine was significantly enhanced (ANOVA, F(1,36) = 31.70, P < 0.0001). Furthermore, phenylxanthine significantly stabilized FF at 2 Hz (Fig. 6D) and enabled an LTD that was significantly larger and more stable (ANOVA, F(1,35) = 44.35, P < 0.0001, n = 5) (Fig. 6B).

We examined whether we could replicate these findings using another adenosine-A1 receptor antagonist. We found a concentration of 76 μg DPCPX to be effective in the occlusion of FF at mossy fibers in vivo (Klausnitzer and Manahan-Vaughan 2008). Application of this compound at the same concentration also effectively occluded FF in the present study. Similar to the application of phenylxanthine, DPCPX (76
l, n = 6) showed a significant increase of responses, evoked with test-pulse stimulation, immediately after injection compared with controls (ANOVA: \( F(1,56) = 75.76; P < 0.0001 \)) (Fig. 6E). The maximal level of FF correlated significantly to the subsequent degree of synaptic depression. (C), LFS at 0.25 (n = 9), 0.3 (n = 7), or 0.5 Hz (n = 6) does not result in lasting synaptic depression at mossy fiber-CA3 synapses in the intact freely behaving rat. LFS at 1 Hz (n = 10) or 2 Hz (n = 9) significantly induces lasting depression (>24 h) of synaptic responses evoked at mossy fiber-CA3 synapses in the intact freely behaving rat (n = 10). LTD elicited by 2-Hz LFS is weaker and less stable than that elicited by 1-Hz LFS (P < 0.01). (F) Analogs represent responses obtained pre-LFS (i) and 5-min post-LFS (ii) using stimulation at 0.25, 0.3, and 0.5 Hz or (G) pre-LFS (i), 5-min post-LFS (ii), and 24h post-LFS (iii) evoked using stimulation at 2 Hz. Vertical scale bar: 4 mV; horizontal scale bar: 8 ms.

**Discussion**

These data indicate that FF at mossy fiber-CA3 synapses may comprise an intrinsic component of the induction of LTD in the intact freely behaving rat. The occurrence of prolonged FF correlated with the successful induction of LTD, indicating that FF may play a role in both short-term and long-term information storage at these synapses. This LTD was reversed by HFS supporting that synaptic plasticity genuinely occurred. Antagonism of the adenosine-A1 receptor occluded FF, rendered unstable FF more stable, and facilitated LTD suggesting that the adenosine-A1 receptor may play a critical role in regulating the thresholds for FF-dependent information storage.

FF has been discussed as a means for holding synaptic information online (Bischofberger et al. 2006), as in in vitro studies FF endures only for as long as LFS is given (Salin et al. ...
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encoding in the dentate gyrus might translate into FF and LTD induction at mossy fiber synapses in the CA3 region, similar to that induced by afferent stimulation in our study.

PPF is a form of short-term synaptic plasticity, which is mediated by an increase in the probability of neurotransmitter release of the presynaptic synapse (Thomson et al. 1993). PPF is considerably enhanced at the mossy fiber-CA3 synapse (Salin et al. 1996; Henze et al. 2000). Paired-pulse stimulation at an interstimulus interval of 50, but not 20, 40, 100, 300, 500, or 1000 ms, elicited facilitation of the second evoked fEPSP compared with the first. This is in line with in vitro findings that reported that PPF was most effective at an ISI of 50 ms (Salin et al. 1996; Kamiya and Ozawa 1998).

It was striking that this effect was evident before application of LFS to induce LTD and not afterward. An ISI of 50 ms enhances action potential–driven Ca\textsuperscript{2+} influx into the presynaptic terminal, a process mediated by presynaptic kainate receptors (Kamiya et al. 2002), which are abundant at the mossy fiber–CA3 synapse.

