Large Amplitude Miniature Excitatory Postsynaptic Currents in Hippocampal CA3 Pyramidal Neurons Are of Mossy Fiber Origin

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Henze, Darrell A., J. Patrick Card, German Barrionuevo, and Yezkiel Ben-Ari. Large amplitude miniature excitatory postsynaptic currents in hippocampal CA3 pyramidal neurons are of mossy fiber origin. J. Neurophysiol. 77: 1075-1086, 1997. Neonatal (P0) γ-irradiation was used to lesion selectively the mossy fiber (MF) synaptic input to CA3 pyramidal cells. This lesion caused a >85% reduction in the MF input as determined by quantitative assessment of the number of dynorphin immunoreactive MF boutons. The γ-irradiation lesion caused a reduction in the mean number of miniature excitatory postsynaptic currents (mEPSCs) recorded from CA3 pyramidal cells (2.292 vs. 1,429/min; n = 10). The lesion also caused a reduction in the mean mEPSC peak amplitude from 19.1 ± 0.45 to 14.6 ± 0.49 pA (mean ± SE; peak conductance 238.8 ± 5.6 to 182.0 ± 6.1 pS). Similarly, there was a reduction in the mean 10–85% rise time from 1.72 ± 0.02 ms to 1.42 ± 0.04 ms. The effects of the γ-irradiation on both mEPSC amplitude and 10–85% rise time were significant at P < 0.002 and P < 0.005 (2-tailed Kolmogorov-Smirnov test). Based on the selectivity of the γ-irradiation, MF and non-MF mEPSC amplitude and 10–85% rise-time distributions were calculated. Both the amplitude and 10–85% rise-time distributions showed extensive overlap between the MF and non-MF mediated mEPSCs. The MF mEPSC distributions had a mean peak amplitude of 24.6 pA (307.5 pS) and a mean 10–85% rise time of 2.16 ms. The non-MF mEPSC distributions had a mean peak amplitude of 12.2 pA (152.5 pS) and 10–85% rise time of 1.26 ms. The modes of the amplitude distributions were the same at 5 pA (62 pS). The MF and non-MF mEPSC amplitude and 10–85% rise-time distributions were significantly different at P ≈ 0.001 (1-tailed, large sample Kolmogorov-Smirnov test). The data demonstrate that the removal of the MF synaptic input to CA3 pyramidal cells leads to the absence of the large amplitude mEPSCs that are present in control recordings.

INTRODUCTION

One useful tool in investigations of the basic mechanisms of neurotransmission is the study of the spontaneous miniature excitatory postsynaptic currents (mEPSCs). mEPSCs are signals produced by the sodium action potential independent spontaneous release of neurotransmitter from the presynaptic terminal. The properties of mEPSCs, in particular frequency of occurrence and relative amplitude, offer a means to study the otherwise inaccessible presynaptic terminals within the CNS (Malgaroli and Tsien 1992; Manabe et al. 1992). However, the major drawback in the use of mEPSCs to study the presynaptic element is the difficulty in identifying the synaptic origin of an individual synaptic current. This is particularly problematic when studying complex neurons of the CNS that receive anatomically and physiologically heterogeneous populations of synapses such as is the case for CA3 pyramidal neurons (Amaral and Witter 1989; Blackstad 1956; Blackstad et al. 1970; Hjorth-Simonsen 1973; Hjorth-Simonsen and Jeune 1972).

CA3 pyramidal cells receive a major glutamatergic synaptic input from the granule cells of the dentate gyrus via the mossy fibers (MFs) (Neuman et al. 1988). The MFs make synaptic contacts on to the proximal dendrites of the CA3 pyramidal cells and, therefore, are believed to play a critical role in modulating activity in these cells. Numerous studies already have demonstrated that the MF to CA3 pyramidal cell synapse has unique features that distinguish it from other cortical synapses. For example, the MF synaptic terminal has a large presynaptic bouton (3–10 μm diam) with small finger-like extensions (Amaral 1979; Blackstad and Kjaerheim 1961; Hamlyn 1962) that completely envelopes a large, complex, postsynaptic “thorn.” Ultrastructural studies have revealed that a single presynaptic MF bouton has ≈35 separate active sites (Chicurel and Harris 1992). The MF presynaptic terminal also has a distinctive complement of neurotransmitters with the opioid dynorphin being colocalized with glutamate (Gall 1988). Finally, the presynaptic terminal of MFs is unique in that it can express at least two distinct forms of plasticity: a prolonged posttetanic potentiation (Langdon et al. 1995) and a N-methyl-D-aspartate (NMDA) receptor independent form of long-term potentiation (Castillo et al. 1994; Johnston et al. 1992; Katsuki et al. 1991; Langdon et al. 1995; Urban and Barrionuevo 1996; Xiang et al. 1994; Zalusky and Nicoll 1990).

