SPROUTING OF MOSSY FIBERS AND PRESYNAPTIC INHIBITION BY GROUP II METABOTROPIC GLUTAMATE RECEPTORS IN PILOCARPINE-TREATED RAT HIPPOCAMPAL SLICE CULTURES

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Abstract—Mossy fibre sprouting (MFS) is a phenomenon observed in the epileptic hippocampus. We have studied MFS, in 7, 14 and 21 day *in vitro* (DIV) organotypic slice cultures, or in slice cultures treated with pilocarpine (0.5 mM) or pilocarpine and atropine (0.1 mM or 0.5 mM) for 48–72 h at 5 DIV and tested at 21 DIV.

Acute application of pilocarpine directly activated hilar neurons and elicited epileptic-like discharges in CA3 pyramids and mossy cells of 5–8 DIV cultures, without causing substantial cell death, as assessed by lactate dehydrogenase measurements.

Timm staining revealed increases in MFS in chronic pilocarpine-treated cultures, which was prevented by prior application of atropine. Extracellular synaptic responses were recorded in the granule cell layer and elicited by antidromic mossy fibre stimulation. The GABA$_A$ antagonist 6-imino-3-(4-methoxyphenyl)-1(6*H*)-pyridazinebutanoic acid (1 μM) induced a greater increase in the coastline bursting index in pilocarpine-treated cultures than in 21 DIV controls. However, there was no significant increase in the frequency of spontaneous or miniature synaptic events recorded in granule cells from pilocarpine-treated cultures. Granule cells were filled with biocytin and morphometric analysis revealed that the length of axon collaterals in the granule and molecular layer was longer in pilocarpine-treated cultures than in 21 DIV controls.

Dual recordings between granule cells and between granule and hilar neurons showed that pilocarpine-treated cultures had a larger proportion of monosynaptic and polysynaptic connections. The group II metabotropic glutamate receptor (mGluR) agonist LY354740 (0.5 μM) suppressed excitatory but not inhibitory monosynaptic currents. LY354740 also inhibited antidromically evoked action currents in granule cells from pilocarpine- and to a lesser extent in pilocarpine and atropine-treated cultures, suggesting that group II mGluRs can reside along the axon and suppress action potential invasion.

We provide direct evidence for the development of functional MFS and suggest a novel, axonal mechanism by which presynaptic group II mGluRs can inhibit selected synapses. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: granule cell, interneuron, paired-recording, spontaneous synaptic transmission, unitary synaptic transmission, metabotropic glutamate receptor.

A major form of plasticity seen in the epileptic hippocampus is mossy fibre sprouting (MFS; Margerison and Corsellis, 1966; Engel et al., 1997; McNamara, 1999; Scharfman, 2002), which is characterised by an abnormal projection of the mossy fibre axon collaterals of granule cells into the inner molecular layer of the dentate gyrus of the hippocampus (Sutula et al., 1989; Scharfman, 2002). These rearrangements could lead to the formation of recurrent excitatory connections between granule cells and thus hyperexcitability within the cell population.

MFS has been reported in animal models of temporal lobe epilepsies (e.g. Tauck and Nadler, 1985; Represa et al., 1987; Cronin and Dudek, 1988; Sutula et al., 1988; Buckmaster and Dudek, 1999). In these models seizures are typically induced by application of neurotoxins such as kainic acid or pilocarpine, and experiments are performed either *in vivo*, or on acute slices prepared from treated animals, weeks to months after the treatment. With this approach, a substantial amount of data has been collected (Sperk, 1994; McNamara, 1999). For example, it has been shown anatomically that sprouted mossy fibers do actually form synapses on granule cell dendritic spines and shafts (Buckmaster and Dudek, 1997; Wenzel et al., 2000). Interestingly, it has also been observed that MFS can target dentate basket cells or hilar interneurons, hence promoting a net inhibition rather than excitation and providing further targets for the sprouted fibers (Ribak and Peterson, 1991; Sloviter, 1992; Kotti et al., 1997). Recent evidence, however, suggests that MFS leads to the formation of a novel recurrent excitatory circuit (Buckmaster et al., 2002), and is also associated with the degeneration of some parvalbumin and/or somatostatin positive GABAergic interneurons (Kobayashi and Buckmaster, 2003).

Aside from its implications in epilepsy, MFS itself is an interesting phenomenon, as it shows that the adult brain does have some capacity for plasticity and that the molecular cues required are present within mature brain tissue.

The developing mammalian brain has a marked capacity for plasticity, and in fact, the sprouting of fibers is an integral part of CNS development (Haydon and Drapeau, 1995). However, with the exception of a few studies (for example, Jacobs et al., 2000; Ben-Ari, 2001), the development and consequences of MFS in the immature brain have been less investigated than those in the adult brain, and are consequently less understood.

The present study aimed to characterise the changes in granule cell excitability and anatomy that occur during the initial stages of MFS in developing brain tissue. Organotypic slice cultures are ideal for studying MFS *in vitro* as one can observe plastic changes occurring within weeks in an anatomically organised space (Gutierrez and Heinemann, 1999; Routbort et al., 1999; Bausch and McNamara, 2000). Interestingly, the slice culture preparation produces denervation of granule cells by eliminating most of the afferent inputs, which leads to a propensity of MFS (Zimmer and Gähwiler, 1984). Therefore, we initially assessed the extent of MFS in slice cultures incubated for different times *in vitro*. Then, to enhance MFS, 5 days *in vitro* (DIV) slice cultures were treated with pilocarpine for 48–72 h and tested 2 weeks later. After ascertaining the level of MFS in slice cultures, we investigated the changes in dentate circuitry underlying MFS with paired recordings.

Mossy fibres release glutamate as their primary transmitter, and thus the expression of presynaptic metabotropic glutamate receptors (mGluRs) at the newly sprouted synapses could counteract the development of a hyperexcitable network. Thus, we have investigated the locus of action of group II mGluRs within the dentate network in our model of developing MFS. A preliminary report of this work has been published in abstract form (Thomas et al., 2003).

EXPERIMENTAL PROCEDURES

Preparation of organotypic slice cultures

All procedures involving animals were performed using methods approved by the UK Home Office and according to The Animals (Scientific Procedures) Act, 1986. Every effort was made to minimize the number of animals used and their suffering. Interface-type organotypic slice cultures were prepared using the method of Stoppini et al. (1991). Briefly, hippocampi from postnatal day 7 male Sprague–Dawley rats were removed and placed in cold minimal essential medium under sterile conditions. Slices of 400 μm thickness were cut using a McIllwain tissue chopper and placed on microporous membrane inserts (Millicell-CM; Millipore, Watford, UK) in a well containing 1 ml of culture medium consisting of: 50% minimal essential medium, 25% Hanks' balanced salt solution (Invitrogen, Paisley, UK), 25% horse serum (Invitrogen), 1% Penstrep (stock: 10,000 U/ml penicillin and 10,000 μg/ml streptomycin; Invitrogen), and 0.003% $NaHCO_3$ (Sigma-Aldrich Co. Ltd., Poole, UK), pH 7.3 with Tris base (Sigma). Cultures were maintained for 4 days at 37 °C/5% CO_2 and at 33 °C/5% CO_2 for the remainder of the incubation period. Culture medium was changed twice a week. Five DIV cultures were either treated with 0.5 mM pilocarpine (Tocris Cookson Inc, Avonmouth, UK) or 0.5 mM pilocarpine plus either 0.1 mM or 0.5 mM atropine (Sigma) for 48–72 h. Finally, cultures were placed back in the original culture medium until testing at 21 DIV.

Electrophysiology

Slice cultures were submerged and superfused with artificial cerebrospinal fluid (ACSF), containing: (in mM) 130 NaCl, 3.5 KCl, 3 $CaCl_2$, 1.5 $MgSO_4$, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 10 glucose (all from VWR International, Poole, UK), pH 7.4, (bubbled with 95% O_2, 5% CO_2) in a 2 ml chamber mounted on the stage of an upright microscope (60× objective; Axioskop; Zeiss, Jena, Germany). The flow rate was maintained at 1–2.2 ml/min, except in experiments measuring ictal-like bursts after pilocarpine application, where this was increased to 4.5 ml/min to ease the detection of such events (Schuchmann et al., 2002).

Extracellular recording and stimulation. Extracellular potentials were elicited with bipolar stimuli delivered through an isolation unit to a platinum–iridium electrode placed in the stratum lucidum at least 300 μm from the hilus, and recorded with a patch pipette filled with 2 M NaCl and placed in the granule cell layer. Field postsynaptic potentials (fPSPs) and antidromic population spikes were evoked in response to rectangular pulse stimuli (0.1 ms width, 250 μA intensity). Field PSPs were also recorded in the presence of the $GABA_A$ antagonist 1 μM 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid (SR95531; Tocris) and were abolished upon application of 20 μM dinitroquinoxaline (DNQX: Tocris). In each culture, the average of 20 responses was obtained in control or with SR95531. fPSPs were also recorded in the CA3 area during mossy fibre stimulation, and 0.5 mM pilocarpine was added to assess any acute epileptogenic effects. Extracellular epileptiform discharges were analysed using the coastline bursting index (CBI; Korn et al., 1987), which measures the total length of the waveform of multiple population spikes. Specifically, one cursor was set immediately after the end of the antidromic response and another after the end of the synaptic response. The total coast length of the line between the two cursors was calculated with a user-defined programme (Matlab; The Mathworks Ltd., Cambridge, UK). The CBI of each average response was measured and normalised to the area of the preceding antidromic population spike.

Whole cell patch clamp recording. Whole cell patch clamp recordings from visually identified granule cells were performed in current or voltage clamp mode (holding potential=−65 mV) using borosilicate glass capillaries (4–7 MΩ), filled with an intracellular solution consisting of: (in mM) 126 K-gluconate, 4 KCl, 4 ATP-Mg, 0.3 $GTP-Na_2$, 10 Na_2-phosphocreatine, 10 HEPES and 0.5%w/v biocytin (all from Sigma) or 0.2% w/v Lucifer Yellow (Molecular Probes Inc, Eugene, OR, USA), osmolarity 270–280 mOsm without biocytin/Lucifer Yellow, pH 7.3 with KOH. Spontaneous inhibitory postsynaptic currents (sIPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) were recorded with a patch solution containing (in mM): 130 KCl, 2 $MgCl_2$ and 10 HEPES (pH 7.3 with KOH, 270–280 mOsm). The series resistance was monitored throughout the experiments to cheque for stability and the mean value was 14±1.4 MΩ S.E.M. (n=62). The resting potential, as recorded in current clamp but not voltage clamp, was corrected after the experiment for liquid junction potential. I/V protocols were performed in current clamp mode to assess the firing pattern of recorded cells in response to depolarising rectangular current pulses (range,−40 pA to 60 pA). Hyperpolarising rectangular current pulses (range,−30 to −80 pA) were injected to assess the input resistance of the recorded cells during the acute application of pilocarpine (0.5 mM). Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded at−65 mV in control saline and miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 30 μM bicuculline and 1 μM tetrodotoxin (TTX; Tocris). sIPSCs were recorded in the presence of 20 μM DNQX and 40 μM α-2-amino-5-phosphonovaleric acid (D-AP-5) and mIPSCs were recorded after the addition of 1 μM TTX and ensuring that action potentials/currents were fully blocked. Mem-

brane currents were amplified (10 mV/pA, EPC9/2; HEKA Elektronik, Lambrecht/Pfalz, Germany) filtered at 2.9 kHz and digitised at 5 kHz. The currents were acquired online with 'Pulse' software (HEKA) and analysed off-line with MiniAnalysis (Synaptosoft, Decatar, GA, USA) and Pulsefit (HEKA). Spontaneous and miniature events were recorded in files of 1 min duration and analysed with a threshold that corresponded to four times the mean square root of the noise (range 7–12 pA, kept constant for each cell).