PPF seen in this study, before application of LFS, may thus be mediated by the activation of presynaptic kainate receptors and facilitation of Ca\textsuperscript{2+} influx into the mossy fiber terminal, which

Figure 6. Adenosine-A1 receptor blockade increases evoked fEPSPs and enhances LTD. Solid lines show the original potentials after application of phenylxanthine in 1 Hz (A) and 2 Hz (B) experiments. Dashed lines show the potentials normalized to levels corresponding to field potentials prior to application of phenylxanthine (12.5 μg). Evoked responses obtained during 1 Hz (C) and 2 Hz (D) stimulation (600 pulses) after application of phenylxanthine. Phenylxanthine elevated evoked responses to approximately 120% of basal levels. FF elicited by LFS in the presence of phenylxanthine was no larger than that seen in controls, providing evidence of occlusion. FF became nondecremental and more stable however in the presence of the adenosine-A receptor antagonist. (C) in the presence of the adenosine-A1 receptor antagonist DPCPX (76 μg), evoked synaptic responses are significantly increased. Normalization of the responses to values obtained before application of DPCPX indicate a significant LTD that lasts over 24 h (n = 6). (D) Analogs represent responses obtained in the presence of phenylxanthine during application of 1 Hz (top row), 2 Hz (middle row), and during the DPCPX experiment (bottom row) (i) 5-min pre-sLFS, (ii) 5-min post-sLFS, and (iii) 24-h post-sLFS. Vertical scale bar: 4 mV, horizontal scale bar 2 ms.
results in an increase of probability of neurotransmitter release. After LFS, the ready-releasable pool of transmitters may be depleted such that paired-pulse stimulation may lead to an enhanced Ca\textsuperscript{2+}-influx but not to a higher probability of transmitter release. Subsequent presynaptic plastic changes may then lead to a sustained decrease in the amount of released neurotransmitter, which in turn enables the persistency of LTD, as observed in our study. Our findings with regard to PPF support the involvement of presynaptic mechanisms mediated by kainate receptors in the formation of FF and subsequent LTD at mossy fiber synapses in the freely behaving rat.

The adenosine-A1 receptor plays a critical role in the regulation of mossy fiber FF (Moore et al. 2003; Klausnitzer and Manahan-Vaughan 2008). We investigated to what extent the adenosine-A1 receptor is involved in the induction of LTD by prolonged FF. Consistent with previous reports, antagonism of adenosine-A1 receptors occluded FF (Klausnitzer and Manahan-Vaughan 2008). Interestingly, receptor antagonism was also associated with a stabilization of FF and facilitation of LTD. This suggests that the adenosine-A1 receptor may not only regulate FF but may also set the thresholds for long-term information storage through LTD in the CA1 region after inhibition of adenosine-A3 receptors (Pugliese et al. 2006). To our knowledge, no study so far has addressed the effects of adenosine-A3 receptor antagonism on mossy fiber plasticity. Recently, it was proposed that adenosine-A3 receptor activation reduced the sensitivity of presynaptic adenosine-A1 receptors in area CA1 in hippocampal slices of rats although no effect on synaptic plasticity was observed (Dunwiddie et al. 1997). In our study, antagonism of adenosine-A3 receptors reduced fEPSPs at mossy fiber-CA3 synapses but had no direct effect on LTD.

Two observations support that recordings derived predominantly from the mossy fiber synapse: Firstly, evoked potentials could be profoundly suppressed by application of the mGlu receptor agonist DCG-IV (Kamiya et al, 1993; Yeckel et al, 1999; Goussakov et al, 2000). Secondly, LTD evoked at these synapses was not sensitive to application of the NMDA receptor antagonist, AP5. The same concentration of AP5 was used that is effective in blocking LTP at medial perforant path-dentate gyrus and Schaffer collateral-CA1 synapses in the freely behaving rat. Finally, postmortem histological analysis was used to confirm correct electrode localization. Taken together, these findings suggest that recordings were obtained from the mossy fiber synapse in vivo.

Conclusion

This study provides the first observation that FF at mossy fiber-CA3 synapses of freely behaving adults rats is closely correlated with the appearance of persistent (>24 h) LTD. This suggests that FF may comprise an important functional component of long-term information storage at these synapses. The finding that antagonism of adenosine-A1 receptors occludes FF and facilitates mossy fiber LTD suggests that this receptor is an intrinsic element in the mechanisms underlying these effects.
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Address correspondence to Denise Manahan-Vaughan, PhD, Department of Experimental Neurophysiology, Medical Faculty, Ruhr University Bochum, MABF 01/551, Universitaetsstrasse, 150, 4780 Bochum, Germany. Email: dmv-igsn@rub.de.

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