Coincident with the presence of MF synapses, mEPSCs recorded from CA3 pyramidal cells are often an order of magnitude larger in amplitude than those observed from other cells that are similar anatomically and physiologically (e.g., CA1 pyramidal cells) (compare Jonas et al. 1993 with Malgaroli and Tsien 1992 or Manabe et al. 1992). To determine whether the MF synapses are the source of the large mEPSCs recorded from CA3 pyramidal cells, in the present study, we have taken advantage of a hippocampal slice preparation in which the granule cells and their axons, the MFs, are lesioned selectively (Dessi et al. 1991; Repressa et al. 1991). This selective lesion of the MFs was accomplished by focal γ-irradiation of neonatal rats; this has been shown to remove the majority (>85%) of MFs from the hippocampal formation. The selectivity of the γ-irradiation induced lesion is supported by a number of studies: CA3 dendritic arbors and spine density are not affected (Gaiarsa et al. 1992; Repressa et al. 1991), the number of NMDA binding sites in area CA3 is not affected (Dessi et al. 1991), and bicuculline...
induces “normal” epileptic bursting (Gaiarsa et al. 1994).
Using this approach, we have determined that the presence of the large mEPSCs is correlated with the presence of a MF synapse onto CA3 pyramidal cells.

METHODS

Irradiation

Newborn Wistar rats (postnatal day P0) were irradiated at 6 Gy (600 rad) by a cobalt bomb (Centre d’études nucléaires, Fontenay-au-Roses) using parameters previously shown to induce pronounced degeneration of granule cells in the dentate gyrus (Dessi et al. 1991; Repressa et al. 1991). The γ-rays were collimated to irradiate only one side of the brain. The nonirradiated contralateral side was used for control recordings.

Lesion characterization

TISSUE PROCESSING. The extent of γ-irradiation-induced cell and fiber loss in the hippocampus was characterized in six animals that were irradiated unilaterally as neonates (see above) and prepared for morphological analysis. These animals were anesthetized and killed by transcardiac infusion of buffered aldehyde fixative 28 days after γ-irradiation exposure. The perfusion procedure included an initial infusion of phosphate buffered saline (PBS; 0.1 M, pH 7.4) into the ascending aorta followed by 200–300 ml of paraformaldehyde-lysine-periodate fixative (McLean and Nakane 1974). The brain then was removed, postfixed for 2 h, washed in PBS, and cryoprotected in 20% phosphate buffered sucrose for ~72 h, all at 4°C. The forebrain was sectioned (35 μm/section) in the coronal plane through the rostro-caudal extent of the hippocampus using a freezing microtome. Sections were collected serially in six wells of phosphate buffer such that each well contained a one-in-six series of sections through the hippocampus. One well of tissue was mounted immediately on gelatin-coated slides, stained with cresyl violet, dehydrated, cleared, and coverslipped using standard procedures. This tissue was used to determine the relative amount of cell loss in the dentate gyrus of the irradiated hippocampus. Remaining tissue was transferred to an ethylene glycol-based cryopreservant (Watson et al. 1986) and stored at −20°C to preserve antigenicity for subsequent immunohistochemical processing.

The extent of MF degeneration in the stratum lucidum of the γ-irradiated hippocampus was evaluated by immunohistochemical localization of dynorphin immunoreactivity. Previous studies have demonstrated that MF boutons in the rat hippocampus contain high levels of dynorphin and that this peptide is a reliable marker of MF boutons. No effort was made to quantitate the degree of cell loss within the dentate gyrus of the irradiated hippocampus. Quantitative determinations of the extent of cell loss in the γ-irradiated hippocampus, and dynorphin immunoreactivity was used to evaluate the degree of MF loss in the afflicted stratum lucidum. In each case, the dentate gyrus ipsilateral and contralateral to the irradiated side of the brain were compared within each animal. As noted above, dynorphin immunoreactivity in the stratum lucidum was used as the measure of MF bouton loss in the irradiated hippocampus. Subjective determinations of the extent of the lesion were made by comparing the density of staining on the control and lesion sides. Quantitative determinations of the magnitude of MF bouton loss on the lesioned side also were conducted using the following procedure. Three comparable 100 μm2 segments of stratum lucidum were identified in multiple changes of PBS to remove the cryopreservant, and the tissue was incubated in primary antiserum diluted in PBS to a final concentration of 1:2,000 with PBS, normal donkey serum (1%), and Triton X-100 (0.3%). The incubation was conducted at 4°C for 24–48 h. Antigen localization was visualized with the avidin-biotin modification of the immunoperoxidase procedure (Hsu et al. 1981) using an affinity-purified donkey anti-rabbit secondary antibody (Jackson ImmunoResearch) and Vectastain Elite reagents (Vector Laboratories). Further details regarding application of this procedure in our laboratory have been published (Card and Enquist 1994). Processed tissue was mounted on gelatin coated slides, dehydrated, cleared, and coverslipped.