Unitary excitatory and inhibitory currents. Unitary events, EPSCs (uEPSCs) or IPSCs (uIPSCs), were studied in synaptically coupled cell pairs (voltage clamped to -65 mV). An action current was elicited in one cell by a depolarising current step and the corresponding unitary synaptic current was measured in the other. The latency of unitary events was determined as the time between the peak amplitude of the presynaptic action current and the onset of the postsynaptic response. Both were visually identified and measured using Pulsefit software, where the onset of the postsynaptic unitary response was defined as the point at which the amplitude could be visualised to deviate from background noise levels. A failure was recorded as a trial in which no event could be visually detected and the failure rate was calculated as the number of failures divided by the number of trials. The unitary event peak amplitude was visually delimited and then measured using Pulsefit. One or more files of 50 trials were analysed for each unitary response. Latency and amplitude values (failures included as 0pA) were averaged for the 50 (or 100/150) trials, and the jitter of the response was defined as the standard deviation of the latency. In most cases a paired-pulse protocol was used, whereby two stimuli were evoked with a 35 ms interval. The paired-pulse ratio was calculated as the mean peak amplitude of the response to the second stimulus divided by the mean peak amplitude of the response to the first stimulus. The selective group II mGluR agonist LY354740 (kindly donated by Eli Lilly & Co., Indianapolis, IN, USA) was perfused in ACSF at a concentration of 0.5 μM.

Antidromic granule cell action currents. Antidromic stimuli were evoked in granule cells (whole cell patch clamped to -65 mV) with a bipolar stimulating electrode placed in the stratum lucidum. Rectangular pulse stimuli (range=22.5–200 μA) were applied and the stimulus intensity was adjusted such that an action current was evoked approximately 100% of the time. After obtaining a control file, 20 μM DNQX, 30 μM bicuculline and 40 μM D-AP-5 were bath applied to abolish synaptic responses. More control files were taken before 0.5 μM LY354740 was bath applied for 6 min and then washed out. In some experiments the mGluR antagonist LY341495 (1 μM; Tocris) was applied after LY354740 for >5 min.

Intracellular labelling and visualisation of recorded cells

Following electrophysiological recordings, slice cultures were immersed in a fixative composed of 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (PB; pH 7.4) for 1–4 h. In order to reveal biocytin-filled cells, cultures (with membrane still attached) were washed in PB and PB+0.1% Triton X-100 (VWR Int.) prior to overnight incubation in a 1:100 solution of avidin–biotin–horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Cultures were further washed in 0.05 M Tris buffer (TB; pH 7.4) before incubation in a 0.5 mg/ml solution of diaminobenzidine (Sigma). Hydrogen peroxide (0.01%) was the substrate for the peroxidase reaction, which was carried out in TB. Cultures were mounted (culture side down) on gelatine-coated slides and left to air-dry for approximately 1 h. Subsequently, slides were dehydrated in graded ethanol (EtOH) up to 95%, where membranes could be removed from cultures. Cultures were rehydrated, washed in PB, incubated in a solution of 0.08% osmium tetroxide, further washed in PB, dehydrated and

permanently mounted on slides. Five to nine neurons from each experimental group were reconstructed using a drawing tube. A 40$\times$ objective was used to draw each cell. Drawings were subsequently converted to a digital image and scaled to a size feasible for morphometric analysis with the MCID image analysis system (Imaging Research Inc., Ontario, Canada). The axon collaterals of each cell were reconstructed at high resolution using a 100$\times$ oil objective, which allowed better resolution of small branches and axon terminals. A primary collateral was defined as one that branched off from the main mossy fibre axon, a secondary collateral as one that branched off from a primary collateral, and so on. The length and type of collaterals and number of terminals for each cell was calculated and is reported in Table 1.

Immunofluorescence

Cultures were fixed in paraformaldehyde as described above. After extensive washes in Tris-buffered saline (TBS; 0.9% NaCl), non-specific protein binding was blocked by incubation in 20% normal goat serum (NGS) for 1 h. Cultures were then incubated overnight (4 °C) with streptavidin conjugated to Cy3 (Amersham Pharmacia Biotech UK Ltd., Bucks, UK) diluted 1:1000 in a solution containing 1% NGS, 0.3% Triton X-100, TBS, in order to reveal biocytin. The following day, cultures were extensively washed in TBS, mounted onto gelatine-coated slides, dehydrated through graded ethanol up to 95% to remove the filter membrane, rehydrated and coverslipped with Vectashield (Vector Laboratories). Immunofluorescence was studied using a Leitz DMRB microscope equipped with epifluorescence illumination and an L5 filter block (excitation filter 480/40 nm, dichroic mirror 505 nm, suppression filter 527/30 nm) to view Lucifer Yellow, and a Y3 filter block (excitation filter 545/30 nm, dichroic mirror 565 nm, suppression filter 610/75 nm) to view the strepavidin-Cy3. Images, recorded through a CCD camera (Hamamatsu C4742-95), were analysed and displayed using Openlab software (version 3.0.2; Improvision, Coventry, UK). Brightness and contrast were adjusted for the whole frame and no part of a frame was modified in any way.

Timm staining

Slice cultures were processed according to a modification of the Timm silver sulphide technique. Cultures were treated with a 1% sodium sulphide solution (Sigma) for 10 min, fixed and stored in 96% and 70% EtOH respectively and then kept at 1–5 °C for up to 2 months. After rehydration, cultures were placed in a solution which consisted of a 12:6:2:1 mixture of gum arabic (50% w/v), hydroquinone (5.67% w/v), citric acid–sodium citrate buffer (26% citric acid, w/v; 24% sodium citrate, w/v) and silver nitrate (17% w/v; all from Sigma) and developed for 45 min in a darkroom at 25 °C. Furthermore, cultures were rinsed in water, fixed in EtOH, mounted on slides, dehydrated and coverslipped. Timm stain relative optical density (ROD) was assessed in cultures with the MCID image analysis system. Only cultures that displayed clear Timm staining in the stratum lucidum were used for densitometric measurements. This staining served as a positive control to show that the stain procedure had been successful. MCID was calibrated for area using a graticule, and optical density using a microscale of different grey values. A standard curve obtained from the microscale was produced for each series of measurements to ensure that light intensity was identical. Using the manual-outlining mode, two readings were taken: one from a large section of the hilus and another from the molecular and granule cell layers together. Only the inner blade of the dentate gyrus was analysed by densitometry. The area taken for measurement of both regions was consistent both within and between groups (S.E.M.<10% mean area, data not included). Background staining levels in the subiculum, a region consistently showing low staining levels were measured to account for the variability in

Table 1. The effects of time *in vitro* and pilocarpine treatment on the anatomy of single granule cells in hippocampal slice cultures[a]

Cell	Group	Dendrite length	Axon length	Collateral hilus	Collateral GC&mol.	Primary collaterals		Other collaterals	
						Number	Terminals	Number	Terminals
1	7 DIV	0.64	2.12	2.42	0.00	6	582	5	33
2[b]	7 DIV	0.77	2.15	1.42	0.00	7	255	3	83
3	7 DIV	0.85	1.15	1.31	0.04	8	233	3	13
4	7 DIV	1.77	1.29	0.13	0.18	11	118	9	21
5	7 DIV	0.58	1.63	0.98	0.09	8	409	6	19
Average		0.92	1.67	1.25	0.06	8.0	319.4	5.2	33.8
S.E.M.		0.22	0.21	0.37	0.04	0.8	80.4	1.1	12.7
1	14 DIV	0.54	0.85	0.82	0.41	6	108	7	95
2	14 DIV	1.06	1.46	1.51	0.32	8	443	7	45
3[b]	14 DIV	0.73	1.77	1.58	0.10	11	421	6	15
4	14 DIV	1.09	1.42	0.59	0.17	7	404	6	13
5	14 DIV	1.14	2.23	0.28	0.20	7	136	7	95
Average		0.91	1.54	0.96	0.24	7.8	302.4	6.6	52.6
S.E.M.		0.12	0.23	0.26	0.06	0.9	74.0	0.2	18.2
1	21 DIV	0.54	1.96	1.13	0.43	6	290	4	41
2	21 DIV	0.86	0.59	1.74	0.00	11	337	10	101
3	21 DIV	0.68	1.17	1.49	0.13	7	376	6	116
4[b]	21 DIV	1.03	1.12	0.94	0.13	9	731	23	194
5	21 DIV	0.96	1.08	2.03	0.48	10	561	8	77
6	21 DIV	0.64	1.56	0.76	0.15	5	356	7	28
7	21 DIV	0.68	1.68	2.79	0.00	5	623	14	415
8	21 DIV	0.88	1.42	2.18	0.34	7	588	12	275
Average		0.79	1.32	1.63	0.21	7.5	482.8	10.5	155.9
S.E.M.		0.06	0.15	0.24	0.07	0.8	57.4	2.1	46.9
1	Pilocarpine	0.85	1.24	2.13	0.38	8	539	11	173
2	Pilocarpine	1.50	1.58	2.42	0.63				
3	Pilocarpine	0.86	1.36	1.76	0.12	9	439	11	92
4	Pilocarpine					10	443	15	210
5	Pilocarpine	0.72	1.35	1.37	0.77	13	790	14	232
6	Pilocarpine	1.18	1.64	1.12	0.37	7	344	8	85
7[b]	Pilocarpine	1.05	1.68	2.75	0.69	11	1044	6	459
8	Pilocarpine	0.84	1.51	0.97	1.33	8	855	13	163
9	Pilocarpine	0.58	1.47	0.92	0.62	7	490	10	147
Average		0.95	1.48	1.68	0.61[b]	9.1	618.0	11.0	195.1
S.E.M.		0.10	0.05	0.25	0.13	0.7	87.4	1.1	41.8

[a] The numbers indicate (from left to right): the total dendritic length (mm), length of the main axon (mm), length of axon collaterals in the hilus (mm), length of axon collaterals in the granule cell (GC) and molecular layers (mol., mm), number of primary axon collaterals or terminals, the number of other (i.e. secondary and tertiary) axon collaterals and terminals. X indicates the cells that are displayed in Fig 4 Statistics: [b]represents significance with respect to 7 and 21 DIV ($P<0.05$).

Timm staining seen in cultures. The ROD within a fixed area of subiculum was determined for each culture and this was subtracted from the previously determined ROD obtained in the hilus and granule and molecular layers. This procedure was completed for several cultures of each age and the mean ROD of stain in the hilus and granule and molecular layers was obtained for each group.

Lactate dehydrogenase (LDH) assessment of cell death in slice cultures

We measured LDH activity to quantify cell injury, according to the method outlined in Noraberg et al., 1999. Briefly, cultures were placed in serum-free, neurobasal medium (98 ml [Invitrogen] with 2 ml B-27 supplement [Invitrogen] and 0.5 ml 200 mM L-glutamine [Sigma] and 25 mM glucose [Sigma]) at 5 DIV for 2 h prior to commencement of the protocol. At time point zero, samples of 150 μl culture medium were collected from each well with six cultures plated on each membrane, whilst maintaining the total volume of medium at 1 ml. Subsequently, a subset of cultures was treated with either pilocarpine (0.5 mM) or pilocarpine+atropine (0.5 mM and 0.1 mM), respectively. Further samples were taken 48 and 72 h after treatment. Finally, 1% Triton X-100 was applied to all cultures to induce total neuronal death and a further sample was taken 24 h later. Samples were stored at −20 °C until analysis.

Samples of media were added to pyruvate (Sigma) and nicotinamide adenine dinucleotide (Sigma) in TBS, and the absorbence of the mixture was measured, using a spectrophotometer (DU 530; Beckman, High Wycombe, UK), at 30 °C. LDH activities were measured as units/l.

