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Biocompatibility of silicon-based arrays of electrodes coupled to organotypic hippocampal brain slice cultures

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Abstract

In this study we examined the passive biocompatibility of a three-dimensional microelectrode array (MEA), designed to be coupled to organotypic brain slice cultures for multisite recording of electrophysiological signals. Hippocampal (and corticostriatal) brain slices from 1-week-old (and newborn) rats were grown for 4–8 weeks on the perforated silicon chips with silicon nitride surfaces and 40 μm sized holes and compared with corresponding tissue slices grown on conventional semiporous membranes. In terms of preservation of the basic cellular and connective organization, as visualized by Nissl staining, Timm sulphide silver-staining, microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) immunostaining, the slice cultures grown on chips did not differ from conventionally grown slice cultures. Neither were there any signs of astrogliosis or neurodegeneration around the upper recording part of the 47-μm-high platinum-tip electrodes. Slice cultures grown on a separate set of chips with platinum instead of silicon nitride surfaces also displayed normal MAP2 and GFAP immunostaining. The width of the GFAP-rich zone (glia limitans) at the bottom surface of the slice cultures was the same (~20 μm) in cultures grown on chips with silicon nitride and platinum surfaces and on conventional insert membranes. The slice cultures grown on chips maintained a normal, subfield differentiated susceptibility to the glutamate receptor agonist *N*-methyl-D-aspartate (NMDA) and the neurotoxin trimethyltin (TMT), as demonstrated by the cellular uptake of propidium iodide (PI), which was used as a reproducible and quantifiable marker for neuronal degeneration. We conclude that organotypic brain slice cultures can grow on silicon-based three-dimensional microelectrode arrays and develop normally with display of normal subfield differentiated susceptibilities to known excito- and neurotoxins. From this it is anticipated that the set-up, designed for recording of electrophysiological parameters, can be used for long-term studies of defined neuronal networks and provide valuable information on both normal, neurotoxicological and neuropathological conditions.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Microelectrode array; Silicon nitride; Platinum; Neurotoxicological screening; Propidium iodide; Excitotoxicity

1. Introduction

Many chemical substances designed for use in industry or daily life can be expected to have neurotoxic effects. In order to advance screening methods to the level where they, besides detecting toxic effects resulting in neurodegeneration, can also detect minor effects at the network or synaptic level, microelectrode arrays (MEA) have been developed and combined with conventional in vitro culture technology to be used for in vitro neurotoxicological testing. Recordings of changes in complex activity over time may more precisely depict what is occurring in the brain than conventional electrophysiological recordings on acute slice preparations or living animals. The use of such

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devices will also improve our understanding of how electrophysiological activity patterns develop and reorganize in the brain, given that each brain slice culture in principle can be used for several weeks instead of the 12 to 16 h which is usual for conventional acute brain slice techniques.

There are existing microelectrode arrays that can be combined with hippocampal slice cultures [5,6,31]. For use with the Physiocard, the slice cultures are grown on a semiporous membrane in an incubator ad modum Stoppini [30] and thereafter transferred to the Physiocard, where the stimulating and recording electrodes are inserted into the cultures from above [31]. In a recent modification of the Physiocard, the array supports the slice culture rather than being placed on top of the culture, since the latter procedure results in tissue deterioration [5]. The modified system seems to be useful for studies lasting a maximum of 3 days [31]. For most of the available modified culturing techniques basic histological validations are limited and none of the systems have been proven to be useful for long-term exposures and recordings lasting for weeks.

In relation to existing arrays using planar microelectrodes, there is room for improvements in terms of obtaining higher signal amplitudes and signal-to-noise ratios. For this purpose, the array tested for support of brain slice cultures in the present study was fabricated with three-dimensional tip-shaped electrodes, which allow electrophysiological recordings inside the cultured tissue slices [32,33]. We have previously reported that hippocampal slice cultures can grow on these perforated silicon chips with silicon nitride surfaces, just as initial electrophysiological experiments have demonstrated that stimulation and recording from CA1 pyramidal cells produced signals with a signal-to-noise ratio superior to planar arrays [7,8,12]. In this study, hippocampal slice cultures were grown for up to 8 weeks on the perforated chips with the tip-shaped microelectrodes to investigate the detailed arrangement of neuronal and glial elements around the electrodes and in relation to the chip surface in general and to compare the effects of the excitotoxic glutamate receptor agonist *N*-methyl-D-aspartate (NMDA) and the neurotoxin trimethyltin (TMT) on the chip-based and regularly grown slice cultures. As we primarily were interested in the basic mechanical and material interaction effects, the chips were passive, i.e. they were not intended to be used for stimulations or recordings in this study. For comparison of toxic effects, we therefore mostly used perforated chips without electrodes. In the parts of the study devoted to potential glial reactions around the electrodes, the arrays with tip-shaped electrodes were used. The uninsulated tips of the electrodes had a platinum-surface, and to test this more generally we also used chips with the entire surfaces made of platinum instead of silicon nitride.

Results from parts of this study have appeared in abstract form [8,12].

2. Materials and methods

2.1. Microelectrode array

The detailed design and fabrication process of the

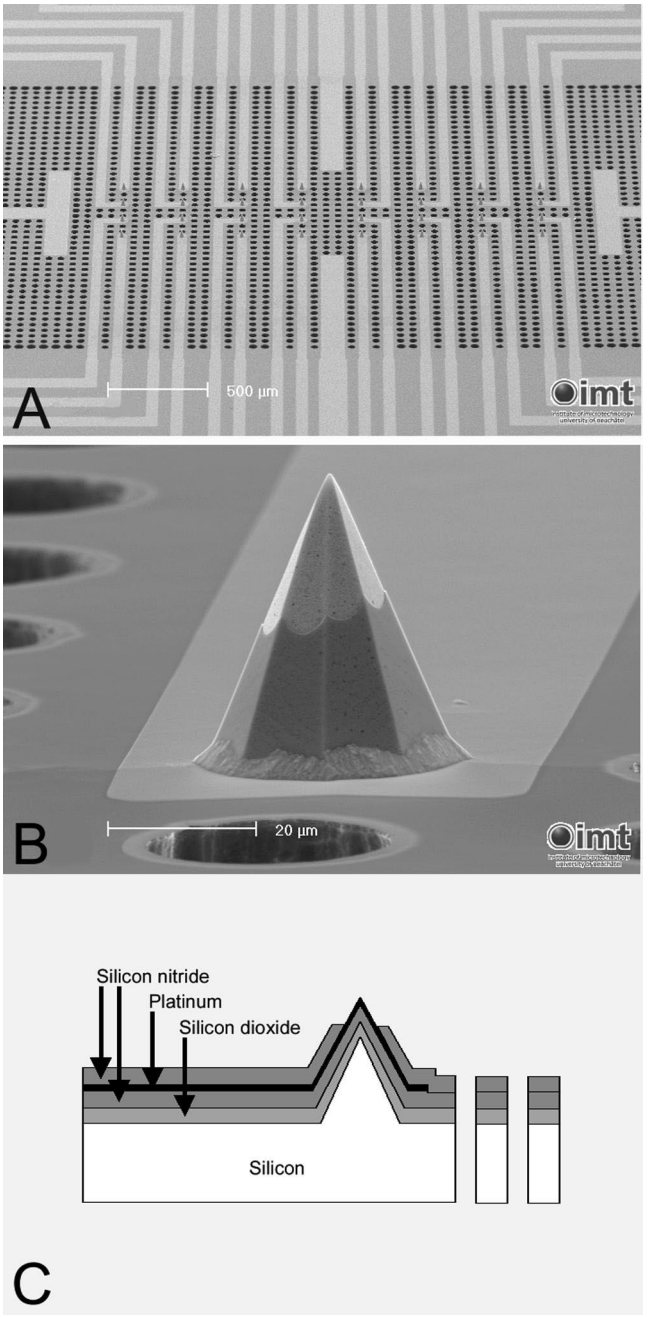


Fig. 1. (A) Scanning electron micrograph of a microelectrode array with a quadratic arrangement of 47-µm-high tip-shaped electrodes embedded on a perforated substrate with holes 40 µm in diameter. (B) Higher magnification of a tip-shaped electrode, where the lower part (32 µm) is covered with silicon nitride and the apical part (15 µm) covered with platinum. (C) Illustration of the different layers of the chip which form the upper surface and downwards consist of silicon nitride, platinum, silicon dioxide and for the rest of the thickness silicon.

microelectrode arrays or chips have been described previously [32] (Fig. 1). In summary, the devices were fabricated on a 390-μm-thick silicon wafer. The silicon tips were formed by anisotropic etching in a 40% KOH solution at 60°C [36]. Following the deposition of 1000 Å of silicon dioxide (SiO_2) and 2000 Å of silicon nitride (Si_3N_4), a 200-Å-thick adhesion layer of Ta and a 1300-Å-thick layer of Pt were evaporated and patterned using a lift-off process. The top passivation layer of 2000 Å silicon nitride was patterned by an SF_6/O_2 RIE plasma [26]. Via-hole photolithography and then etching, using a DRIE (Surface Technology systems, UK) process, and an aluminium mask finished the device fabrication sequence.