Tissue analysis

Cresyl violet stained preparations were used to determine the qualitative extent of cell loss in the γ-irradiated hippocampus, and dynorphin immunoreactivity was used to evaluate the degree of MF loss in the afflicted stratum lucidum. In each case, the dentate gyrus ipsilateral and contralateral to the irradiated side of the brain were compared within each animal. As noted above, dynorphin immunoreactivity in the stratum lucidum was used as the measure of MF bouton loss in the irradiated hippocampus. Subjective determinations of the extent of the lesion were made by comparing the density of staining on the control and lesion sides. Quantitative determinations of the magnitude of MF bouton loss on the lesioned side also were conducted using the following procedure. Three comparable 100 μm2 segments of stratum lucidum were identified in the control and irradiated sides of the brain, midway through the rostro-caudal extent of the hippocampus. The relative position of these three regions are illustrated in the schematic diagram shown in Fig. 1. Varicose boutons in each of these regions displaying dynorphin immunoreactivity were mapped using a drawing tube and a ×40 objective on a Nikon Optiphot microscope. The number of boutons were counted in each area and quantitative comparisons of counts of lesion and control sides were made to determine the degree of MF bouton loss in the irradiated hippocampus. No effort was made to quantitate the degree of cell loss within the γ-irradiated dentate gyrus.

In vitro slice preparation

Slices were prepared from the γ-irradiated rats 27 to 40 days old. Briefly, animals were anesthetized with equithesin and their chest cavities were opened rapidly. The rats then were transcardially perfused with a cold oxygenated sucrose solution with the following composition (in mM): 229 sucrose, 1.9 KCl, 1.2 Na2PO4, 11 glucose, 25 NaHCO3, 3.0 MgCl2, and 3.0 CaCl2. The ACSF was bubbled with 95% O2-5% CO2 to adjust the pH to 7.4. After 1 min of transcardial perfusion, the rats were decapitated and the hippocampi dissected from the brain. Each hippocampus then was sliced into 400-μm-thick slices perpendicular to its long axis using a vibrotome. This sucrose perfusion technique originally was described as a method to improve the viability of facial motoneurons in in vitro slices taken from rat brain stem (Aghajanian and Rasmussen 1989). We found that the sucrose perfusion also improves the viability neurons of area CA3 in hippocampal slices.

After sectioning, slices were maintained at room temperature (19–23°C) in an artificial cerebrospinal fluid (ACSF) consisting of (in mM) 126 NaCl, 3.5 KCl, 1.2 Na2PO4, 11 glucose, 25 NaHCO3, 3.0 MgCl2, and 3.0 CaCl2. The ACSF was bubbled with 95% O2-5% CO2 (pH to 7.4). Individual slices were transferred as

FIG. 1. Areas examined to quantify dynorphin labeling of mossy fibers (MFs). A schematic diagram of the transverse hippocampal slice illustrating 3 regions used to quantify the effect of γ-irradiation on dynorphin labeled MF boutons.

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Automated mEPSC detection algorithm from the same animals. The cell loss was apparent in both mEPSC foot and peak detected by the search algorithm. The analysis changes in either cell morphology, number, or distribution.

mEPSCs were detected by scanning for peaks in the first derivative comparing Fig. 3, long recording epochs. The algorithm first calculated the first and other derivatives using boxcar averaging. Peaks in 1st derivative exceeding 5 pA/ms indicate an event has occurred (1). After detection of an mEPSC, its amplitude and rise time is determined as follows: peak of mEPSC is determined by scanning the 1st derivative forward for a 0 crossing (2). Foot of mEPSC is determined by scanning backward in the 3rd derivative for a 0 crossing (3). Amplitude change between points 2 and 3 represents mEPSC amplitude; 10–85% rise times also are calculated using points 2 and 3 as 0 and 100%.

needed (between 2 and 12 h after slice preparation) to a submerged chamber where they were constantly superfused with oxygenated ACSF at 33°C.

Electrophysiological recording

Spontaneous mEPSCs were recorded using the “blind” whole cell recording technique (Blanton et al. 1989). Recordings for both control and γ-irradiated groups were from CA3 pyramidal cells (verified in some cases by filling cells with biocytin) in the same area used to quantify the amount of dynorphin immunoreactivity (see Fig. 1). The mEPSCs were recorded using a voltage-clamp amplifier, filtered at 3 kHz, digitized at 10 kHz, and then stored on computer disk in 3-min epochs. The recordings also were collected simultaneously to DAT tapes, which provided a continuous record of the experiment. The analysis presented is based on one 3-min epoch per cell selected for having a relatively stable baseline and stable series resistance. Whole cell pipettes were filled with a solution consisting of (in mM) 50 CsF, 80 CsCl, 10 HEPES, and 1.0 bis-(o-aminophenoxy)-N,N,N′,N′-tetraacetic acid·K2, with a pH of 7.0 and final osmolarity of 270 mOsm. Pipettes had a resistance of 3–6 MΩ. Membrane potentials were voltage clamped at −80 mV. Data were rejected if the access resistance was >20 MΩ or a significant change (>15%) in whole cell series resistance was observed. All recordings were carried out in the presence of 1 μM tetrodotoxin and 10 μM bicusculine methiodide (Sigma).