Statistical analysis

Data throughout the text are given as mean±S.E.M., unless otherwise stated. Where the number of observations was greater than 25 we applied a Levene test to determine the normality of the data, and where data were shown to be normal we used a parametric statistical test. The mean values for each age/treatment group gained from Timm staining, LDH cell death analysis, intra-
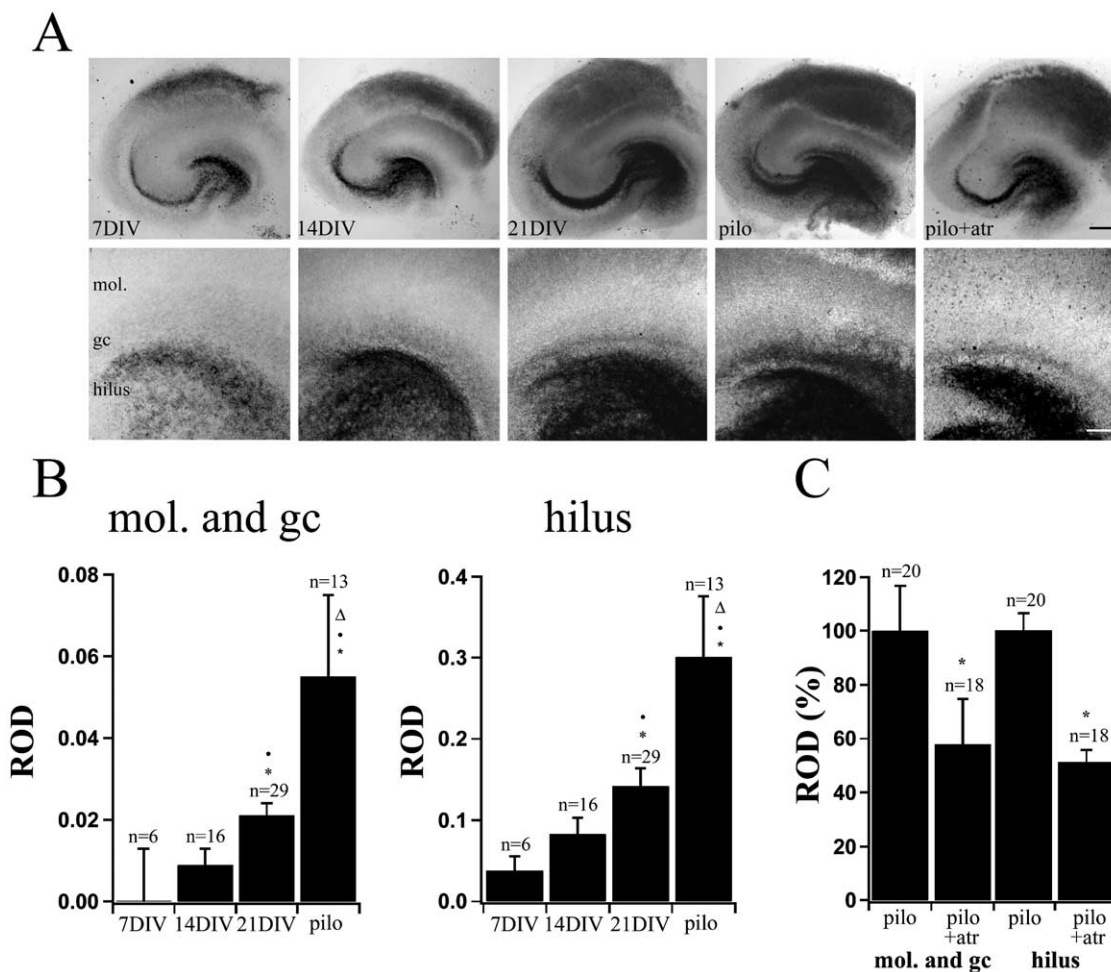
Fig. 1. The density of Timm stain in the hilus and molecular and granule cell layers increased with time in culture, and was greatest in pilocarpine-treated cultures. (A) Upper panel: Timm-stained control 7 DIV, 14 DIV, 21 DIV, pilocarpine-treated and pilocarpine and atropine-treated organotypic slice cultures. Scale bar=300 μm. Lower panel: The dentate region has been enlarged for clarity. Scale bar=100 μm. (B) ROD of Timm stain detected in the granule cell and molecular layers, left graph, and the hilus, right graph, in control 7, 14 and 21 DIV and pilocarpine-treated cultures. Bars represent mean ROD±S.E.M. (of 'n' cultures, as labelled on graph). *, ● and Δ represent significance compared with ROD measured in 7, 14 and 21 DIV cultures, respectively ($P<0.05$). (C) ROD, expressed as a percentage of pilocarpine value of Timm stain in pilocarpine-treated and pilocarpine (0.5 mM) and atropine-treated (0.5 mM) cultures. * Represents significance with respect to pilocarpine-treated cultures ($P<0.05$). atr, atropine; gc, granule cell layer of dentate gyrus; mol., molecular layer of dentate gyrus; pilo, pilocarpine.

cellular labelling analysis, sEPSC, mEPSC, sIPSC and mIPSC protocols were compared with a one-way analysis of variance test combined with a Bonferroni post hoc comparison. Timm staining in pilocarpine versus pilocarpine+atropine-treated cultures, the normalised CBI in the presence of SR95531 in 21 DIV and pilocarpine-treated cultures, and the length of bursts in different cell types were compared using an unpaired t-test. The χ^2 test was used to determine whether the frequency of each type of connection observed with dual recordings was significantly different in each experimental group, to investigate any significant changes in the failure rate after LY354740 application and also to test changes in action current success rate after LY354740 for each cell studied. In paired recordings, a paired t-test investigated any significant changes in uEPSC/uIPSC amplitude or paired pulse ratio upon application of LY354740 compared with the control values for each individual cell. A paired t-test was also used to compare the mean frequency of sEPSCs and mEPSCs before, during and after LY354740 application, and also in comparing fPSP CBI before and after acute pilocarpine application. A one-sample t-test was used to investigate significant changes in rest-

ing membrane potential (from current clamp mode) or holding current (from voltage clamp) after acute application of pilocarpine. A Wilcoxon signed ranks test was also applied and the results were consistent with the t-test.

RESULTS

Population analysis of sprouted mossy fibers: Timm staining and extracellular recording

The Timm heavy metal stain has been used to label hippocampal mossy fibre terminals (Zimmer and Gähwiler, 1984). In seven DIV control cultures, a dark brown/black stain revealed the presence of mossy terminals in the hilus and the CA3, where it appeared in a compact band along the stratum lucidum (Fig. 1A, upper panel). A significant increase in stain ROD with time *in vitro* in both the hilus and molecular and granule cell layers was

observed (Fig. 1). A more robust degree of sprouting was evident in pilocarpine-treated cultures, where the ROD of Timm stain within the molecular and granule cell layers and in the hilus was significantly greater than in all other groups ($P<0.05$). This effect was blocked in both regions by the competitive muscarinic antagonist atropine (0.5 mM, $P<0.05$, Fig. 1C).

The sprouted fibers appeared to form functional synaptic connections since extracellular recording in the granule cell layer, during stimulation in the stratum lucidum, revealed an antidromic population spike superimposed on a fPSP from the activation of mono- and polysynaptic pathways. The fPSP was greater in pilocarpine-treated cultures, and the application of a low concentration of the GABA$_A$ antagonist SR95531 (1 μM), uncovered more multiple peaks in the synaptic response than in control cultures (Fig. 2). The synaptic responses were abolished by the application of DNQX (20 μM). Multiple population spikes were assessed by means of normalised CBI. The increase in normalised CBI caused by SR95531 was greater in pilocarpine-treated than 21 DIV control cultures ($P<0.001$, $n=9$ for each group).

What are the short-term effects of pilocarpine, and can these lead to cell death?

It is believed that *in vivo* application of pilocarpine generates both acute repetitive seizures and excitotoxicity of several hippocampal cell types, especially hilar neurons, and these phenomena could contribute to the genesis of MFS (Obenaus et al., 1993). We have further investigated this issue in slice cultures of 5–8 DIV, the age at which pilocarpine was applied to cultures in our chronic treatment protocol, using both electrophysiological and biochemical techniques. All recorded cells were filled with biocytin and anatomically identified using light microscopy.

Acute bath application of pilocarpine (0.5 mM, 3 min) led to clear-cut and reversible depolarisations in the presence of TTX, often associated with a change in input resistance in the recorded neurons (Fig. 3A, B). This was observed in hilar mossy cells, and hilar interneurons, but not in granule cells recorded in current clamp (see Fig. 3B). Likewise, a pilocarpine-induced inward current was observed in hilar mossy cells (five of six) and hilar interneurons (five of six), but only in one of five granule cells recorded in voltage clamp (not shown). These effects observed in hilar mossy cells and interneurons, but not granule cells, were significant ($P<0.01$) and were reversed by subsequent application of 0.5 mM atropine in three of three mossy cells (data not shown). When pilocarpine was applied to cultures in the absence of TTX, the depolarisations led to long lasting, prolonged bursts of action potentials in all mossy cells and pyramidal cells studied ($n=6$ for both cell types, flow rate=4.5 ml/min, Fig. 3C). These ictal-like discharges were preceded by an increase in the occurrence of spontaneous synaptic activity, and persisted throughout the 20 min recording period until atropine was co-applied (50 μM). Furthermore, the epileptic-like activity
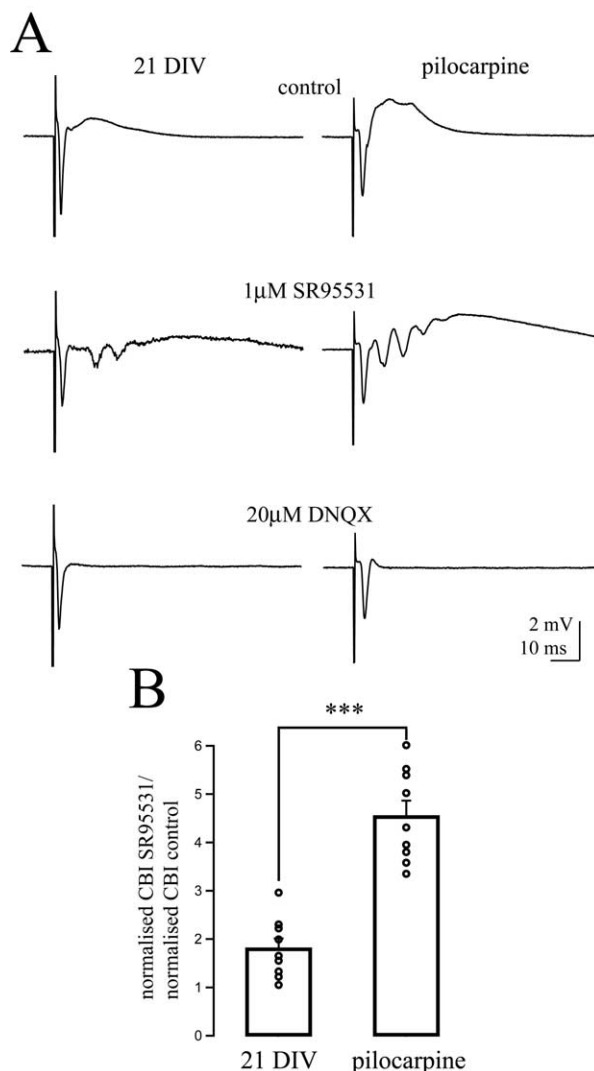

Fig. 2. Epileptiform activity elicited by the GABA$_A$ antagonist SR95531 (1 μM) was greater in pilocarpine-treated than in 21 DIV cultures. (A) Examples of extracellularly recorded traces from the granule cell layer in 21 DIV or pilocarpine-treated cultures after bipolar stimulation (100 μA, 0.1 ms) in the stratum lucidum. Stimulation evoked a stimulus artefact, an antidromic population spike and a fPSP. Note that stimulation evoked a larger fPSP in a pilocarpine-treated culture. Application of a low concentration of SR95531 elicited more antidromically driven synaptic responses in pilocarpine-treated than 21 DIV cultures. Subsequent application of 20 μM DNQX abolished the synaptic responses. (B) Plot of the ratio of the CBI normalised to the area of the antidromic population spike after and before SR95531 in 21 DIV and pilocarpine-treated cultures. Each value (open circles) refers to one experiment in one slice culture. The average value (bars in the graph±S.E.M.) was higher in pilocarpine than in 21 DIV cultures. *** Represents significance between the two groups ($P<0.001$, $n=9$ for each group).

observed in hilar mossy cells had a longer mean duration as compared with CA3 pyramids ($P<0.01$, Fig. 3D).