Silicon [27], silicon dioxide [29], silicon nitride [6] and platinum [29], are in general considered to be biocompatible materials, meaning that the materials do not induce toxic reactions in tissues they are placed in intimate contact with. The final device has a perforated square-shaped region of 4 mm×3 mm perforated by holes of 40 μm in diameter, between which there is either a double elliptical or quadratic arrangement of 32–34 pointed platinum-tip microelectrodes (Fig. 1A). Each microelectrode has a total height of 47 μm of which the top 15 μm has an uninsulated platinum surface. The remaining bottom parts are covered and insulated by a layer of silicon nitride (Fig. 1B and C).

2.2. Organotypic slice cultures

Hippocampal slice cultures were prepared as previously described [20,30]. In brief, 5–7-day-old rat pups of Wistar strain were killed by instant decapitation and the dorsal hippocampus rapidly dissected out and sectioned transversely at 350–400 μm by a McIlwain tissue chopper. In addition a few corticostriatal slice cultures were prepared from newborn rats as previously described [11]. For growth of hippocampal slice cultures as regular controls the hippocampal slices were placed on porous insert membranes (Millipore Corp., Bedford, MA, USA, cat. no. PICM 030 50). For growth on the microelectrode arrays, a 4×5 mm hole was first cut in the central part of the Millipore membrane and an array was positioned on top of the membrane, covering the hole and thereby leaving the perforated, central part of the chip in direct contact with the culture medium (Fig. 2A). A tissue slice was thereafter placed on the perforated part of the chip, while other tissue slices for conventional growth and comparisons were placed on the intact membrane around the chip. In a few experiments the chips were placed on the intact membrane (no hole made) to test for effects of having both the chip and the membrane as a support interposed between the tissue and the growth medium below (Fig. 2B). Such an arrangement might be appropriate for maintaining the cultures between sessions of electrophysiological measurements, where the chip is mounted in a printed circuit board. The membrane inserts were transferred to 6-well culture trays (Corning Costar, Corning, NY, USA), where each well contained 1 ml culture medium, composed of 50% Opti-MEM (cat. no. 31985-047), 25% horse serum (cat. no. 26050-047) and 25% Hank's BSS (HBSS; cat. no. 24020-091) (all from Gibco Brl, Life Technologies Ltd., Paisley, Scotland), supplemented by D-glucose (Merck, Darmstadt, Germany, cat. no. 8342) to a final concentration of 25 mM. The culture trays were kept in an incubator at 36°C with 5% CO_2 and 95% humidified air. After 4 days of incubation, the culture medium was replaced with 1 ml of chemically defined, serum-free Neurobasal medium (Gibco Brl, cat. no. 21103-049) with 25 mM D-glucose, 1 mM L-glutamine (Sigma, Vallensbæk Strand, Denmark, cat. no. 25030-024), and 2% B27 supplement (Gibco Brl) [3]. The medium was thereafter changed twice a week for the next 3.5–8 weeks. No antimitotic drugs or antibiotics were added at any stage.

2.3. Propidium iodide uptake

Propidium iodide (PI or 3,8-diamino-5-(3-(dieth-

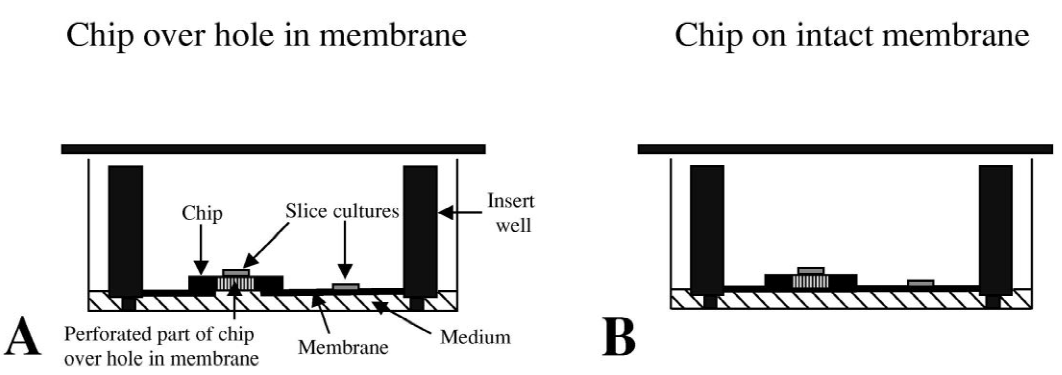


Fig. 2. Illustration of placement of silicon-based chips with cultured brain tissue slices either on top of a hole cut in the underlying semiporous membrane (A) or on the intact membrane (B). For comparison of cell survival and histological organization corresponding hippocampal slices were positioned directly on the semiporous membrane in the same wells.

ylmethylamino)propyl)-6-phenyl phenanthridinium diiodide; Sigma, cat. no. P4170) is a very stable fluorescent dye absorbing blue–green light (493 nm) and emitting light with red fluorescence (630 nm). As a polar compound, PI only enters dead cells with a damaged cell membrane and yields a brightly red fluorescence upon interaction with DNA [34].

For comparing the toxic effects of *N*-methyl-D-aspartate (NMDA) and trimethyltin (TMT) between slice cultures grown as conventional membrane cultures and slice cultures grown on chips, PI was used in accordance with a previously developed protocol known to be suitable for detecting regional and quantitative differences [19,20,38] (Fig. 3). In brief, PI was added to the culture medium of 3–4-week-old cultures 3 h before the start of the NMDA- and TMT-exposures in order to monitor basic levels of PI uptake and cell degeneration in the cultures. The cellular uptake of PI was recorded by a digital camera (Sensys KAF 1400 G2, Photometrics) mounted on an upright fluorescence microscope (Olympus Vanox-T, 4X), using a standard rhodamine filter set. After digital recording of the basic PI uptake (day 0 in Fig. 3), the cultures were exposed to either 10 μM NMDA (Sigma, cat. no. M-3262) or 20 μM TMT (trimethyltin chloride; Aldrich-Europe, cat. no. 14, 649-8) and PI uptake in the individual cultures was recorded again after 24 h (day 1 in Fig. 3) and 48 h (day 2 in Fig. 3). All neurons were finally killed by exposure to 50 mM of glutamate for 1 h followed by 24 h in normal medium before a final set of digital pictures recording the maximal PI uptake (total neuronal degeneration) were taken (day 3 in Fig. 3). This allows the NMDA- and TMT-induced PI uptake at day 1 and day 2 to be expressed as a percentage of the maximal PI uptake (see above). The digital photos were analyzed densitometrically in NIH image 1.62 image analysis program (National Institute of Health, USA).

2.4. Histological procedures

The general organization of cell layers and major intrinsic fiber projections in the cultures was analyzed in toluidin blue and Timm stained cryostat sections of 3.5–8-week-old cultures. The Timm sulphide-silver method, which histochemically detects and by its staining precipitate visualizes zinc associated in particular with glutamatergic projections, is commonly used to monitor the distribution of hippocampal pathways [37]. In brief, the cultures to be processed for Timm staining were soaked in a sodium sulphide solution with sucrose. Thereafter the cultures were gently removed from the supporting membrane or chip and frozen and cut in two series of 20-μm-thick cryostat sections, which where then stained by toluidin blue and Timm staining as previously described [7].

Microtubule-associated protein 2 (MAP2) is a cytoskeletal protein primarily found in dendrites and as such it has been used as an immunocytochemical marker for neuronal integrity [19]. Glial fibrillary acidic protein (GFAP) is on the other hand commonly used as an immunohistochemical marker for astroglial cells [13], and it was used as such to look for details of astroglial processes and for astroglial reactions or possible astrogliosis in the slice cultures. Four-week-old slice cultures were fixed in paraformaldehyde and transferred to a sucrose solution for cryoprotection. Thereafter the cultures