Automated mEPSC detection algorithm

The detection and measurement of the mEPSCs was accomplished using an automated routine to permit the rapid analysis of long recording epochs. The algorithm first calculated the first and third derivatives of the raw data trace with respect to time. Then mEPSCs were detected by scanning for peaks in the first derivative which corresponds to the rising slopes of the mEPSCs. For all recordings, an mEPSC was detected if its rising slope >5 pA/ms. After the detection of a mEPSC, the mEPSC peak was determined from the first 0 crossing after the peak in the first derivative. The foot of the mEPSC was determined from the 0 crossing in the third derivative preceding the peak in the first derivative (Fig. 2). The amplitude and 10–85% rise time were determined using the mEPSC foot and peak detected by the search algorithm. The analysis of all mEPSCs included “smoothing” the raw data and its subsequent derivatives using boxcar averaging with an 11-point window. The detection algorithm was developed and run using Igor Pro (Wavemetrics) on an accelerated Macintosh Ici. This algorithm is similar to a published automated detection algorithm for detecting miniature inhibitory postsynaptic potentials (Ankri et al. 1994). All values given in the text are means ± SEM, unless otherwise indicated. The accuracy of the mEPSC detection algorithm was evaluated by comparing the automated results with results collected manually. We considered three types of error: 1) the number of false positives. The false positives were determined for a single 60-s period where the algorithm found 1,341 events. Of the detected 1,341 events, only 7 of those events (0.52%) could be rejected as events not having an mEPSC-like appearance. 2) The number of overlapping doublets that were not resolved. The overlapping doublets for the same 60-s period was 57. The lack of detection of these events can be explained by the fact that these doublets, although reflected on the rising phase, did not reverse direction and therefore did not have a 0 crossing in the first derivative. 3) The number of false negatives. There were numerous small events that had an mEPSC-like appearance that were not detected by the algorithm. These undetected events were always very small (<5 pA) and, by definition, had rising slopes <5 pA/ms. Although many of these small mEPSCs are recognizable by eye, it is impossible to establish a set of criteria to identify all of them because they recede into the noisy baseline and therefore this error is not quantifiable.

RESULTS

Anatomic effects of γ-irradiation lesion

Clear changes in the cellular organization of the hippocampus were apparent in cresyl violet-stained coronal sections passing through the same portion of the rostrocaudal axis that was selected for electrophysiological analysis in other animals. As previously reported (Dessi et al. 1991; Gaiarsa et al. 1992, 1994; Repressa et al. 1991), the most obvious alterations were a profound cell loss in the dentate gyrus and a decreased packing density of pyramidal cells in the CA1 subfield of each γ-irradiated hippocampus. These changes were quite consistent between animals and were distinguished easily when comparing the γ-irradiated and control hippocampus within the same animal (Fig. 3, A and B). Below, we focus on the γ-irradiation induced cell loss in the dentate gyrus and the resultant alterations of the MF projection to stratum lucidum.

γ-irradiated tissue exhibited extensive cell loss in the granule cell layer of the dentate gyrus and a corresponding decrease in the thickness of the molecular cell layer, changes that were not present in the contralateral, nonirradiated tissue from the same animals. The cell loss was apparent in both the supra- and infrapyramidal blades of the granule cell layer and occurred throughout the rostrocaudal axis of hippocampus. The characteristic pattern and magnitude of cell loss in the dentate gyrus of all experimental animals is apparent in comparing Fig. 3, C and D. These photomicrographs of cresyl violet-stained preparations from a single animal illustrate the extensive cell loss in the irradiated hippocampus (Fig. 3D) compared with the nonirradiated control hippocampus (Fig. 3C). In contrast, the immediately adjacent polymorphic neurons of the hilus and the pyramidal cells of area CA3 exhibited no apparent γ-irradiation-induced changes in either cell morphology, number, or distribution,
FIG. 3. Effect of \( \gamma \)-irradiation on dentate gyrus and stratum lucidum of hippocampus. Morphology of control (A and C) and irradiated (B and D) hippocampus are illustrated in cresyl violet stained coronal sections. The same, which received unilateral neonatal irradiation, was the source for all of photomicrographs. Comparison of A and B reveals profound \( \gamma \)-irradiation-induced cell loss in dentate gyrus and a splaying of the CA1 pyramidal cell layer (small open block arrows in B). The extent of granule cell loss and the accompanying reduction in thickness of the molecular layer (demarcated by bars with arrowheads) resulting from irradiation is more readily apparent in comparing C and D. Scale bars: 440 \( \mu \)m in B for A and B; 160 \( \mu \)m in D for C and D.