When granule cells were voltage clamped, acute application of 0.5 mM pilocarpine caused an increase in the frequency of sEPSCs and sIPSCs that often appeared in a burst-like manner. These effects were blocked by 1 μM TTX or 0.5 mM atropine ($n=4$, not
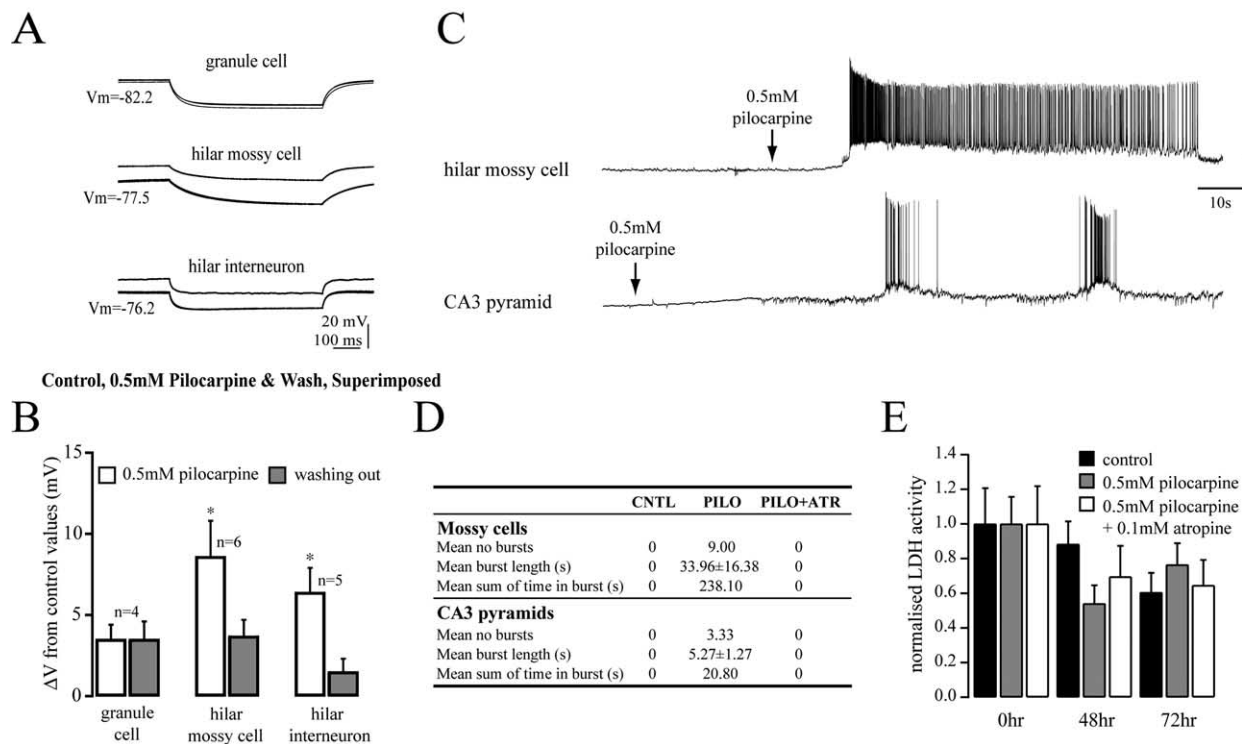
Fig. 3. Acute actions of 0.5 mM pilocarpine in hippocampal slice cultures. (A) Average current clamp traces in response to hyperpolarising current pulses at the indicated resting membrane potentials (Vm) before, during and after pilocarpine. Note that hilar mossy cells and interneurons, and not granule cells are depolarised. 1 μM TTX was present throughout the experiments. (B) Pooled current clamp data. Data are expressed as ΔV, the change in resting membrane potential evoked by application of pilocarpine and after washout of the drug (* represents significance as compared with before pilocarpine application, $P<0.05$). (C) Example current clamp recordings showing bursts of action potentials in a hilar mossy cell and a CA3 pyramidal cell after application of 0.5 mM pilocarpine, in the absence of TTX. (D) Table of the mean number of bursts, burst length and mean total bursting length for mossy cells ($n=6$) and pyramidal cells ($n=6$) in control ACSF, 0.5 mM pilocarpine and 0.5 mM pilocarpine+50 μM atropine. Pilocarpine was applied for 20 min and pilocarpine+atropine were applied for 10 min. (E) Bar graph of normalised absorbance units/l at 340 nm in samples from control, pilocarpine-treated, or pilocarpine and atropine-treated cultures at time=0, 48, and 72 h ($n=9$, for each experimental condition).

shown). A clear-cut blockade was also observed by application of a lower concentration (0.1 mM) of atropine ($n=3$, not shown). However, in current clamp no ictal-like discharges were observed in granule cells in the absence of TTX ($n=3$, not shown). Furthermore, acute application of 0.5 mM pilocarpine to 5–8 DIV cultures (15 min) evoked multiple peaks in the synaptic responses following stimulation of the mossy fibers and recorded extracellularly in the CA3 pyramidal cell layer. On average, 0.5 mM pilocarpine enhanced the coastline length of the synaptic waveform by 148%±14.5 ($n=7$, $P<0.05$, not shown).

We also investigated whether application of 0.5 mM pilocarpine could lead to cell injury within our slice cultures. We found no significant increase in LDH activity up to 72 h after pilocarpine application (Fig. 3E, $n=9$ each experimental condition, $P>0.05$). Total cell death was measured after application of 1% Triton X-100 for 24 h, resulting in LDH activities of 67.63±4.11 U/l ($P<0.001$ as compared with experimental groups).

These results, taken together, indicate that pilocarpine can produce epileptic-like discharges in hilar neurons and CA3 pyramidal cells, but does not cause substantial cell death.

Unitary analysis of sprouted mossy fibres: a) granule cell anatomy

Granule cells were patched for electrophysiological experiments (see below) and filled with biocytin to investigate single granule cell anatomy. Five to nine cells from each experimental group (7 DIV, 14 DIV, 21 DIV and chronic pilocarpine-treated 21 DIV cultures) were reconstructed using a drawing tube attached to a microscope. Reconstruction of granule cells from the inner blade of the dentate gyrus revealed the typical anatomy associated with granule cells in culture (Fig. 4; Zimmer and Gähwiler, 1984). Dendrites projected in a conical manner from the granule cell layer into the molecular layer. A single main mossy fibre axon arose from the granule cell soma and entered the hilus, where it gave rise to several collaterals. The main axon always extended to the stratum lucidum of the CA3 where giant varicosities were present in all labelled cells. Table 1 illustrates the quantification of several morphometric parameters. The mean length of collaterals entering the granule and molecular layer in pilocarpine-treated cultures was greater than in 7 and 21 DIV control cultures ($P<0.05$). With respect to 14 DIV control cultures, the results were close to reaching significance ($P<0.07$).
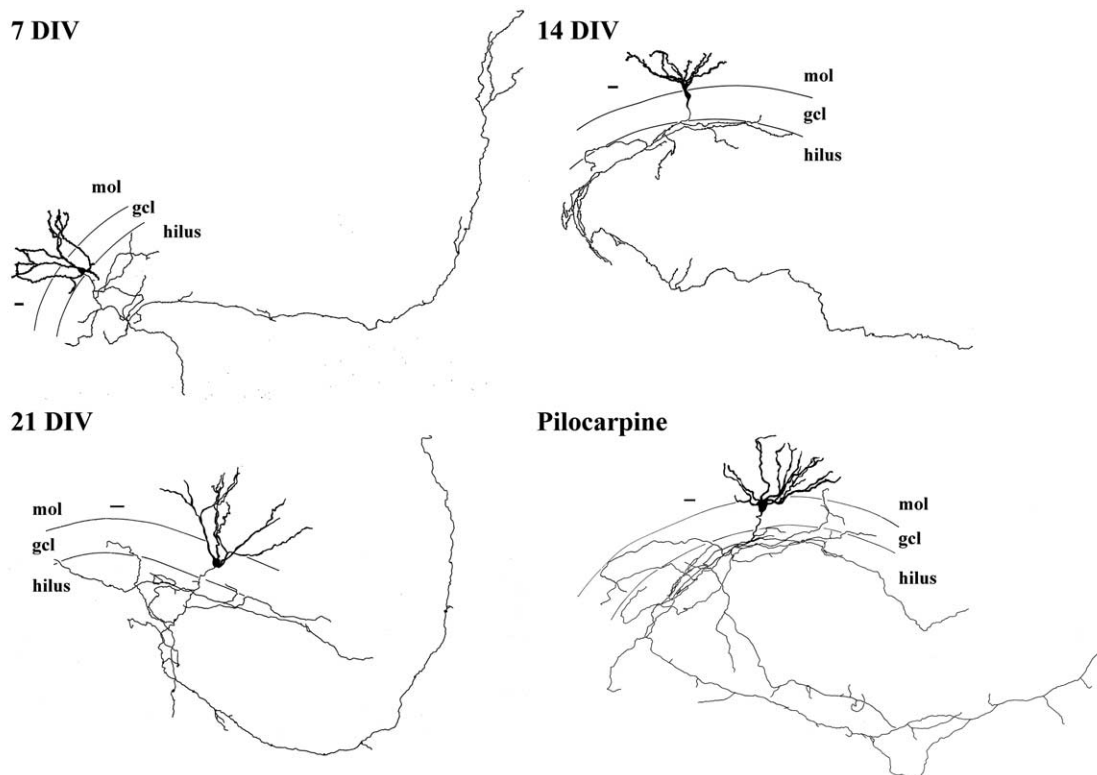
Fig. 4. Drawing tube reconstructed images of representative granule cells from 7, 14, 21 DIV and pilocarpine-treated cultures. The solid line indicates the border of the granule cell layer. The total length of collaterals in the granule and molecular cell layers is greater in pilocarpine-treated than in control cultures. gcl, granule cell layer; mol, molecular layer.

These results show that granule cells from pilocarpine-treated cultures exhibit more MFS.

The mean length of the main mossy fibre axon did not vary significantly amongst the different groups ($P > 0.1$, Table 1). The length of collaterals in the hilus was greatest in 21 DIV and pilocarpine-treated cultures, but no significant differences were found amongst the groups (Table 1). No significant increases were observed in the mean number of secondary and tertiary collaterals or the total number of putative boutons associated with these collaterals with age or pilocarpine treatment.

Previous studies have reported the presence of basal dendrites in models of MFS (Ribak et al., 2000; Dashtipour et al., 2002) and it is thought that they could provide another postsynaptic target for the abundant mossy fibers in the hilus and thus contribute to recurrent excitation. In our model basal dendrites were seen in one culture from 7, 14 and 21 DIV untreated groups and in two pilocarpine-treated cultures.

Unitary analysis of sprouted mossy fibers: b) spontaneous synaptic events

Next, we compared spontaneous excitatory or inhibitory synaptic inputs in different slice culture groups, by recording from granule cells with whole cell patch clamp. All recordings included in the analysis were from patched neurons anatomically identified as dentate granule cells. All cells displayed stereotyped action potential kinetics and

discharge patterns similar to whole cell current clamp recordings obtained from granule cells in acute hippocampal slices (not shown; Staley et al., 1992). The mean resting membrane potential and input resistance of the granule cells were -84.3 ± 1.9 mV ($n = 62$) and 475 ± 19 MΩ ($n = 12$), respectively.

A positive trend between time *in vitro* or pilocarpine treatment and the frequency of spontaneous events was observed (Fig. 5). A significant difference was found in sEPSC and mEPSC frequency between 7 DIV and pilocarpine-treated cultures and in mEPSC frequency between 7 DIV and 21 DIV cultures ($P < 0.05$). Furthermore, the amplitude of mIPSCs was different in 21 DIV and 14 DIV cultures ($P < 0.05$). Taken together, these data suggest that time *in vitro* and/or pilocarpine-treatment only exerts a modest influence on spontaneous excitatory or inhibitory transmitter release onto granule cells.