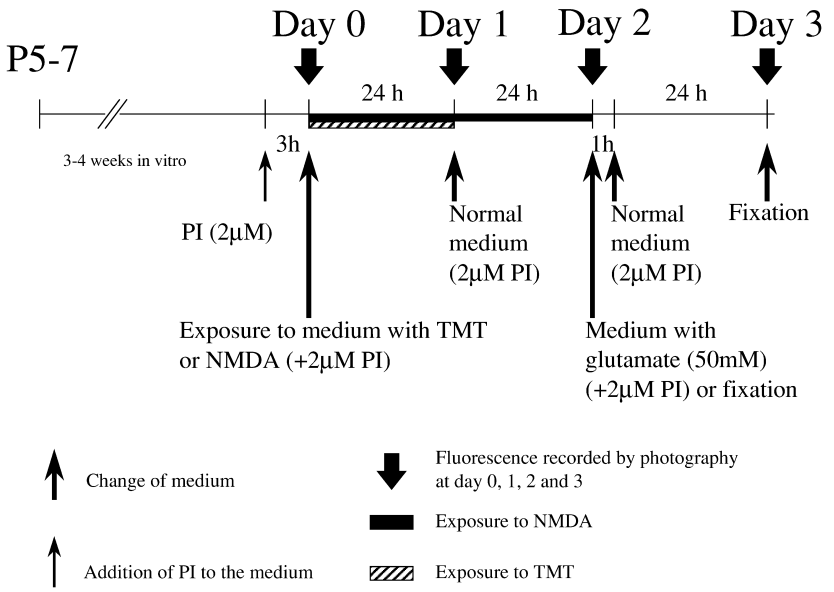
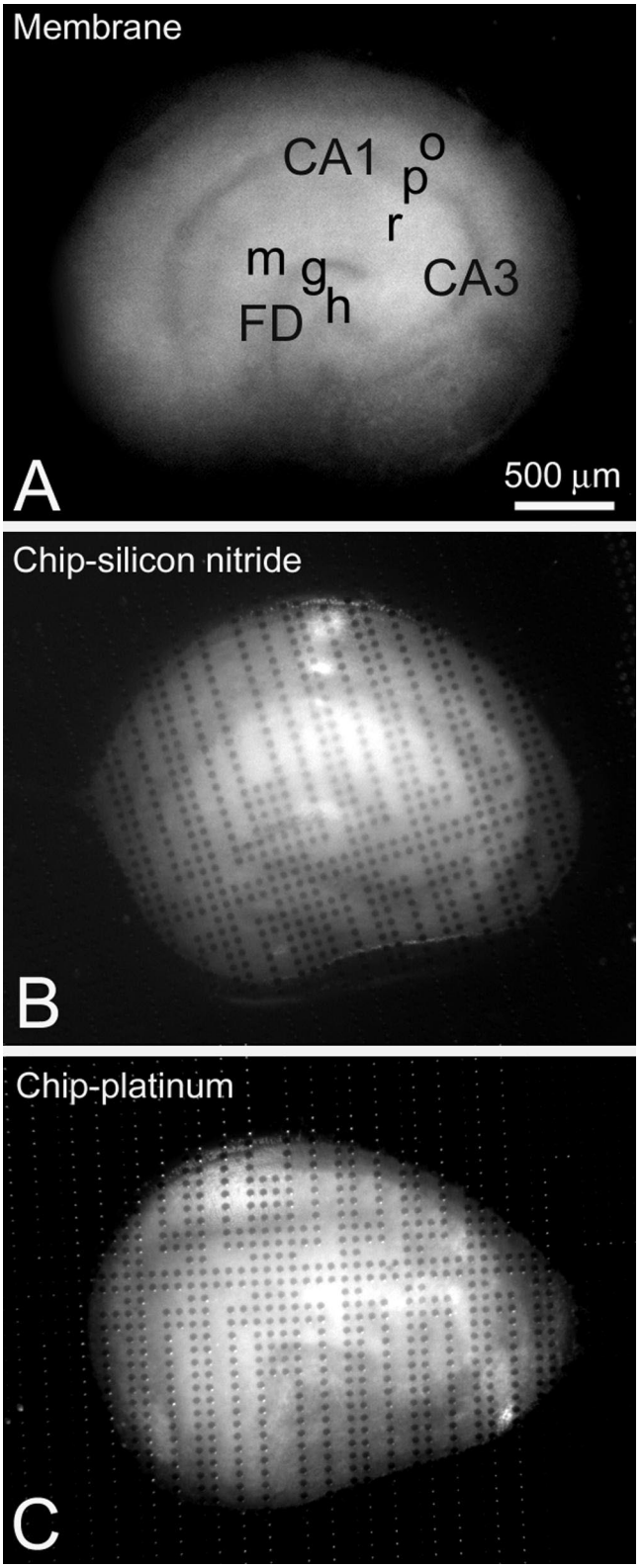


Fig. 3. Protocol for the use of propidium iodide (PI) uptake as a marker of neuronal cell death in hippocampal slice cultures exposed to the glutamate receptor agonist NMDA and the neurotoxin TMT. For further explanation, see text.

were gently removed from the supporting membrane or chip and frozen and cut in two series of 20-μm-thick cryostat sections. One of the series was stained immunocytochemically for MAP2, the other for GFAP according to earlier used protocols [7]. Omission of the primary antibody abolished all staining reaction. Double staining for MAP2 and GFAP was performed to demonstrate the mutual distribution of MAP2- and GFAP-immunoreactive processes around the microelectrodes and in the tissue processes extending into the holes of the chip. The double staining protocol was essentially a combination of the protocol used for conventional MAP2 and GFAP immunostaining except for the use of fluorescent dyes for visualization. Also normal goat serum was used to reduce unspecific staining, because goat secondary antibodies were used. The sections were incubated simultaneously with a mouse anti-MAP2 and rabbit anti-GFAP antibody [7] and later with a secondary Cy3 labeled goat anti-mouse (Jackson ImmunoResearch, West Grove, PA, USA, cat. no. 115-165-146) and Cy5 labeled goat anti-rabbit (Jackson ImmunoResearch, cat. no. 111-175-144) antibody. Omission of primary antibodies abolished all staining reaction. The sections were analyzed in a Leica confocal laser scanning microscope (Heidelberg, Germany) with an argon/krypton mixed gas multiline laser. Confocal microscopical data were acquired with a 25× oil immersion objective lens and the Cy3 and Cy5 fluorochromes were excited by the laser beam at 568 and 647 nm, respectively. The resulting emissions were detected after passing through 590 and 665 nm emission filters. For control, immunostainings were also made with either the MAP2 primary antibody and the secondary Cy3 antibody, or the GFAP primary antibody and the secondary Cy5 antibody. When the Cy3 filter settings were used for Cy5 and vice versa no signal was obtained from the respective fluorochromes.



2.5. Statistics

All densitometric data were expressed as means+ standard error of mean (S.E.M.). Statistical significance was assessed in GraphPad Instat (GraphPad Software, San Diego, CA, USA), using paired or unpaired *t*-test or single factor analysis of variance (ANOVA) with Bonferroni correction for comparison of the groups of interest. Differences were considered significant at $P<0.05$.

Fig. 4. Photomicrographs with epi-light of organotypic hippocampal slice cultures located on the semiporous insert membrane (A) or on silicon chips (without microelectrodes) with a silicon nitride surface (B) or a platinum surface (C). In both membrane- (A) and chip-based cultures (B, C) the hippocampal CA1 and CA3 pyramidal cell layers and the granule cell layer of fascia dentata are clearly visible during the culture period. On photographs of chip-based cultures, the pores stand out as black dots and the electrode connectors as light bands. Since the electrodes are situated at the ends of the light bands, it is even possible directly to localize the electrodes inside the tissue. FD, fascia dentata; g, granule cell layer; m, molecular layer; h, dentate hilus or CA4; p, pyramidal cell layer; o, stratum oriens; r, stratum radiatum.

3. Results

3.1. Microscopical inspection of cultures during culture period

The slice cultures were regularly examined by stereomicroscopy. Both in cultures grown conventionally on the interface membrane and cultures grown on silicon-based chips with surfaces of silicon nitride or platinum, an overall, normal organotypic organization was evident with distinct presence of the main neuronal cell and neuropil layers (Figs. 4 and 7A). Corticostriatal slice cultures grown on chips also appeared normal, allowing a clear distinction between the striatum and cortex even after several weeks (Fig. 5).

3.2. Toluidin blue cell staining

In ordinary cell staining hippocampal slice cultures grown for 4–8 weeks on the interface membrane (Fig. 6A) or on the silicon nitride-coated chips with (Figs. 6C and 7B) or without (Fig. 6E) a hole in the underlying membrane displayed the same distinct organotypic organization with clear identification of the main cell and neuropil layers of the hippocampal and dentate subfields. The yield of good quality cultures with intact hippocampal and dentate subfields and layers was essentially the same in the different culture groups.