A finding consistent with previous reports (Dessi et al. 1991; Gaiarsa et al. 1992, 1994; Repressa et al. 1991).

\( \gamma \)-Irradiation-induced changes in the number of dynorphin immunoreactive MF boutons in the s. lucidum were dramatic and consistent across animals. In the nonirradiated control hippocampus, a broadband of dynorphin immunoreactive varicose fibers filled the stratum lucidum adjacent to the CA3 pyramidal neurons (Fig. 4, A and C). Scattered individual immunoreactive fibers also were observed within the stratum pyramidale. This was in marked contrast to the decrease in immunoreactive varicosities in comparable regions of the contralateral, \( \gamma \)-irradiated hippocampus (Fig. 4, B and D). The immunoreactive boutons in the \( \gamma \)-irradiated hippocampus were reduced substantially throughout the full extent of CA3. Comparative counts of the number of immunoreactive varicosities within each of three 100-\( \mu \)m\(^2\) areas (see Fig. 1) of the stratum lucidum in control and irradiated hippocampus confirmed that there was a substantial decrease of these profiles on the irradiated side. These counts revealed that there was an \( \sim 84\% \) decrease in the number of immunoreactive varicosities irrespective of whether the data were analyzed in individual 100 \( \mu \)m\(^2\) bins or grouped together (Table 1).

Electrophysiological effects of \( \gamma \)-irradiation lesion

The present analysis compares recordings from cells in 10 slices from control and 10 slices from \( \gamma \)-irradiated animals. All recordings were collected under voltage-clamp conditions with the holding potential set to \(-80\) mV. Application of 10 \( \mu \)M 6-cyano-7-nitroquinoxaline-2,3-dione completely abolished all observed mEPSCs, indicating that they are mediated by glutamatergic \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation (data not shown). Figure 5 illustrates the effect of \( \gamma \)-irradiation on the overall frequency of mEPSCs recorded from two selected CA3 cells. When the results from the 10 recordings from each condition are considered together, the mean frequency of mEPSCs decreased from \( \sim 12.5 \) to \( 7.8 \) Hz. This decrease was not statistically significant for \( P < 0.05 \) (1-tailed, Student’s \( t \)-test, \( P = 0.082 \)) primarily due to the large variation in mEPSC frequency between recordings (standard deviations were \( 7.5 \) and \( 7.0 \) Hz for control and \( \gamma \)-irradiated tissue, respectively). The number of mEPSCs detected at all amplitudes and rise times were reduced by the \( \gamma \)-irradiation treatment, however, larger amplitude (Fig. 6A) and slower rising (Fig. 6B) mEPSCs showed a greater decrease. The mean amplitude decreased from 19.1 ± 0.45
FIG. 4. Effect of $\gamma$-irradiation on dynorphin labeled MF boutons. Dynorphin immunoreactivity in MFs of control (A and C) and irradiated (B and D) tissue is illustrated. The extent of dynorphin immunoreactive profiles in coronal sections through control and irradiated hippocampus are shown in A and B, respectively. C and D are higher magnification views of dynorphin-immunoreactive fibers in stratum lucidum of control and irradiated tissue. A thick band of dynorphin immunoreactivity adjacent to proximal portions of apical dendrites of CA3 pyramidal cells defines stratum lucidum in control tissue (A and C). In contrast, irradiation-induced degeneration of dentate granule cells causes a pronounced reduction in both density and thickness of stratum lucidum, as defined by dynorphin immunoreactive fibers (B and D). The densest concentration of dynorphin immunoreactive fibers in control and irradiated tissue is defined by regions between arrowheads on right-hand border of C and D. Scattered immunoreactive boutons outside of this zone in irradiated animal are indicated by open block arrows in D. Scale bars: 440 $\mu$m in B for A and B; 14 $\mu$m in D for C and D.

pA in control to 14.6 $\pm$ 0.49 pA ($P < 0.05$, 1-tailed Student’s $t$-test) in $\gamma$-irradiated tissue (corresponding to mean peak conductances of 238.8 $\pm$ 5.6 pS and 182 $\pm$ 6.1 pS, respectively). The median peak amplitude also was decreased in irradiated records, going from 13.9 to 11.2 pA (173.8 to 140 pS). The distributions of mEPSC amplitudes

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FIG. 5. Effect of γ-irradiation on mEPSCs recorded from hippocampal CA3 cells. Examples of raw data collected from control and γ-irradiated slices. A, top: 3 min of a voltage-clamp (V_h = −80 mV) whole cell recording from a control CA3 pyramidal cell (1 μM tetrodotoxin and 10 μM bicuculline present). A, bottom: similar recording from a γ-irradiated CA3 pyramidal cell. B: an example of raw data from a control recording at a higher gain. Synaptic currents were abolished by application of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (data not shown).