Unitary analysis of sprouted mossy fibers: c) evoked currents

In the next series of experiments, we analysed in more detail some functional and pharmacological properties of the synaptic network in the dentate area especially in 21 DIV and pilocarpine-treated groups, as these cultures displayed the greatest MFS. To this end, we have performed paired-recordings ($n = 100$) in which the anatomical identity of each recorded cell was ascertained (see Fig. 7B, and
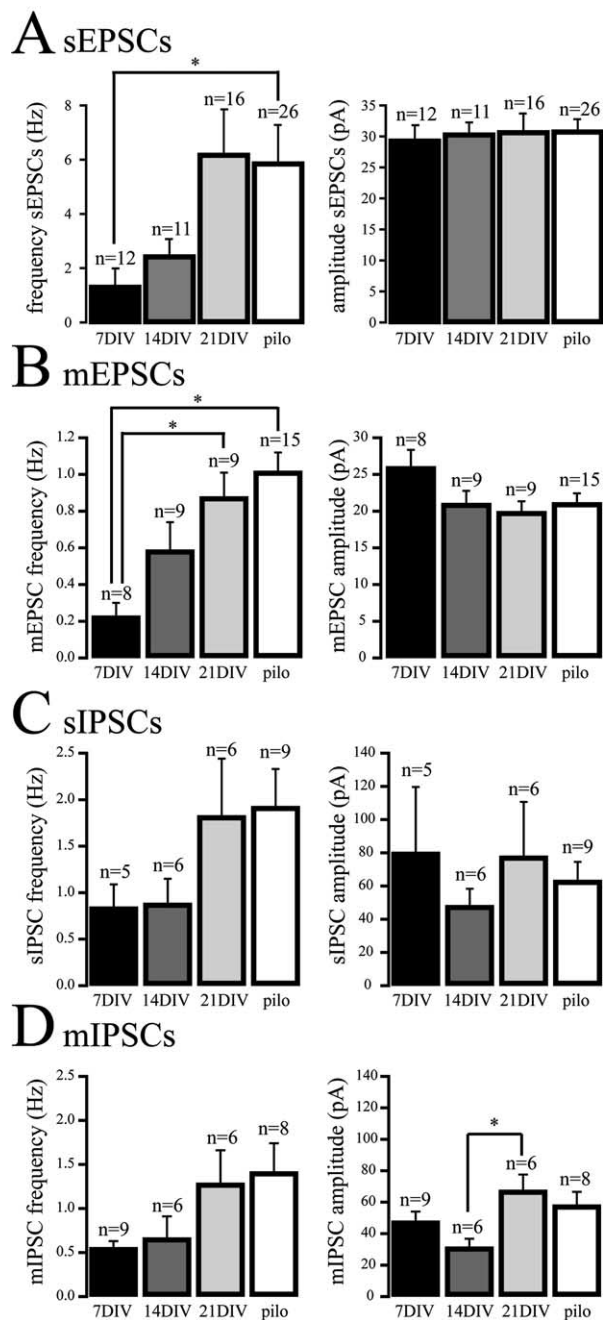
Fig. 5. Mean frequency and amplitude of sEPSCs, mEPSCs, sIPSCs and mIPSCs in untreated and pilocarpine-treated cultures. The histograms show the mean frequency, left graphs, and mean amplitude, right graphs, for 'n' granule cells±S.E.M. for sEPSCs (A), mEPSCs (B), sIPSCs (C) and mIPSCs (D). * Denotes significance, $P<0.05$. pilo, pilocarpine.

Fig. 8D for number of granule cell pairs and granule-hilar cell pairs, and types of connections).

Monosynaptic granule cell pairs. First, granule cell paired recordings were performed and one example of such experiment from a pilocarpine-treated culture is shown in Fig. 6. One cell was filled with biocytin, the other with Lucifer Yellow. Immunofluorescence images subse-

quently revealed a presynaptic cell, shown in red (Lucifer Yellow), and a postsynaptic cell in green (biocytin). Both cells had spiny apical dendrites in the molecular layer (Fig. 6A), and possessed a long, thick axon, which projected to the stratum lucidum and emitted large varicosities. Numerous smaller collaterals entered the granule cell layer, and several apparent sites of contact between the two cells could be observed. The inset in Fig. 6A shows two putative boutons (in red) apposed to a primary dendrite of the postsynaptic cell (in green). Whenever an action potential was evoked in the presynaptic cell, it was closely followed by a reliable (1.3% failure rate) uEPSC in the postsynaptic cell (Fig. 6B). The average latency of this uEPSC obtained from 150 trials was 1.59 ms and the jitter was 0.16 ms (Fig. 6C), suggesting a monosynaptic connection (Doyle and Andresen, 2001). The mean amplitude of the uEPSC was 39.5±1.8 pA. When two action potentials were evoked in short succession, the second uEPSC tended to have smaller amplitude than the first (uEPSC$_1$=39.9pA, uEPSC$_2$=19.9pA, paired pulse ratio=0.87±0.79). Therefore this synapse had apparent high release probability and displayed paired-pulse depression. When the group II mGluR agonist LY354740 (0.5 μM) was applied, the frequency of failures markedly increased (Fig. 6B, D; $P<0.05$), the mean uEPSC amplitude was significantly reduced (uEPSC$_1$=12.8 pA, uEPSC$_2$=13.6 pA, $P<0.05$) and the mean paired-pulse ratio significantly increased to 1.13±0.65 ($P<0.05$; Fig. 6D, lower graph). Application of 20 μM DNQX abolished the uEPSC (not shown). This paired data shows that monosynaptic connections exist between granule cells in our culture model of MFS, and group II mGluRs are present at the presynaptic terminal of these synapses.

Polysynaptic granule cell pairs. Monosynaptic connections were rarely found and only two monosynaptic connections were established from 60 attempts, one from a control 21 DIV culture, the other from a pilocarpine-treated culture (Fig. 7B). Instead, recordings from pairs of granule cells often showed polysynaptic connections, which were seen as either inward or outward currents in the postsynaptic cell. Example traces from two anatomically identified pilocarpine-treated granule cells are illustrated in Fig. 7A, and reveal a reciprocal polysynaptic connection between the pair. When an action current was evoked in cell 2, it was followed by a long latency (14.33 ms), excitatory glutamatergic current in cell 1. This response was reliable (4% failure rate), but had a large jitter (0.92 ms) and was thus likely to be polysynaptic in nature. The mean amplitude was 35.3 ±10.7 pA. Similarly, stimulation of cell one evoked a glutamatergic current (Fig. 7A (i)) in cell 2, and this response had a comparable latency and failure rate (latency, 14.76 ms; jitter, 0.59 ms; failure rate, 6%; mean amplitude, 79.6 ±24.2 pA). Furthermore, the same stimulus also evoked a longer latency outward GABAergic current in cell two (Fig. 7A (ii)). This was a less reliable connection with a large jitter and almost certainly represents a polysynaptic connection (latency, 27.7 ms; jitter, 1.1 ms; failure rate, 64%; mean amplitude, 33.4
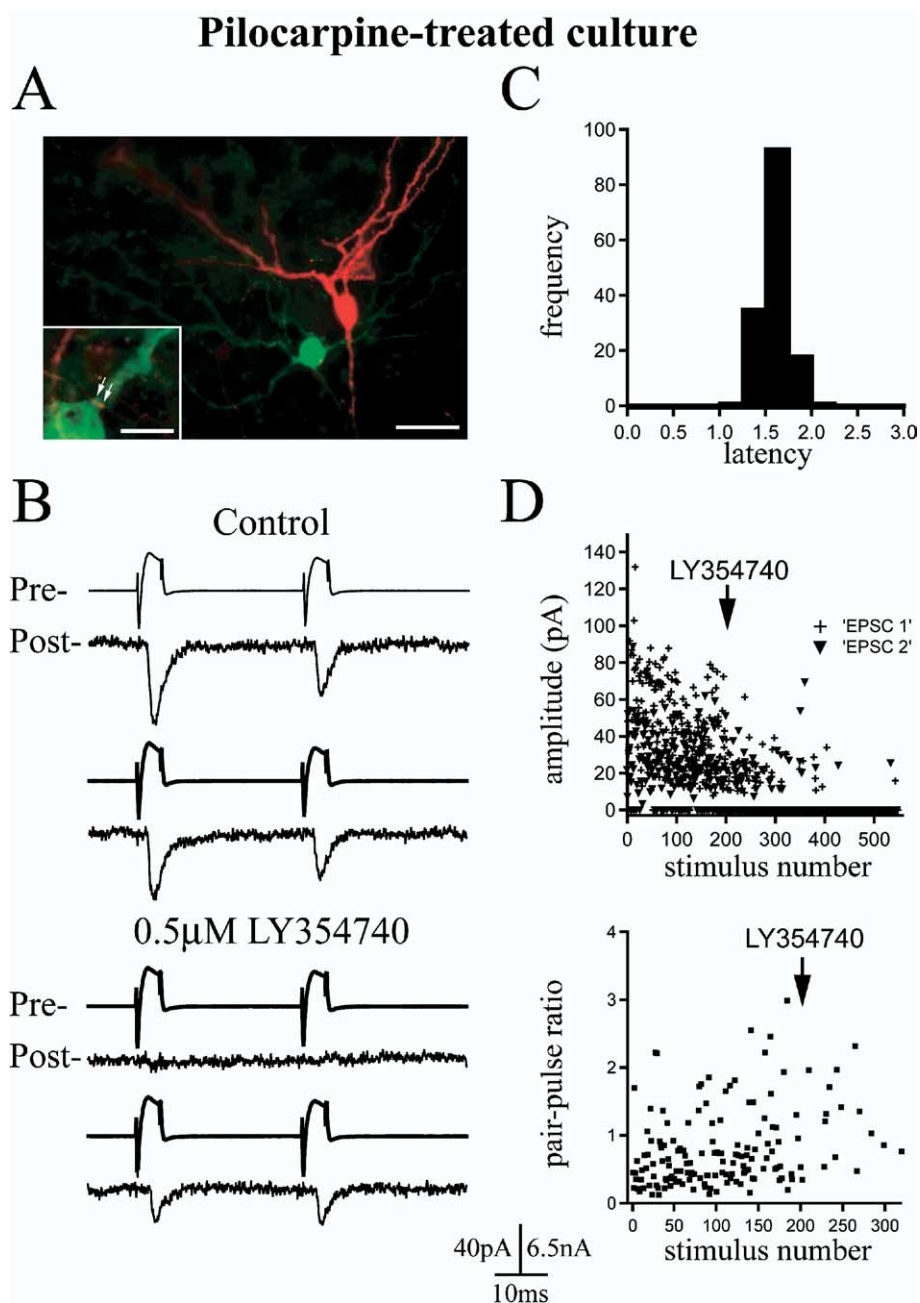
Fig. 6. Monosynaptic connections exist between granule cells and activation of group II mGluRs inhibits unitary monosynaptic currents in a pilocarpine-treated culture. (A) Immunofluorescence image of a granule cell pair (red cell, presynaptic; green cell, postsynaptic), scale bar=50 μM. Inset is an enlargement of a region containing putative synapses (arrows), scale bar=20 μM. Note that the dendritic arborisation of the green cell is not in the plane of focus in this image. (B) Electrophysiology of the granule cell pair. An action current in the presynaptic cell evoked a reliable, short latency response in the postsynaptic cell. Paired pulse depression predominated in the control. Bath application of the group II mGluR agonist LY354740 (0.5 μM) induced a pronounced increase in the number of failures, a decrease in uEPSC amplitude and paired pulse facilitation. (C) The histogram showed a unimodal distribution of uEPSCs with a short latency and jitter. This is consistent with a monosynaptic connection. (D) Upper panel: uEPSC amplitude was markedly decreased and the number of failures increased after the addition of LY354740 (arrow). Lower panel: paired-pulse ratio increased after LY354740 addition.