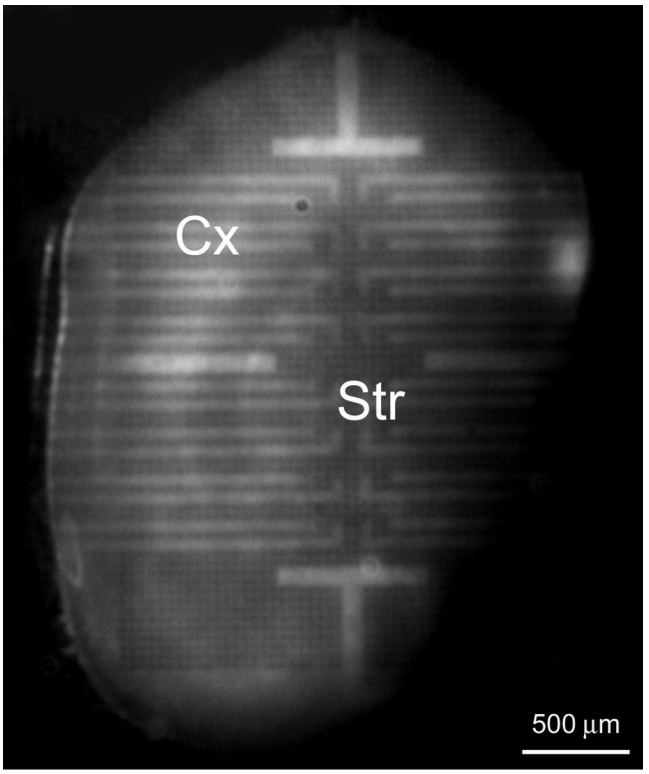


Fig. 5. Organotypic corticostriatal slice culture derived from newborn rat and grown for 4 weeks on a silicon chip with a silicon nitride surface (without microelectrodes). Corticostriatal slice cultures appeared normal with well defined cortical (Cx) and striatal (Str) parts.

3.3. Timm sulphide silver staining

Sections of cultures grown 4–8 weeks on the interface membrane (Fig. 6B) and the silicon nitride-coated chips with (Figs. 6D and 7C) or without (Fig. 6F) a hole in the underlying membrane displayed similar staining patterns. Densely Timm stained mossy fiber terminals were found in the dentate hilus and extending along and mostly above the CA3 pyramidal cell layer towards the CA3–CA1 transition. Smaller aberrant mossy fiber terminals were also found between and just above the granule cell layer, representing mossy fiber collaterals, known to sprout in response to the absence of the normal entorhinal perforant path input to the dentate molecular layer [37]. As part of this reorganization, which also included associational projections within CA3 and the Schaffer collateral projection from CA3 to CA1, the otherwise distinctive perforant terminal zones in the dentate molecular layer were absent, and a clear distinction between stratum lacunosum-moleculare and stratum radiatum in CA3 and CA1 was difficult.

3.4. Immunocytochemical staining for MAP2 and GFAP

Cultures grown on the semiporous membrane and chips with silicon nitride and platinum surfaces displayed similar patterns of intense MAP2 staining of primarily dendrites, corresponding to the staining pattern in vivo (Figs. 8, 11A and B). In sections towards the bottom of the slice cultures, a clear systematic pattern evolved in relation to the localization of microelectrodes and the holes perforating the chip (Figs. 10 and 11). MAP2 positive processes were in close contact with the surface of the holes in the tissue left by the upper parts of the electrodes, when the slice cultures were removed from the chips for histological processing (Fig. 11C and E), while they were reduced or absent in a narrow zone around what corresponds to the basal part of the electrodes (Fig. 11F and H). From the bottom surface of the cultures, where tissue processes extended into the holes perforating the chips, MAP2 positive neuronal processes converged in bundles towards the base of these tissue extensions, keeping a central position within these (Figs. 10A, 11I and K).

The GFAP staining of the hippocampal slice cultures grown on chips with silicon nitride and platinum surfaces did not differ from the staining of the cultures grown on the interface membrane (Figs. 9, 11A and B). GFAP-positive fibers in the neuronal layers were in general oriented perpendicular to these layers in the cultures (Fig. 9A, C and E), and several astroglial cell bodies were present in stratum radiatum of CA3 and CA1 with a more stallate, radiating orientation of their processes (Fig. 9B, D and F). Most importantly, there was no glial encapsulation of the upper parts of the electrodes (Fig. 11D and E),

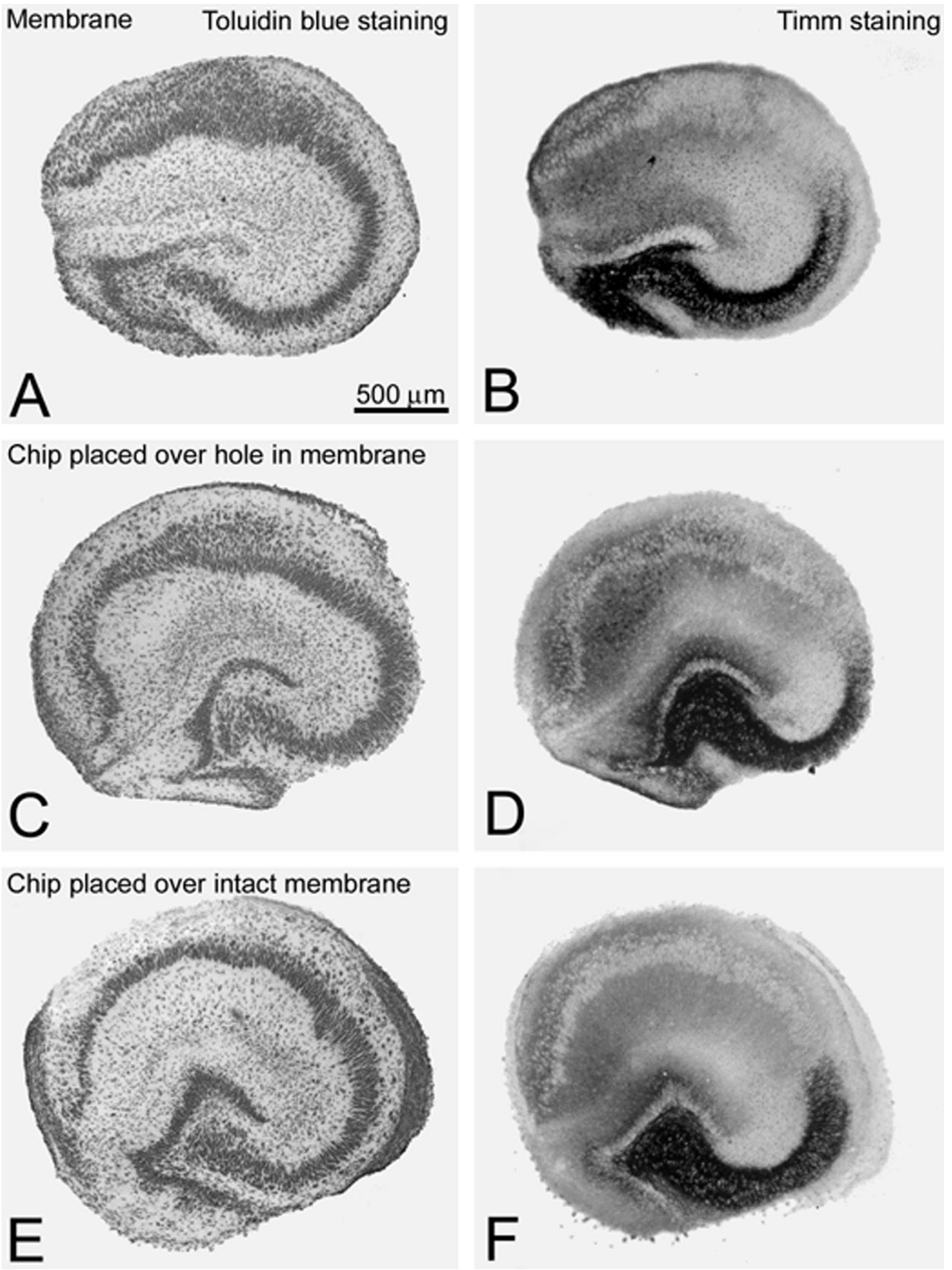


Fig. 6. Organotypic hippocampal slice cultures grown for 4 weeks on membrane (A, B), or on chip (with silicon nitride surface and without microelectrodes) placed over a hole in the membrane (C, D) or on an intact membrane (E, F). The cultures were later processed for histology and stained by toluidin blue (A, C, E) and the histochemical Timm's sulphide-silver method for zinc (B, D, F). Note the similar staining of the membrane-based and the two types of chip-based cultures. For abbreviations, see Fig. 4.