...are not normally distributed as reflected by the high skew values of 4.3 and 5.5 for control and γ-irradiated tissue, respectively. The mean distribution of mEPSC amplitudes from γ-irradiated animals was significantly different from the mean distribution from control animals (P < 0.002, 2-tailed Kolmogorov-Smirnov test, 1 pA bins). It can be seen from both the standard and cumulative probability histograms that the relative differences between the amplitudes of mEPSCs from control and γ-irradiated tissue are greatest for larger amplitudes. With respect to the 10–85% rise-time distributions, the mean 10–85% rise time decreased from 1.72 ± 0.02 ms in control to 1.42 ± 0.04 ms (P < 0.05, 1-tailed Student’s t-test) in γ-irradiated tissue. γ-Irradiation also decreased the median 10–85% rise time from 1.44 to 1.16 ms. The distributions of mEPSC 10–85% rise time also are not normally distributed as reflected by the high skew values of 2.3 and 2.5 for control and γ-irradiated tissue, respectively. The mean distribution of mEPSC 10–85% rise times from γ-irradiated animals was significantly different from the mean distribution from control animals (P < 0.005, 2-tailed Kolmogorov-Smirnov test, 0.1-ms bins). It can be seen from both the standard and cumulative probability histograms that the relative differences between 10–85% rise times of mEPSCs from control and γ-irradiated tissue differ the most for longer rise times. Figure 7 illustrates the amplitude and rise-time distributions for those mEPSCs that were absent from recordings from γ-irradiated tissue. These distributions were obtained by simply subtracting the respective irradiated histogram from the respective control histogram. To the extent that the γ-irradiation-induced lesion is selective for MFs, these histograms nominally represent the distributions for mEPSCs arising from MF synapses removed by the lesion (see DISCUSSION). Figure 8 illustrates the general appearance of the large and/or slow mEPSCs that are reduced greatly or absent from γ-irradiated tissue. The examples shown in Fig. 8 are also representative of the few large mEPSCs that were observed in γ-irradiated tissue.

DISCUSSION

Previous work has shown that destruction of the granule cells by postnatal γ-irradiation selectively reduces the MF input to area CA3 (Dessi et al. 1991; Gaiarsa et al. 1992, 1994; Repressa et al. 1991). Using this preparation, we have demonstrated that the large amplitude and/or slow rising mEPSCs recorded from CA3 pyramidal cells are correlated with the presence of normal, intact MFs.

Specificity of the γ-irradiation-induced lesion

In the brain of the postnatal rat, there are only a few populations of cells that have not finished their final division and therefore are sensitive to γ-irradiation. These cells include the granule cells of the olfactory bulb, the granule cells of the cerebellum, and the granule cells of the dentate gyrus (Bayer and Altman 1975b). Focal application of the radiation permits the specific lesioning of the granule cells of the dentate gyrus (Bayer and Altman 1975a). Other studies have shown that within the γ-irradiated zone, only the dentate gyrus granule cells and their MF input to area CA3 pyramidal cells are affected, whereas all other ionotropic synaptic inputs, both excitatory and inhibitory, remain unchanged (Dessi et al. 1991; Gaiarsa et al. 1994). The best
FIG. 6. Effect of $\gamma$-irradiation on observed mEPSC amplitude and rise-time distributions. Average histogram of mEPSC amplitudes (A) and 10–85% rise times (B) recorded from control and $\gamma$-irradiated tissue ($n = 10$ group; $\pm$SE). Note $\gamma$-irradiation affects all amplitudes and rise times. Both histograms are truncated for presentation purposes as illustrated by broken axes. For amplitude, single events measuring $\leq 400$ pA were recorded (an average of 16.9 mEPSCs with amplitudes $>100$ pA in control and 5.4 mEPSCs in $\gamma$-irradiated recordings, single bins after axis break). For rise time, single events measuring $\leq 14$ ms were recorded (an average of 7.6 mEPSCs with 10–85% rise times $>7$ ms in control and 0.7 mEPSCs in $\gamma$-irradiated recordings, single bins after axis break). Amplitude and 10–85% rise-time histograms are significantly different at $P < 0.002$ and $P < 0.005$, respectively (2-tailed Kolmogorov-Smirnov test). Insets: control vs. $\gamma$-irradiated cumulative probability histograms. mEPSCs from $\gamma$-irradiated slices display narrower cumulative distributions of amplitude and rise time.