±52.5 pA). Pilocarpine-treated cultures displayed a higher frequency of these granule cell reciprocal polysynaptic connections then their control counterparts (12.5%, 21 DIV untreated cultures; 42%, pilocarpine-treated cultures). Connections were considered polysynaptic when the unitary current had one or more of the following characteris-

tics: jitter>0.5 ms, latency>9 ms, failure rate>0.6. The frequency of the different types of connections observed in each experimental group is summarised in Fig. 7B, and the frequency of connections in 7, 14 and 21 DIV and pilocarpine-treated cultures were significantly different from each other ($P<0.05$). Therefore, there was a larger degree

A Pilocarpine-treated cultures

10pA 2nA
10ms

60pA 1nA
10ms

B

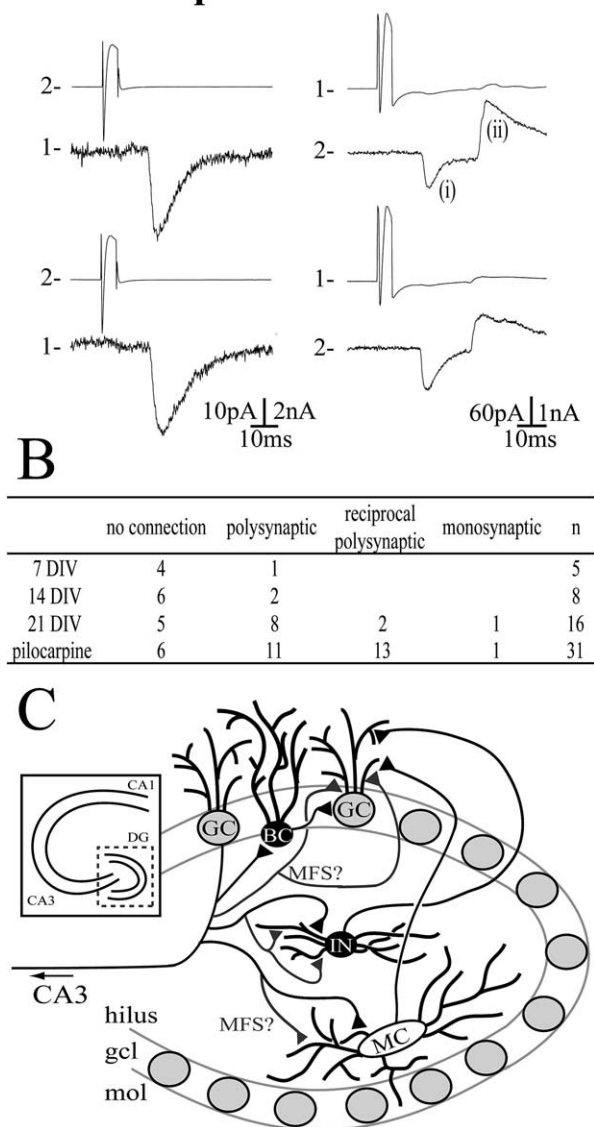|	no connection	polysynaptic	reciprocal polysynaptic	monosynaptic	n
7 DIV	4	1			5
14 DIV	6	2			8
21 DIV	5	8	2	1	16
pilocarpine	6	11	13	1	31

C

Fig. 7. Granule cells are frequently connected via polysynaptic interactions in pilocarpine-treated cultures. (A) Paired recordings from two pilocarpine-treated anatomically identified granule cells revealed reciprocal polysynaptic connections. Left hand panel: stimulating cell two evoked a long latency, reliable EPSC in cell 1. Right hand panel: stimulating cell one produced a long latency, reliable EPSC in cell 2 (i) followed by an even longer latency IPSC (ii). (B) Table of the number and type of connections between recorded granule cell pairs. No connection, granule cells not connected; polysynaptic, one-directional polysynaptic connection; reciprocal polysynaptic, both granule cells are polysynaptically connected to each other; monosynaptic, one-directional monosynaptic connection. (C) Schematic drawing of likely connections present in the dentate area of pilocarpine-treated cultures. Some granule cells are connected to each other via monosynaptic connections. However, polysynaptic connections (with either an interneuron, mossy cell or possibly other granule cells intercalated between recorded granule cells) are also present. gcl, granule cell layer; IN, interneuron; MC, mossy cell; mol, molecular layer; 1, granule cell 1; 2, granule cell 2.

of connectivity between granule cells in pilocarpine-treated cultures. Such polysynaptic pathways may arise from an

intercalated granule cell, i.e. a chain of two granule cell monosynaptic connections, between the two recorded cells. However, it was also possible that the intercalated cell was an interneuron or mossy cell of the hilus or even that more than one cell was intercalated between the two recorded neurons, thus creating a more complex network (Fig. 7C).

Granule–hilar cell pairs. The polysynaptic data combined with the low incidence of failures, implied that a granule cell presynaptic action potential reliably evoked an action potential in the hilar neuron intercalated between the recorded cells. This issue was directly investigated in pilocarpine-treated cultures by studying monosynaptic connections between granule cells and hilar neurons in current clamp mode. As expected, an action potential elicited in granule cells evoked an action potential riding on the uEPSP in hilar cells ($n=3$ pairs) recorded at resting membrane potential (data not shown). When recorded in voltage clamp, 12 single monosynaptic (two granule cell–interneuron, two granule–mossy cell, four mossy cell–granule cell and four interneuron–granule cell), five reciprocal monosynaptic (3 granule–mossy cell and two granule cell–interneuron) and 11 polysynaptic connections, out of a total of 32 attempts, were detected in pilocarpine-treated cultures. Only four pairs of cells were not connected in any way (Fig. 8D). In contrast, in 21 DIV control cultures, out of a total of eight attempts, one single monosynaptic connection (interneuron–granule cell) and three polysynaptic connections were observed (Fig. 8D). Four pairs of cells were not connected in any way. Notably, the frequency of single and reciprocal monosynaptic connections in pilocarpine-treated cultures was found to be significantly higher than that observed in control 21 DIV cultures ($P<0.05$, Fig. 8D). All cells from monosynaptic connections were filled with biocytin and visualised. Granule cells, interneurons and mossy cells were identified by synaptic physiology, firing patterns and their characteristic anatomical features.

Granule cell–interneuron synapse: Fig. 8A shows representative traces of a reciprocal monosynaptic connection between a granule cell and a hilar interneuron recorded from a pilocarpine-treated culture. Stimulation of the granule cell evoked a reliable large amplitude, short latency uEPSC with small jitter (Fig. 8A1). Application of LY354740 to all pairs significantly increased the failure rate and decreased the mean amplitude of the first response ($P<0.05$). Conversely, stimulation of the interneuron evoked a uIPSC with a slightly longer latency (Fig. 8A2). The inhibitory unitary response was not polysynaptic, as the response remained unchanged after the addition of DNQX at the end of the experiment (data not shown). In contrast to the effects on the excitatory response, LY354740 had no significant effect on the inhibitory connections recorded at the same time (Fig. 8E), and in all cases the number of failures was not significantly changed.

Granule cell–mossy cell synapse: Example traces from a connection between a granule cell and a hilar mossy cell recorded from a pilocarpine-treated culture are shown in Fig.

8B. Stimulation of the granule cell evoked a large, short latency, reliable monosynaptic uEPSC in the postsynaptic mossy cell with paired-pulse facilitation. In all cells application of LY354740 increased the number of failures, decreased the mean amplitude of the first uEPSC and increased the paired pulse ratio ($P<0.05$). This was partially reversed by washout of the drug. Furthermore, an action current in a mossy cell (Fig. 8C) evoked a uEPSC in a granule cell which had a

longer latency, small jitter and amplitude. In five out of seven pairs LY354740 increased the failure rate, and decreased the mean amplitude ($P<0.05$). In two out of seven pairs LY354740 had no effect on uEPSC mean amplitude, failure rate or paired-pulse ratio. Quantification of the average data is given in Fig. 8E.

These results demonstrate that, in pilocarpine-treated cultures, granule cells are frequently monosynaptically
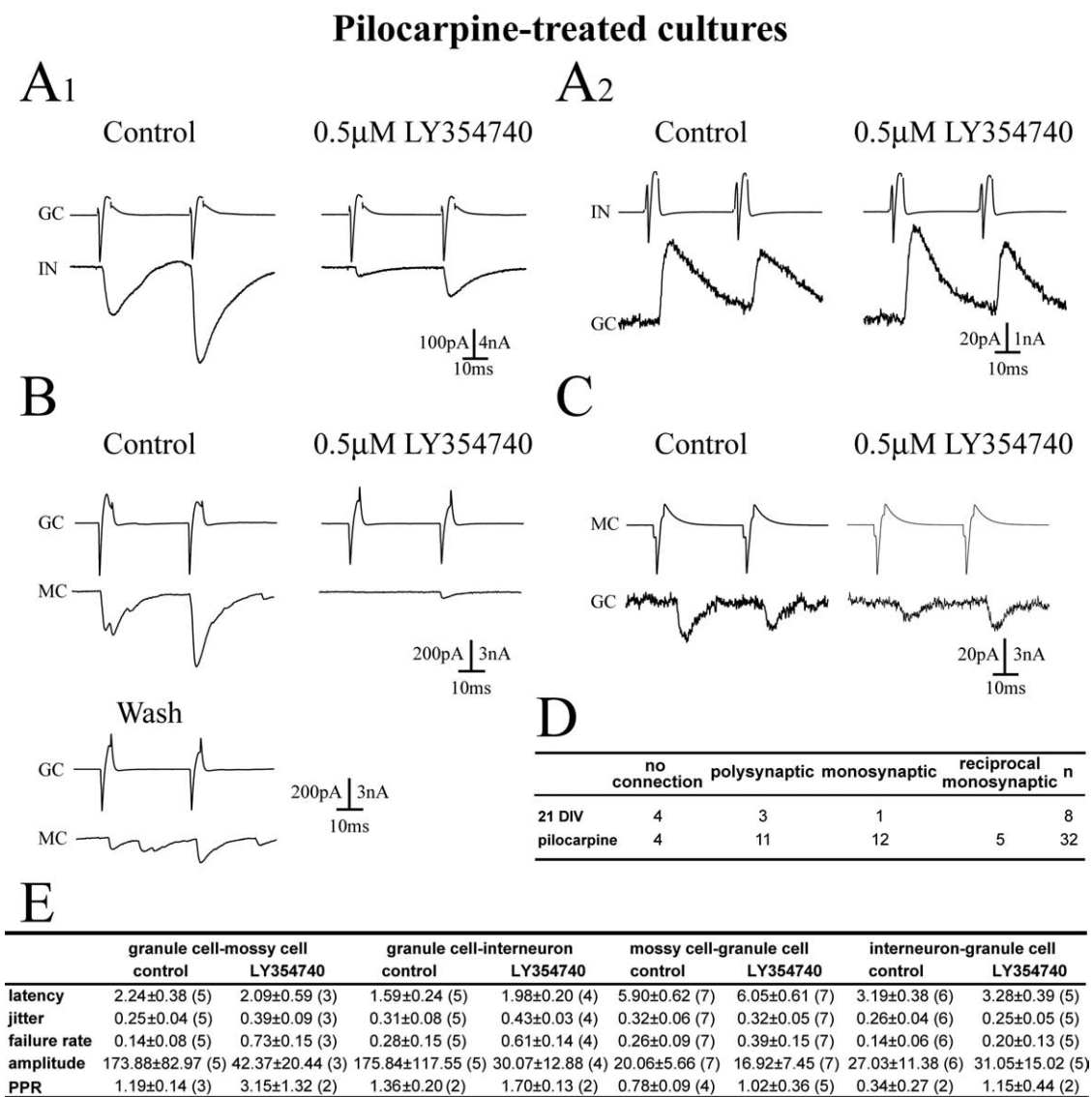
Pilocarpine-treated cultures

	no connection	polysynaptic	monosynaptic	reciprocal monosynaptic	n
21 DIV	4	3	1		8
pilocarpine	4	11	12	5	32

	granule cell-mossy cell		granule cell-interneuron		mossy cell-granule cell		interneuron-granule cell	
	control	LY354740	control	LY354740	control	LY354740	control	LY354740
latency	2.24±0.38 (5)	2.09±0.59 (3)	1.59±0.24 (5)	1.98±0.20 (4)	5.90±0.62 (7)	6.05±0.61 (7)	3.19±0.38 (6)	3.28±0.39 (5)
jitter	0.25±0.04 (5)	0.39±0.09 (3)	0.31±0.08 (5)	0.43±0.03 (4)	0.32±0.06 (7)	0.32±0.05 (7)	0.26±0.04 (6)	0.25±0.05 (5)
failure rate	0.14±0.08 (5)	0.73±0.15 (3)	0.28±0.15 (5)	0.61±0.14 (4)	0.26±0.09 (7)	0.39±0.15 (7)	0.14±0.06 (6)	0.20±0.13 (5)
amplitude	173.88±82.97 (5)	42.37±20.44 (3)	175.84±117.55 (5)	30.07±12.88 (4)	20.06±5.66 (7)	16.92±7.45 (7)	27.03±11.38 (6)	31.05±15.02 (5)
PPR	1.19±0.14 (3)	3.15±1.32 (2)	1.36±0.20 (2)	1.70±0.13 (2)	0.78±0.09 (4)	1.02±0.36 (5)	0.34±0.27 (2)	1.15±0.44 (2)

Fig. 8. Monosynaptic connections exist between granule cells and hilar neurons and mGluRII receptors are functionally present only at excitatory granule cell to hilar neuron synapses. (A1) Paired recordings from a granule cell and a hilar interneuron that revealed a reciprocal monosynaptic connection. Capacitive transients have been removed in some cases for clarity. Stimulation of the granule cell evoked a large, short latency, reliable uEPSC and paired pulse facilitation predominated. Bath application of 0.5 μM LY354740 increased the number of failures and increased the paired-pulse ratio. (A2) Stimulation of the interneuron resulted in a reliable uIPSC that displayed paired pulse depression and was not reduced by LY354740. (B) A granule cell–mossy cell connection. Again, stimulation of the granule cell evoked a large, short latency, reliable uEPSC that displayed paired pulse facilitation. LY354740 (0.5 μM) dramatically increased the number of failures and increased the paired pulse ratio. This effect was partially reversed upon washout of the drug. (C) Stimulation of a mossy cell evoked a small uEPSC. Addition of LY354740 reduced the amplitude of uEPSCs and increased the paired-pulse ratio. All experiments were performed in pilocarpine-treated cultures. (D) Table of the number and type of connections between recorded granule and hilar cell pairs. No connection, cells not connected; polysynaptic, one-directional polysynaptic connection; reciprocal monosynaptic, both cells are monosynaptically connected to each other; monosynaptic, one-directional monosynaptic connection. (E) Table of the synaptic properties and the effects induced by LY354740 on monosynaptic connections between granule and hilar cells in pilocarpine-treated cultures. Data are presented as mean±S.E.M. (n).

connected to mossy cells or interneurons in the hilus and corroborate our findings of frequent polysynaptic connections between pairs of granule cells. Interestingly, application of LY354740 mainly suppressed excitatory but not inhibitory transmission.