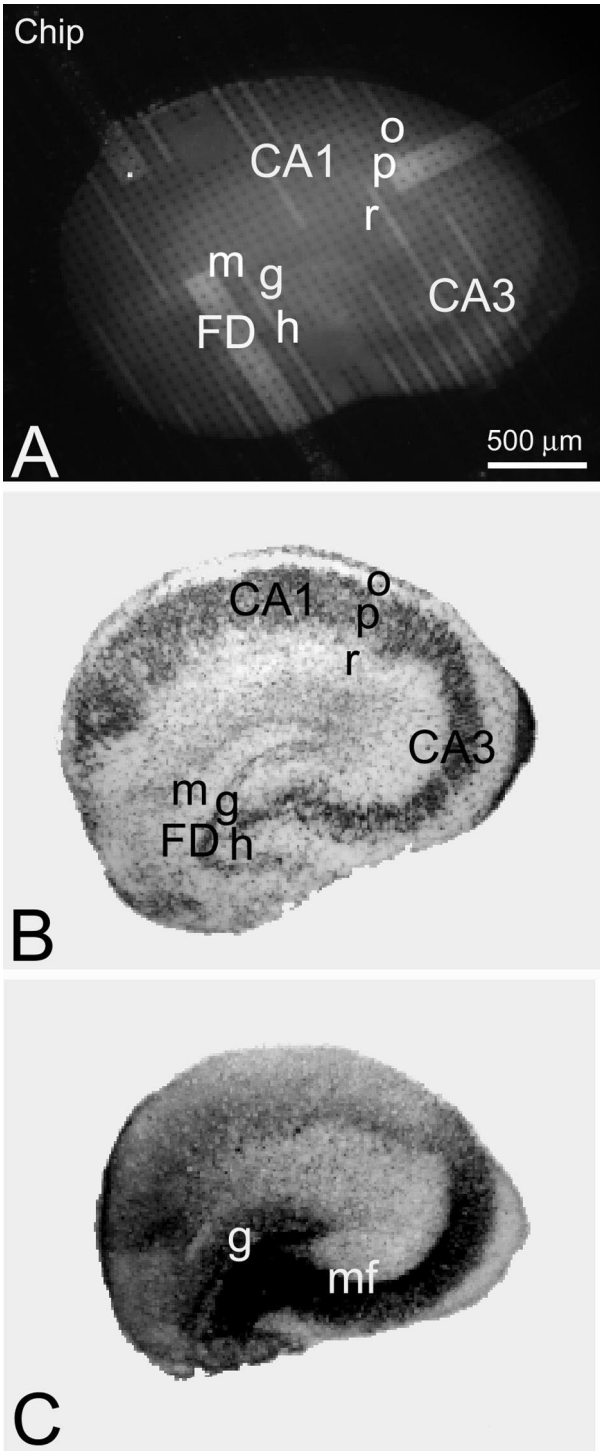


Fig. 7. Organotypic hippocampal slice culture grown for 8 weeks on a chip with a silicon nitride surface and without microelectrodes. The culture was photographed alive (A), before it was processed for histology with pairs of adjacent cryostat sections being stained by toluidin blue as a general Nissl cell stain (B) and by Timm staining (C). (B) Toluidin blue stained section, illustrating the organotypic appearance of subregions and layers of the hippocampus and fascia dentata. (C) Timm stained section next to the section in (B) with characteristic, densely stained mossy fiber zone along the pyramidal layer of CA3, stopping at the CA3–CA1 border. FD, fascia dentata; m, molecular layer; g, granule cell layer; h, dentate hilus or CA4; mf, mossy fiber projection; p, pyramidal cell layers; r, str. radiatum; o, str. oriens.

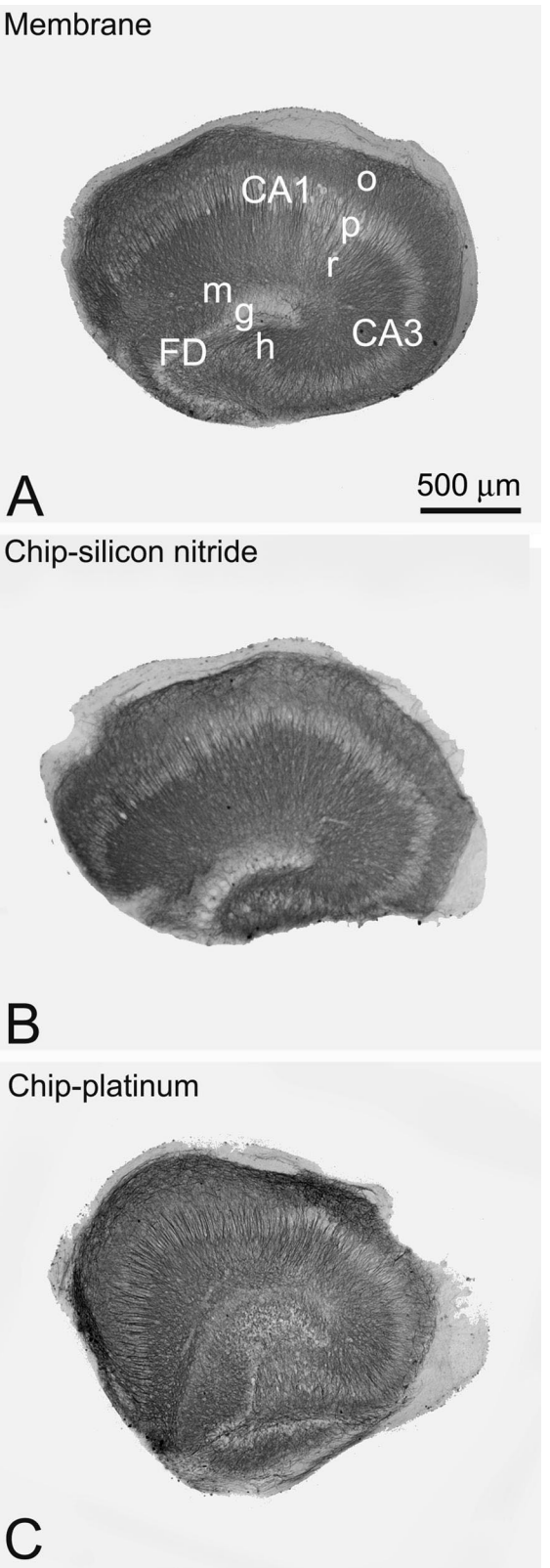


Fig. 8. MAP2 immunostaining of sections from hippocampal slice cultures grown for 4 weeks on the interface membrane (A) or on silicon chips (without microelectrodes) with a silicon nitride surface (B) or a platinum surface (C). Note the intense MAP2 staining of the dendritic regions and the weak staining of cell body layers in both membrane and chip cultures, corresponding to the distribution in vivo. For abbreviations, see Fig. 4.

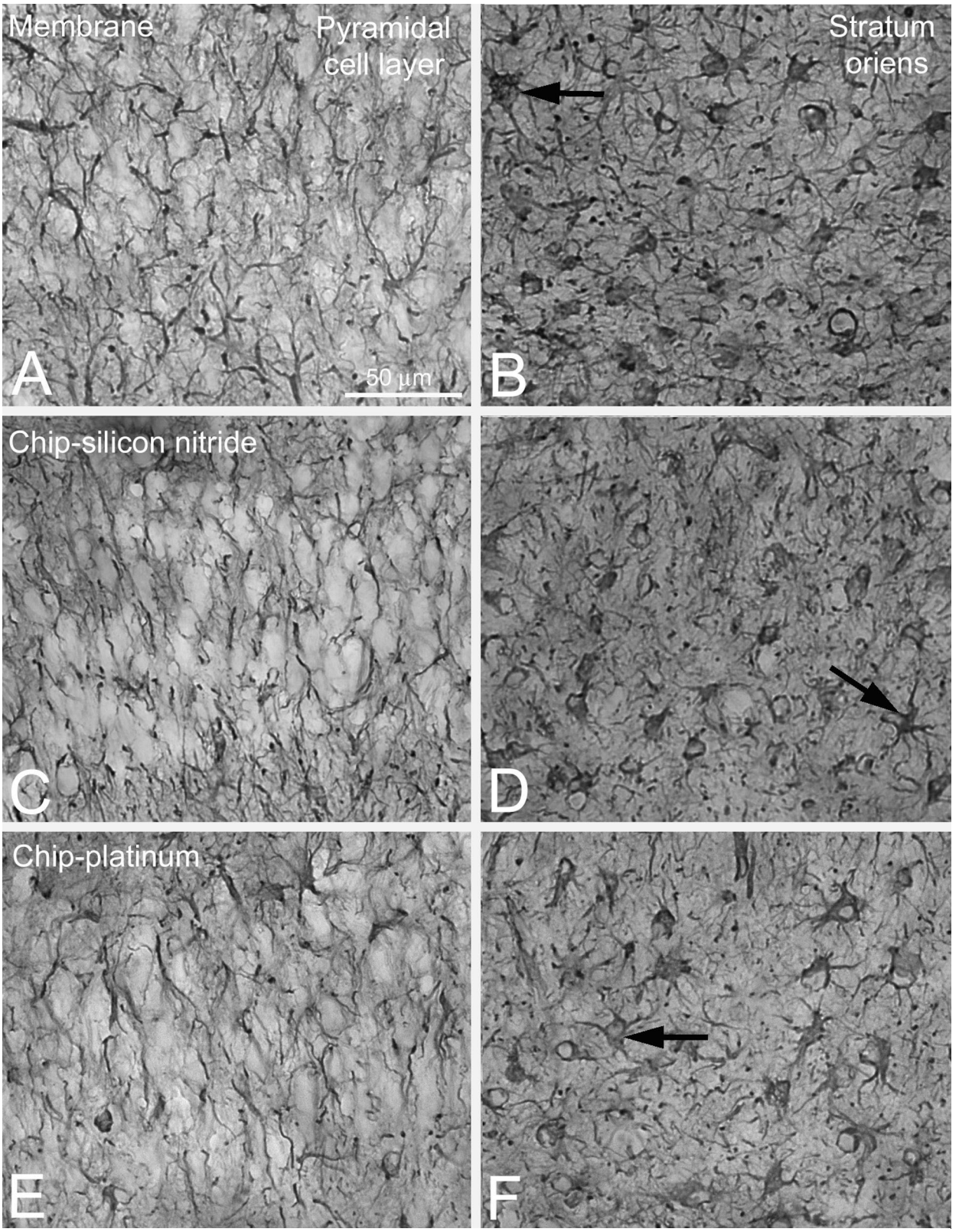


Fig. 9. Astroglial cells and processes demonstrated by GFAP immunostaining of sections from hippocampal slice cultures grown for 4 weeks on the interface membrane (A, B) or on silicon chips (without microelectrodes) with a silicon nitride surface (C, D) or a platinum surface (E, F). Note the GFAP positive radial fibers passing perpendicularly through the pyramidal cell layer (A, C, E), and the stellate astroglial cells (arrows) in stratum radiatum (B, D, F). There was no differences in the GFAP staining pattern between the three cultures.