Evidence that sprouting of associational/commissural synapses is not significant is the observation that there is no change in NMDA receptor density (as measured by immunohistochemistry) in the stratum oriens (Dessi et al. 1991). Other reports also suggest that non-MF synapses do not sprout as reflected by the loss of all spinelike structures on the CA3 pyramidal cell in the stratum lucidum (Gaiarsa et al. 1992; Repressa et al. 1991). It is important to point out that a small amount of sprouting of glutamatergic inputs from the associational collaterals does not affect our conclusion that large and/or slow mEPSCs arise from the MF synapses. Sprouting of associational synapses would only serve to counter the observed decrease in the frequency of mEPSCs and minimize the apparent effect of the $\gamma$-irradiation lesion. With respect to the MFs, the 84% reduction in the number of dynorphin positive boutons in the stratum lucidum is exactly what is predicted given the reduction in granule cells, and can be taken as a strong indication that there is no MF sprouting. Although it is possible that the $\gamma$-irradiation treatment may affect the ability of residual MFs
to express dynorphin, this is unlikely because the residual MF boutons in γ-irradiated tissue appear to express dynorphin at control levels. These data are in agreement with previous reports showing a drastic reduction in Timm staining in the stratum lucidum after γ-irradiation (Repressa et al. 1991). Timm staining reveals Zn²⁺, which has been shown to exist at elevated levels in the mossy fiber terminals.

With respect to changes in nonglutamatergic neurotransmission, we have observed an increase in cholinergic innervation of the γ-irradiated hippocampus (data not shown), as has been observed for colchicine-induced DG lesions (Repressa and Ben-Ari 1989). We determined that the increased cholinergic innervation did not play a role in causing the observed changes in the mEPSC amplitude and rise-time histograms because adding the cholinergic antagonist scopalamine had no effect on mEPSCs recorded from γ-irradiated tissue (data not shown).

A possible nonspecific effect of the γ-irradiation treatment is that it could decrease the amplitude of non-MF mEPSCs. This is highly unlikely for at least three reasons. First, Gaiarsa et al. (1994) have shown that both excitatory AMPA- and NMDA-receptor-mediated neurotransmission at non-MF synapses are unchanged with respect to control. In addition, they have shown that both γ-aminobutyric acid A (GABA_A) and GABA_B-mediated neurotransmission are unaffected. Second, the irradiation treatment induces the lesion by disrupting the mitotic process of granule cells (Hicks and D’Amato 1966). Because most of the non-MF synapses are formed after the γ-irradiation treatment, there can be no direct effect of the treatment on the synapses. Finally, previous work also has shown that the only change in the dendritic morphology of CA3 pyramidal cells after the γ-irradiation lesion is the loss of the large thorny excrescences that normally receive the MF input (Gaiarsa et al. 1992; Repressa et al. 1991). It is not likely that the loss of the thorny excrescences could, in and of itself, account for the observations in this study because the loss of the extra membrane and channels within that membrane would only serve to increase input resistance and therefore increase the observed amplitude and decrease the observed rise time of distal synaptic signals.
The relative probability of an mEPSC of a given amplitude or rise time arising from either the MF or non-MF synapses is illustrated in Fig. 9, B and D.

Examination of these calculated histograms indicate that, on average, MF mEPSCs are larger and slower than non-MF mEPSCs although there is extensive overlap of the distributions for these two classes of synapse. The mean MF mEPSC peak amplitude is twice that of non-MF mEPSCs (24.6 vs. 12.2 pA corresponding to 307.5 vs. 152.5 pS). The median MF mEPSC amplitude is also twice that of the non-MF mEPSC (17.5 vs. 9.0 pA corresponding to 218 vs. 112 pS). The two amplitude distributions are significantly different ($P < 0.001$) using a one-tailed, large sample Kolmogorov-Smirnov test (1-pA bin size). The distributions also indicate that, on average, the MF mEPSCs are slower rising than non-MF mEPSCs. The mean MF mEPSC 10–85% rise time is twice that of the non-MF mEPSCs (2.16 vs. 1.26 ms). The median MF mEPSC 10–85% rise time is also twice that of the non-MF mEPSC (1.8 vs. 0.97 ms). The two amplitude distributions are significantly different ($P < 0.001$) using a one-tailed, large sample Kolmogorov-Smirnov test (0.1-ms bin size).

The mean MF mEPSC amplitude reported above is larger than that (16.1 pA) previously reported by Jonas et al. (1993). This difference is best explained by the fact that in that report, mEPSCs were determined to be of MF origin using a 20–80% rise-time criteria of $\leq 0.8$ ms. This approach to identify MF mEPSCs would exclude many MF mEPSCs that have longer rise times, which can be very large in amplitude (see Fig. 8).