Mechanisms of inhibition mediated by the group II mGluR agonist, LY354740

The results of our paired recordings in the presence of LY354740 suggested that group II mGluRs were present at the presynaptic terminals of mossy fibers. However, although the number of failures usually increased dramatically upon application of the drug, there was not always a parallel increase in the paired-pulse ratio in granule cell–hilar cell pairs, suggesting a non-classical presynaptic mechanism. Thus we investigated in more detail the effects of LY354740 on granule cell excitability.

Bath application of 0.5 μM LY354740 had no effect on the frequency of mEPSCs (n=4), but significantly decreased the frequency of sEPSCs (Fig. 9A; test, P<0.05, n=9) recorded from granule cells in pilocarpine-treated cultures. This effect was reversed upon washout of the drug (n=7).

Stimulation of the mossy fibers in the stratum lucidum evoked an 'all-or-none' antidromic action current in granule cells. Initially, the recording revealed an action current superimposed on synaptic currents (Fig. 9B1). Application of 30 μM bicuculline, 20 μM DNQX and 40 μM D-AP-5 abolished the synaptic currents, whereas the action current remained and thus was not of synaptic origin (Fig. 9B2). In five out of six granule cells from pilocarpine-treated cultures, application of 0.5 μM LY354740 dramatically decreased the number of action current successes (Fig. 9B3; P<0.0001) and in three cells, this effect was fully reversed upon washout of LY354740 (Fig. 9B4). Application of the group II mGluR antagonist, LY341495 (1 μM; Kingston et al., 1998) reversed the effect of LY354740. The mean action potential success rate was initially 1, then 0.43$\pm$0.1 during application of LY354740, and reversed to 1 when LY341495 was added (n=3, not shown). Interestingly, a less pronounced, short-lasting and reversible inhibition was observed in three out of nine cells studied (Fig. 9C; P<0.01) from pilocarpine and atropine-treated cultures (0.5 mM pilocarpine plus either 0.1 mM or 0.5 mM atropine). Action currents were abolished by the addition of TTX (1 μM, not shown).

Taken together, these experiments suggest a novel mGluR-mediated presynaptic mechanism, in which group II mGluRs placed along the granule cell axon membrane act to depress action potential invasion and consequently glutamate release.

DISCUSSION

Development of granule cell anatomy and electrophysiology with time *in vitro*

The analysis of the anatomical and electrophysiological properties of dentate granule cells at different DIV provides a dynamic view of the re-arrangements of synaptic con-

nections that take place with time *in vitro*. This is essential for a correct interpretation of the changes observed upon treatment with pilocarpine.

Our Timm staining results are consistent with Zimmer and Gähwiler (1984), who were the first to note the development of MFS in slice cultures. They suggested that MFS is a consequence of slice culture preparation, resulting from the loss of synaptic contacts made by afferent fibers from extrahippocampal areas that terminate in the dentate molecular layer, and the loss of some hilar mossy cell axons. Two studies using Timm staining in slice cultures have presented contradicting results. Coltman et al. (1995) showed that MFS increased with time *in vitro*, whereas Routbort et al. (1999) observed that MFS in the molecular layer remained constant in control cultures of different ages. The discrepancies between these results may have arisen from the different analysis protocols. Our Timm stain results revealed that the density of sprouted mossy fibers in the hilus and molecular and granule cell layers did increase as a function of DIV.

Anatomical reconstructions of single granule cells from slice cultures at 7 DIV revealed that cells at this stage rarely had large amounts of axon penetrating the granule and molecular layers, confirming previous studies (Bausch and McNamara, 2000). Conversely, in cultures maintained for 14 DIV and 21 DIV, mossy fibre collaterals were more often observed in the granule and molecular cell layers, indicating more MFS in these cultures. From a functional point of view, spontaneous synaptic events showed a trend toward an increase in frequency with time *in vitro*, although this did not reach statistical significance, perhaps due to cell to cell variability. Existing anatomical evidence indicates that excitatory events recorded from granule cells *in situ* arise via polysynaptic innervation from CA3 pyramids (Scharfman, 1996) and the associational/commissural pathway from mossy cells (Amaral, 1978; Buckmaster et al., 1996; Wenzel et al., 1997). Indeed, the trend toward an increase in frequency of excitatory events observed in older cultures might be explained by maturation of these pathways, although our results show a parallel trend of MFS in the older cultures. Sprouting can lead to the formation of recurrent excitatory inputs onto granule cells and thus contribute to the higher sEPSC and mEPSC frequency in older cultures.

Pilocarpine-treated cultures exhibit stronger MFS

We used the muscarinic agonist pilocarpine to induce MFS *in vitro*, extending previous reports of neurotoxin-induced plasticity and MFS in hippocampal slice cultures (Rimvall et al., 1987; Benedikz et al., 1993; Routbort et al., 1999; Koyama et al., 2004). We have studied the acute effects of pilocarpine on slice cultures in order to gain an insight into the mechanism of MFS induction. We found that pilocarpine was capable of inducing ictal-like discharges in mossy cells and CA3 pyramids, due to both direct membrane depolarisation and enhanced network activity, but this appeared insufficient to generate substantial cell injury. Our results are in agreement with the conclusions of Poulsen et al. (2002), who found that despite some signif-
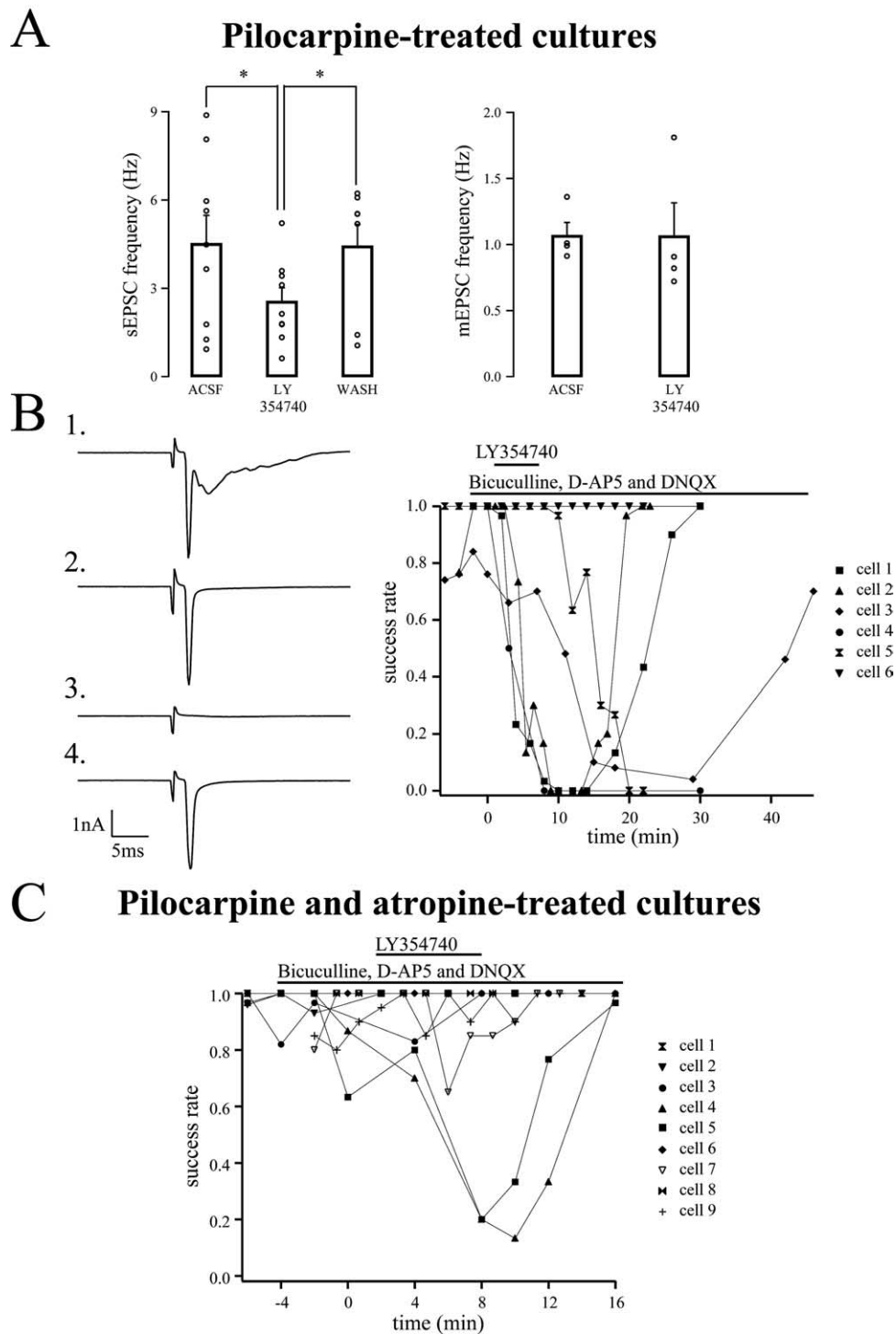
Fig. 9. Effects of LY354740 on axonal group II mGluRs in pilocarpine-treated cultures. (A) Application of 0.5 µM LY354740 significantly reduced the mean frequency of sEPSCs (left graph, $n=9$, $P<0.05$), whereas it had no effect on mEPSC frequency (right graph, $n=4$) in granule cells. * Denotes significance with respect to ACSF and WASH. Bars in the graphs represent mean frequency$\pm$S.E.M., with open circles representing the frequency in individual cells. (B) Application of 0.5 µM LY354740 to pilocarpine-treated cultures significantly decreased the number of successes in obtaining an antidromic action potential in recorded dentate granule cells ($P<0.0001$, $n=5$). The stimulating electrode was placed in the stratum lucidum. Traces on the left represent the response obtained at the various numbered stages of the experiment: 1. Control response. 2. Isolated antidromic action potential in 30 µM bicuculline, 40 µM D-AP5 and 20 µM DNQX. 3. Maximum response to LY354740, i.e. all failures. 4. Response obtained upon wash-out of LY354740. Right graph: Horizontal bars represent the time-scale with which drugs were applied. Each line on the graph represents a different granule cell recording. (C) Similar graph to that in B, obtained from pilocarpine and atropine-treated cultures. Note the faster time scale in plot C than in B. Cells 1–6 are from cultures treated with 0.5 mM atropine and cells 7–9 are from 0.1 mM atropine-treated cultures.

icant cell death in the hilus, very limited cell death occurred after a 48 h pilocarpine application, as measured by propidium iodide uptake. A limited and discrete cell death, for example restricted to a few neurons in hilus, cannot be ruled out at present, based on our LDH data. Granule cells did not display direct depolarisation or ictal-like discharges in response to pilocarpine, but an increase in network-driven spontaneous synaptic activity was observed. Thus, a prolonged pilocarpine-induced enhancement of network activity, coupled with direct effects on specific cell types, could lead to long term plastic changes such as MFS, within the slice culture preparation. This conclusion is consistent with a recent study (Koyama et al., 2004) demonstrating that MFS in slice cultures is activity-dependent.