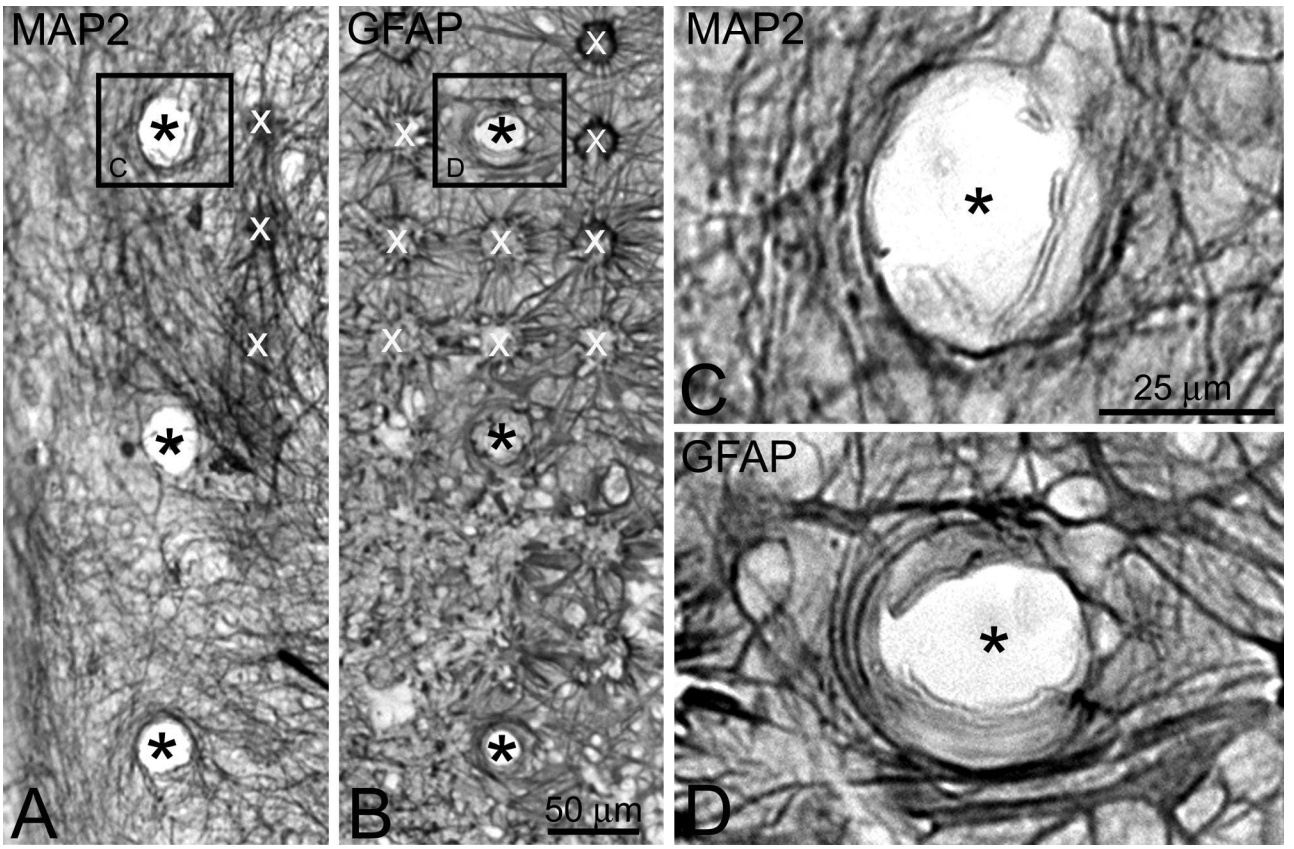
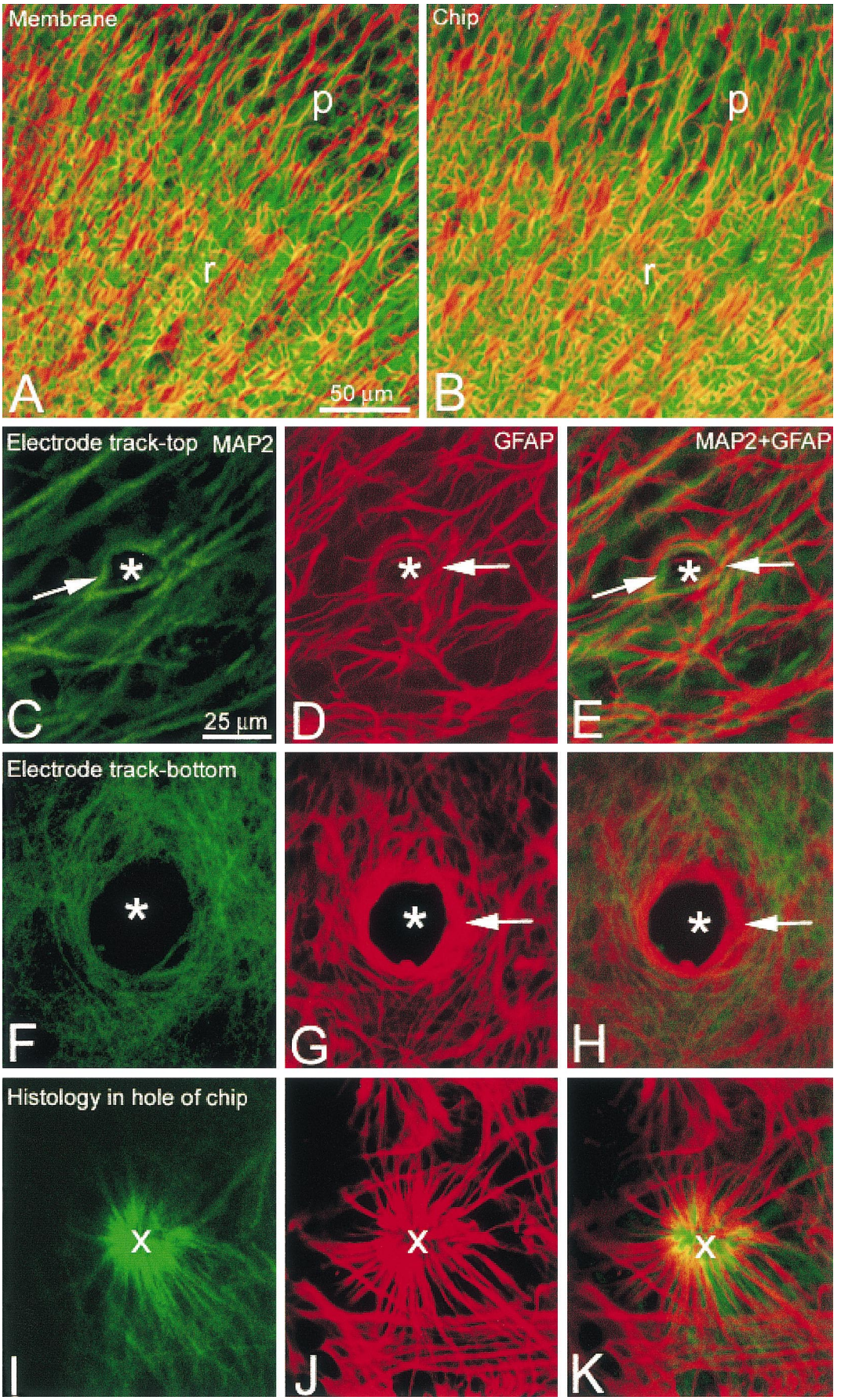


Fig. 10. MAP2 (A, C) and GFAP (B, D) immunostaining from tangentially cut hippocampal slice cultures grown 4 weeks on a chip with a silicon nitride surface and tip-shaped microelectrodes. The upper part of the pictures in (A) and (B) show the bottom surface of the slice culture with a regular histological pattern corresponding to the sites of the chip-electrodes within the tissue (*) and tissue elements growing towards and into the holes of the chip (×). The lower part of the pictures (A) and (B) cut into a slightly higher level of the slice culture reaching directly into the culture neuropil. The framed part of (A) and (B) is shown in (C) and (D), showing MAP2 and GFAP stained neuronal processes, respectively, around the position of the basal part of two electrodes. Scale bars, 50 μm (A, B), 25 μm (C, D).

where individual MAP2- and GFAP-immunoreactive processes were found equally close to the electrode tips, displaying an orientation and shape indistinguishable from processes in the tissue further away from the electrode locations. Around the basal parts of the electrodes, there was, however, a capsule-like zone with condensed GFAP-immunoreactive processes (Fig. 11G and H), corresponding to the zone devoid of MAP2-immunoreactive processes (Fig. 11F and H). At the same bottom levels of the slice cultures, facing the perforated area of the chip, GFAP-immunoreactive processes converged towards the tissue processes extending into the holes of the chips (Figs. 10B, 11J and K). Relative to the MAP2-immunoreactive processes, the astroglial processes have a peripheral position, separating the neuronal elements from the inner surface of the chip holes.