As expected, the mEPSC amplitude histogram for non-MF synapses is very similar to published values for mEPSCs recorded from CA1 pyramidal cells (Malgaroli and Tsien 1992; Manabe et al. 1992). This was expected for two reasons. First, collaterals of the same CA3 pyramidal cell axons provide input to both CA1 and CA3, and second, the morphological structure of CA1 and CA3 pyramidal cells, while not identical, is similar with respect to the overall electronic locations of synapses, particularly for proximal synapses. We found that the maximal non-MF mEPSC amplitude was $\approx 30$ pA, similar to values reported for recordings from area CA1 pyramidal cells (Chavez-Noriega and Stevens 1994; Malgaroli and Tsien 1992; Manabe et al. 1992). This was expected for two reasons. First, collaterals of the same CA3 pyramidal cell axons provide input to both CA1 and CA3, and second, the morphological structure of CA1 and CA3 pyramidal cells, while not identical, is similar with respect to the overall electronic locations of synapses, particularly for proximal synapses. We found that the maximal non-MF mEPSC amplitude was $\approx 30$ pA, similar to values reported for recordings from area CA1 pyramidal cells (Chavez-Noriega and Stevens 1994; Malgaroli and Tsien 1992; Manabe et al. 1992). Thus it is impossible that the approximate 10-fold difference in maximal mEPSC amplitude observed between MF and non-MF mEPSCs is strictly due to the dendritic locations of the synapses. Instead, large mEPSCs recorded from CA3 pyramidal cells are due to some special property of neurotransmission at the MF synapse (see below).

Properties of mossy fiber mEPSCs

The apparent quantitative mismatch between an 85% reduction in MF synaptic input and the lack of a statistically significant change in overall mEPSC frequency can be explained by the anatomy and biophysics of CA3 pyramidal cells and their inputs. Each CA3 pyramidal cell receives $\approx 46$ MF boutons (Amaral et al. 1990) and each bouton has $\approx 15$ release sites (Chicurel and Harris 1992); thus each CA3 pyramidal cell receives $\approx 700$ MF release sites. This
is in contrast to the estimated 12,000 release sites from the commissural/associational collaterals and the perforant path (Amaral et al. 1990). This works out to be that <4% of the total synaptic input to a typical CA3 pyramidal cell is of MF origin. In light of the anatomically small contribution of the MFs to the total number of synapses on a CA3 cell, one might also question why any effect of the \(\gamma\)-irradiation was seen at all. Whereas synapse numbers are negligible the detection of non-MF mEPSCs, the electrotonic filtering properties of the dendrites favor the detection of MF mEPSCs by reducing the contribution of the distal non-MF synaptic inputs to below detectable levels. The interaction between the number of synapses for MFs and non-MFs and the differential electrotonic filtering leads to the observed relative contribution by each of these two types of synapses to the overall level of mEPSC activity.

An unexpected outcome of this study is that MFs give rise to the slowest rising mEPSCs. Because of its close electrotonic location, it has long been held that evoked MF synaptic EPSCs should be of large amplitude and fast rise time, whereas evoked non-MF EPSCs should be smaller and slower (Brown and Johnston 1983; Johnston and Brown 1983; Rall et al. 1967). It is important to point out that the mEPSCs we attribute to the MFs are not necessarily large and slow because both large and fast, as well as, large and slow mEPSCs are reduced by \(\gamma\)-irradiation treatment. This observation is supported by the lack of a correlation between mEPSC amplitude and rise time when plotted against each other (data not shown). The lack of a significant shape-index correlation suggests that the contribution of electrotonic filtering to variations in mEPSC size and shape is relatively minor. Due to the problems inherent in whole cell recording from large neurons of the CNS (e.g., access resistance and dendritic cable filtering), the reported abso-
mEPSC rise times should be considered with care. However, due to the comparative nature of our study, the validity of the conclusion that \( \gamma \)-irradiation removes a population of slower rising mEPSCs is unaffected by these technical issues.

Another explanation for the presence of long MF rise times is that our automated detection method cannot resolve individual mEPSCs that have overlapping rising phases, which it then would count as a single large slow event. This, however, cannot account for the data because we found that the detection algorithm misses relatively few mEPSCs (57 missed, 1,341 detected per 60-s period) that we would have included when inspected by eye with no bias toward missing overlapping events. Although the algorithm does fail to resolve some overlapping mEPSCs, when the data traces are inspected visually, there do exist many monophasic mEPSCs with amplitudes >250 pA and/or rise times >5 ms as shown in Fig. 8.

A possible presynaptic mechanism by which the MF synapse could give rise to the observed large and/or slow mEPSCs (‘‘maxi’’-mEPSCs) could involve multiquantal release from the multiple active zones of the presynaptic bouton. This proposed mechanism requires the presence of a synchronizing signal that would cause the simultaneous or near-simultaneous release of multiple quanta. Experiments searching for the mechanism of the unique maxi-mEPSCs are currently underway.

Summary

The major goal of this study was to test the hypothesis that the large mEPSCs observed in recordings from CA3 pyramidal cell arise from synapses made by the MFs of the dentate gyrus granule cells. We have shown that \( \geq 95\% \) of mEPSCs with amplitudes >45 pA are from the \( \gamma \)-irradiation-sensitive MF synapses. The data also suggest that 95% of the events with 10–85% rise times >4.5 ms are from the MF synapses. These data provide evidence for yet another unique feature of neurotransmission at the MF synapse, specifically that the MFs give rise to a population of unusually large and/or slow mEPSCs. Further work elucidating the mechanisms that give rise to these maxi-mEPSCs will determine the relevance of these events.

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