The Timm staining of chronic pilocarpine-treated cultures revealed a significant increase in MFS in the hilus and the molecular and granule cell layers. Pilocarpine-treated cultures did not show spontaneous ictal activity, but did appear to have a greater propensity for epileptiform activity, as revealed by our experiments using a low concentration of the GABA$_A$ antagonist SR95531. However, the degree of MFS we have observed is less than that seen in acute slices from pilocarpine-treated animals (Okazaki et al., 1999; Buckmaster et al., 2002), despite the fact that the duration of the single systemic injection of pilocarpine normally used in *in vivo* models is shorter than our 48–72 h application of pilocarpine to cultures. It is likely that in *in vivo* models, pilocarpine causes repetitive acute seizures which could lead to more substantial long-term plastic changes and eliminate more hilar cells than in the present *in vitro* conditions (compare Obenaus et al., 1993 with Poulsen et al., 2002). This discrepancy may also arise due to the absence of extrinsic cholinergic inputs in the cultures as compared with the presence of active cholinergic fibers from the diagonal band and medial septal nuclei that target the hilus *in vivo* (Amaral and Witter, 1995). Interestingly, atropine treatment blocked many of the effects observed in pilocarpine-treated cultures, confirming that pilocarpine induces MFS by the specific activation of muscarinic receptors in the dentate gyrus.

In our morphometric analysis, the most striking effect was a significant increase in the total length of axon collaterals in the molecular and granule cell layers of pilocarpine-treated cultures, as compared with 7 and 21 DIV cultures ($P<0.05$). An increase was also observed with respect to 14 DIV, but this did not reach statistical significance ($P<0.07$). When one thinks about differences between the culture groups, it is important to bear in mind that 14 and 21 DIV cultures already show some degree of MFS, which can hamper statistical comparisons with the pilocarpine-treated group. Furthermore, it is possible that, as with the *in vivo* situation, a small subset of cultures did not fully respond to pilocarpine-treatment (for example, see Table 1, cell 3, pilocarpine-treated group). We did not observe a significant increase in the number of terminals associated with the axon collaterals in pilocarpine-treated cultures. When comparing these data with those obtained from Timm staining, it is important to keep in mind that the Timm data labels many axon terminals from many cells

and this tends to masque inter-cell variation. In contrast, in the single cell analysis it is only feasible to label a few cells and the inter-cell variation becomes more evident.

A previous study has observed an increase in the frequency and amplitude of granule cell sEPSCs with time after kainate application *in vivo* (Wuarin and Dudek, 2001). In this study, the effect started 2–4 weeks after the treatment, and progressively strengthened up to 9–50 weeks later. We found that treating cultures with pilocarpine had no significant effect on the frequency of spontaneous or miniature excitatory/inhibitory events. However, the cultures were not tested more than 2 weeks after treatment. We have not extended the time of observation in treated-cultures to greater than 21 DIV for the following reasons. First, in preliminary experiments, we did not observe enhanced MFS from pilocarpine-treated 28 DIV compared with 21 DIV cultures. Second, in cultures maintained several weeks *in vitro*, aberrant excitatory connections can contribute to granule cell hyperexcitability (Bausch and McNamara, 2000).

Unitary synaptic responses between granule cell

Dual whole cell patch clamp recordings have been performed to provide an unequivocal demonstration of functional synaptic connections between granule cells, and to study the functional and pharmacological properties of the newly formed synapses. It is important to bear in mind that our experiments were performed in conditions whereby release probability was slightly enhanced, namely with ACSF containing 3 mM Ca^{2+} and 1.5 mM Mg^{2+}, which ease the detection of synaptically coupled pairs.

The observed monosynaptic responses directly prove the presence of excitatory connections between granule cells in MFS. However, monosynaptic connections between granule cells were rare and polysynaptic connections were much more frequently observed. The monosynaptic responses were invariably abolished by DNQX. Direct evidence for monosynaptic granule cell excitatory connections in MFS has so far been scant. Our finding of a low frequency of monosynaptic granule cell pairs is consistent with data so far obtained from epileptic tissue with strong MFS and is probably due to the large granule cell population. In fact, a recent study observed a few granule cell pairs (six from 903 sharp recordings) in acute slices prepared from pilocarpine-treated rats (Scharfman et al., 2003). Gutierrez and Heinemann (1999) performed some granule cell paired recordings with sharp microelectrodes in slice cultures, and showed that stimulation of one cell could evoke an event in the presence of bicuculline with a latency consistent with a monosynaptic connection. Furthermore, in a previous study, Molnar and Nadler (1999) used granule cell whole cell patch clamp in combination with minimal laser photostimulation of caged glutamate to approximate a 'unitary' situation. They proposed that, by only uncaging glutamate in a small fixed area, extracellular glutamate concentration is sufficient to cause firing only in a few neighbouring cells. Their unitary-like EPSCs were considerably less reliable than those obtained in our hands. This could be due to a decline in the

reliability of transmission during development as our cultures were younger than those used in the above study (see also: Mori-Kawakami et al., 2003). Furthermore, minimal stimulation has been used to reveal the presence of monosynaptic granule cell-granule cell synapses (Feng et al., 2003).

Synaptic circuits in MFS in our model

The sprouted fibers in the molecular layer may form synapses on hilar cells during the development of MFS in the immature hippocampus, as opposed to granule cell dendrites as usually assumed in the adult. In this respect, hilar neurons have dendrites that extend into the molecular layer in both normal (Amaral, 1978) and pilocarpine-treated rats (Scharfman et al., 2001). Indeed, 57% of our labelled hilar neurons had dendrites reaching the molecular layer.

Paired granule cell recordings frequently revealed polysynaptic responses with relatively long latency and large jitter. These currents were inward (excitatory) and outward (inhibitory), and often occurred together in the same cell pair. These results suggest the involvement of a mossy cell and/or a hilar interneuron, respectively, intercalated between the recorded granule cells in pilocarpine-treated cultures. For this to be correct, one would have to assume that the presynaptic granule cell action potential is capable of evoking an action potential within the hilar neuron. We investigated paired granule–hilar cell recordings in current clamp mode and found that this was indeed the case. The long latency observed in polysynaptic connections could be due to a lower conduction velocity in the sprouted collaterals than the 0.67 m/s estimated for the unmyelinated mossy fibre axon (Langdon et al., 1993).

Paired granule–hilar cell recordings in voltage clamp directly investigated the network in more detail. The frequency of monosynaptic connections between granule cells and hilar interneurons or mossy cells was found to be significantly greater in pilocarpine-treated as compared with control cultures of the same DIV. In several cases, pairs were characterised by reciprocal monosynaptic connections, and again, in some cases a postsynaptic action current was evoked in response to a presynaptic stimulus.

In situ, hilar neurons are the major synaptic targets of mossy fibers (Acsady et al., 1998). The positive correlation between MFS *in vitro* (as revealed by Timm staining), the high frequency of polysynaptic granule cell pairs and granule cell to hilar cell connections shows that MFS directly enhances the excitatory drive onto both excitatory and inhibitory cells in the hilus. It is not yet clear whether the same situation exists with MFS *in vivo*. In this respect, it has been recently shown in adult rats that mossy cells in the hilus survive pilocarpine-induced seizures and generate epileptiform burst discharges (Ratzliff et al., 2002).

Presynaptic mGluRs at sprouted synapses

Normally, synapses between granule cells and CA3 pyramidal neurons in the rat are inhibited by the activation of presynaptic group II mGluRs (Henze et al., 2000). Are these receptors also present at the newly formed excitatory synapses? Application of the selective group II mGluR agonist, LY354740 (Schoepp et al., 1999), dramatically increased the number of failures, decreased the uEPSC amplitude, and increased the paired-pulse ratio at granule cell–granule cell synapses. Thus, group II mGluRs are present at the novel, sprouted terminals and act at a presynaptic locus to depress synaptic transmission. The present findings agree with a previous report (Okazaki and Nadler, 2001) showing that the application of the group II mGluR agonist DCG-IV attenuates epileptiform activity evoked by hilar stimulation in acute slices from pilocarpine-treated rats in the presence of bicuculline. More recently, Feng et al. (2003) showed that DCG-IV decreases reliability at the granule cell–granule cell synapse.

Furthermore we observed that LY354740 decreased the frequency of action potential-evoked sEPSCs, but not quantal mEPSCs recorded from granule cells. We have reported in current clamp experiments that granule cells can frequently entrain mossy cell firing, and that mossy cells can in turn elicit EPSCs in granule cells. Therefore, inhibition of the granule cell to mossy cell synapse by group II mGluR activation is consistent with the reduction in granule cell sEPSC frequency. In our experiments the activation of presynaptic group II mGluRs increased the number of failures and significantly reduced the uEPSC amplitude at all granule cell to hilar neuron synapses investigated. LY354740 also caused a weak inhibition in five out of seven monosynaptic connections between mossy cells and granule cells. This effect was reversible and consistent with a presynaptic action. In contrast, at the inhibitory synapses studied, group II mGluRs failed to inhibit uIPSCs recorded in granule cells. This may provide a new rationale for future drug design for temporal lobe epilepsy. Previously, Doherty and Dingledine (2001) found that DCG-IV inhibits release of glutamate from granule cells to hilar border interneurons in acute slices from pilocarpine-treated rats. It seems, therefore, that group II mGluRs do not display target specificity in their distribution at granule cell terminals.

We further investigated the mechanisms of the group II mGluR-mediated inhibition in pilocarpine-treated cultures. The increased number of failures after LY354740 in paired recordings gave a clue as to the mechanism behind this action. This suggested that group II mGluRs located on sprouted mossy terminals and mossy cell axon terminals presynaptically inhibited the release of glutamate by blocking the action potential invasion of the terminals.

This was directly investigated by applying LY354740 whilst recording an antidromic action current in granule cells. We found that the application of LY354740 dramatically inhibited an action current evoked in the stratum lucidum, near the mossy fibre terminals, and recorded at the soma of granule cells. Furthermore, this action was reversed by the application of an mGluRII antagonist, LY341495. This finding represents a novel action for presynaptic mGluRs that is consistent with the detection of group II mGluRs at extrasynaptic sites remote from the active zone in the preterminal portion of hippocampal axon terminals (Yokoi et al., 1996; Shigemoto et al., 1997). In

atropine and pilocarpine-treated cultures, LY354740 mostly failed to suppress the antidromic action currents, suggesting that group II mGluRs could be expressed along the axon of sprouted granule cells only, perhaps at high density at the axonal branching points. Semyanov and Kullmann (2001) presented data showing that kainate receptors can reside along the axons of hippocampal interneurons and activation of these receptors induces spontaneous antidromic action potentials. This non-conventional mechanism does not exclude that more classical mechanisms, such as inhibition of presynaptic calcium channels (Kamiya and Ozawa, 1999), could also contribute to the presynaptic inhibition mediated by group II mGluRs at the synapses that we have studied.

CONCLUSION

Our results point out that MFS *in vitro* involves not only the formation of novel excitatory synapses between granule cells but also between granule cells and hilar neurons. Our data may also reconcile conflicting results obtained with MFS in the adult, where different studies show MFS acting mainly to enhance either excitatory (for example, Buckmaster et al., 2002; Kobayashi and Buckmaster, 2003) or inhibitory (for example, Kotti et al., 1997) inputs to granule cells. We provide insights into important features that have been often neglected in previous studies, such as the initial stages of MFS and the mechanisms of MFS in the developing hippocampus (see also, Holmes et al., 1999; Jacobs et al., 2000; Ben-Ari, 2001). It remains to be seen whether our model using young tissue *in vitro* will be applicable also to the developing hippocampus *in vivo*. Furthermore, we have shown that the activation of group II mGluRs can potently inhibit excitatory synapses within our model of MFS, consistent either with conventional presynaptic sites or non-conventional axonal locations.

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