Fig. 11. Confocal micrographs of MAP2 (green) and GFAP (red) double immunofluorescence staining of sections of hippocampal slice cultures grown 4 weeks on membranes or chips with silicon nitride surfaces and microelectrodes. Images showing the combined images (MAP2+GFAP) from the two sequential scannings are shown in (A), (B), (E), (H) and (K). The histology in the deep part of the cultures grown on a chip is illustrated with MAP2 (C, F, I) and GFAP (D, G, J) single channel images. (A, B) The general histology of the two types of cultures was similar with regard to MAP2 and GFAP immunostaining as illustrated for the pyramidal cell layer (p) and stratum radiatum (r) of CA1 in a membrane-based culture (A) and chip-based culture (B), with several electrodes (not visible) located in this region. (C, D, E) In more basal parts of the chip-based culture both MAP2- and GFAP-immunoreactive processes (arrows, C, D, E) were found close to the electrode tip (electrode track is marked by *), but were otherwise indistinguishable from processes further away from the electrodes. (F, G, H) Further towards the chip-facing surface of the slice culture, there was a capsule-like GFAP-positive zone around the basal part of the electrodes (arrows, G, H). This zone was devoid of MAP2-immunoreactive processes (F, H). (I, J, K) From the bottom-side of the chip-based slice culture, MAP2- (I) and GFAP-immunoreactive processes (J) extended into the holes of the silicon chip (×). The GFAP-immunoreactive processes were located closer to the inner surface of the holes than the MAP2-immunoreactive processes (K). Scale bars, 50 μm (A, B), 25 μm (C–K).


Membrane
p
r
A
50 µm
Chip
p
r
B
Electrode track-top MAP2
*
C
25 µm
GFAP
*
D
MAP2+GFAP
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Histology in hole of chip
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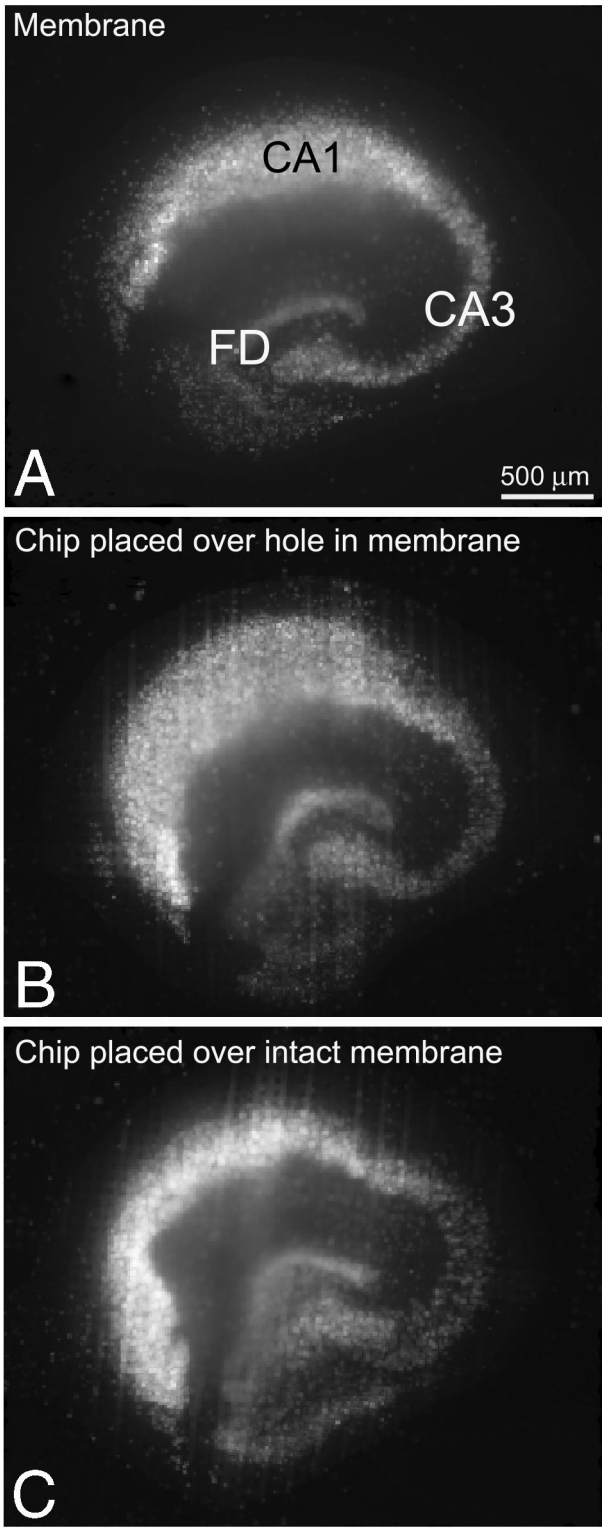

Fig. 12. Digitized fluorescence micrographs showing the cellular uptake of propidium iodide (PI) in hippocampal slice cultures after 48 h exposure to 10 μM NMDA. The cultures were grown for 4 weeks on insert membranes (A), or on chips (with a silicon nitride surface and without microelectrodes) placed over a hole in the membrane (B) or on the intact membrane (C). Note that the PI uptake appears similar in the three cultures, with most uptake in the CA1 region and less in CA3 region and the dentate granule cell layer. For quantitative analysis of the subregional PI uptake, see Fig. 13.

3.5. Differential susceptibility of hippocampal subfields to NMDA and TMT

In hippocampal slice cultures exposed to 10 μM NMDA, CA1 pyramidal cells were the most susceptible in both conventionally grown cultures and cultures grown on chips (CA1 versus CA3 and FD, $P<0.001$) (Figs. 12 and 13). This was valid irrespective of whether the chips were in direct contact with the NMDA-containing medium through a hole in the semiporous membrane or with an intact membrane. Comparison of the PI uptake within a given field between the sets of cultures with different membrane or chip support revealed that there was a slightly, but significantly higher PI uptake, in the CA1 pyramidal cell layer of cultures grown on the membrane compared to cultures grown on the silicon chip (Fig. 13). Regarding the change in PI uptake from 24 to 48 h of exposure, there was a significant increase in the PI uptake for both dentate granule cells and CA3 pyramidal cells in all groups of cultures ($P<0.001$), but within CA1 this increase was only significant for cultures grown on chips with ($P<0.05$) or without ($P<0.001$) a hole in the underlying membrane (Fig. 13).

In conventionally grown cultures and cultures grown on chips exposed to TMT, the dentate granule cells were the most susceptible and CA3ab the least susceptible subfield in terms of PI uptake (Figs. 14 and 15). There was higher PI uptake in all subfields in cultures grown on the membrane compared to cultures grown on the silicon chip at both 24 h and 48 h after start of the TMT exposure (Fig. 15). The increase in the PI uptake from 24 to 48 h was significant for all subfields in both membrane- and chip-based cultures ($P<0.001$) (Fig. 15).

The directly measured arbitrary densitometric values for PI uptake after NMDA and TMT exposure and the final exposure to 50 mM of glutamate were significantly higher for the chip-based than for the membrane-based cultures (Fig. 16). This difference disappeared when the NMDA- and TMT-induced PI uptakes were expressed as a percentage of total uptakes after treatment with 50 mM of glutamate (Figs. 13 and 15). The in general higher densitometric values obtained from the chip-based cultures may include some extra reflected light from the chip, enhancing the recorded fluorescence.

4. Discussion

In this study we have focused on (a) the general biocompatibility of a recently developed three-dimensional microelectrode array [32,33], (b) the detailed structural relation of neuronal and astroglial processes to basal and apical parts of the microelectrodes and (c) possible differences in the susceptibility of the chip-based slice cultures to toxic concentrations of NMDA and TMT compared to conventionally grown slice cultures. The biocompatibility

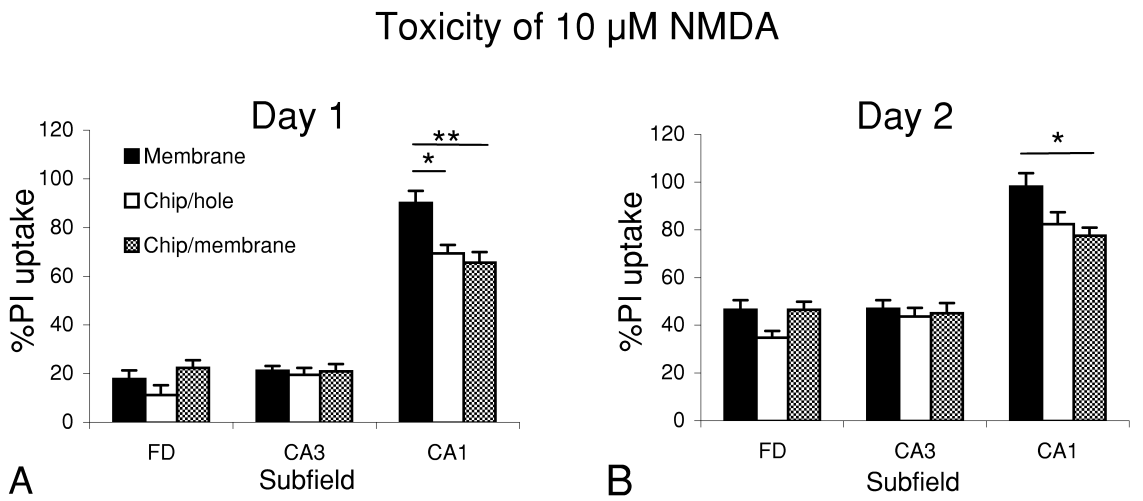

Fig. 13. Densitometric measurements of propidium iodide (PI) uptake in the CA1 and CA3 pyramidal cells and the fascia dentata granule cells (FD) after 24 h and 48 h exposure to 10 μM NMDA. The cultures were grown for 4 weeks directly on the membrane or on chips (with a silicon nitride surface and without microelectrodes) placed over a hole in the membrane (chip/hole) or on the intact membrane (chip/membrane). The PI uptake is expressed as percentage of total PI uptake after a subsequent exposure to 50 mM glutamate, supposed to kill all neurons and hence inducing maximal PI uptake. Note the increase in the PI uptake from 24 to 48 h and the relatively selective uptake of PI in CA1, in particular after 24 h. Between the cultures grown directly on the membrane and on the chip with and without a supporting membrane interposed between the chip and the culture medium, there seems to be a minor difference in terms of less PI uptake (neuronal damage) in CA1 for cultures grown on the chip. Data are shown as means+S.E.M., with $n=22$ for cultures grown on the membrane and $n=11$ for each of the culture groups grown on the microelectrode array. *$P<0.05$, **$P<0.01$, using ANOVA with Bonferroni correction for comparison of the three groups of cultures.

of existing microelectrode arrays [5,6,31] has mainly been tested in terms of electrophysiology, while the histological investigations are poor [6] or non-existing [5,31]. In general, we found that slice cultures grown on the silicon-based microelectrode arrays displayed few or no differences in morphology and susceptibility to the applied toxins compared to conventional slice cultures.

4.1. Nissl and Timm staining

Cultures grown on the silicon-based microelectrode arrays placed over a hole in the underlying insert membrane or on an intact membrane displayed a histological organization, which by stereomicroscopical inspection and in Nissl and Timm stained sections was similar to cultures grown on membranes or by the roller drum method, as previously described [37]. This means that the pores in the perforated part of the chip were sufficient for exchange of nutrients and waste products between tissue and medium for development and survival of the cultures for at least 8 weeks. The results also imply that cultures can be maintained on chips placed on intact membranes for a long period of time before and between sessions of electrophysiological measurements, where the chip is supposed to

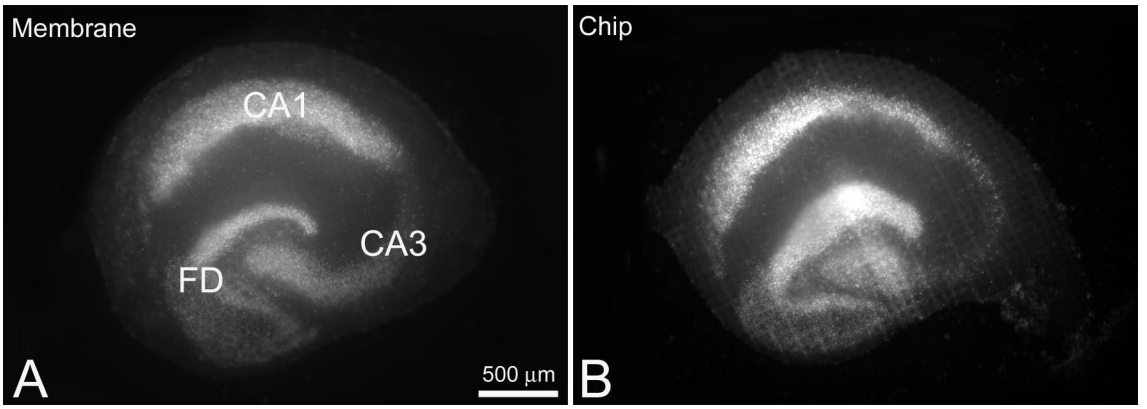

Fig. 14. Digitized fluorescence micrographs showing the cellular uptake of propidium iodide (PI) in hippocampal slice cultures exposed to 20 μM of the neurotoxin trimethyltin (TMT) for 24 h followed by 24 h in normal medium. The cultures were grown for 3.5 weeks on membrane (A), or chip (with a silicon nitride surface and without microelectrodes) placed over a hole in the membrane (B). Note that both membrane- and chip-based cultures displayed the same differentiated vulnerability among subfields with the fascia dentata being the most susceptible and CA3ab the least susceptible subfield. For quantitative analysis of the subregional PI uptake, see Fig. 15.

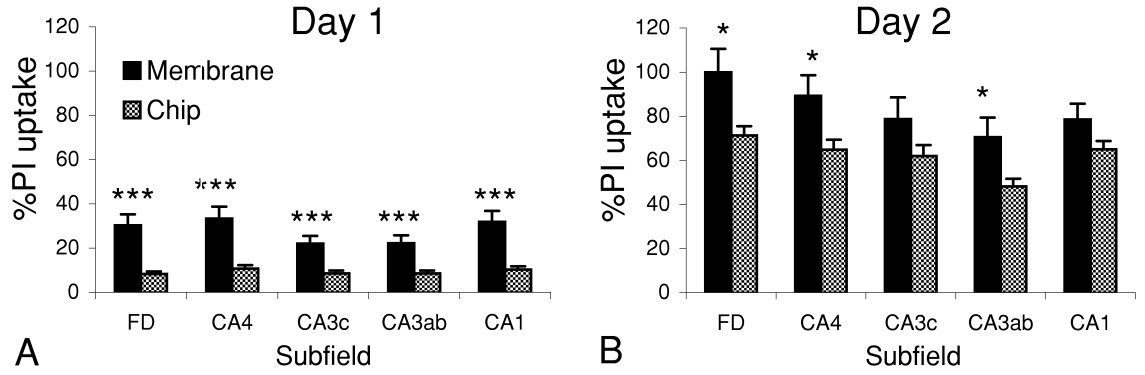

Fig. 15. Densitometric measurements of propidium iodide (PI) uptake in the dentate granule cell layer (FD) and the CA3 and CA1 pyramidal cell layers in hippocampal slice cultures exposed to 20 μM of the neurotoxin trimethyltin (TMT) for 24 h followed by 24 h in normal medium. The cultures were grown for 3.5 weeks on membrane (A) or chip (with a silicon nitride surface and without microelectrodes) placed over a hole in the membrane (B). The PI uptake is expressed as percentage of total PI uptake after a subsequent exposure to 50 mM glutamate. Both membrane- and chip-based cultures display the same differential vulnerability of the different subfields with the dentate granule cells being the most susceptible and CA3ab the least susceptible subfield. There was a higher PI uptake in cultures grown on the membrane compared to cultures grown on the silicon chip, as well as there was a time-dependent increase in the PI uptake from 24 to 48 h for all subfields in both membrane and chip cultures. Data are shown as means+S.E.M., with $n=18$. *$P<0.05$ and ***$P<0.001$, using t-test for comparison of the two groups of cultures.

be mounted on a printed circuit board. The results are best explained by the functioning of the semiporous membrane as a soaked sponge, which allows transfer of medium so easily, that it does not matter, whether the perforated part of the chips is in direct contact with medium or with the membrane. This also explains the similar susceptibility to 10 μM NMDA of chip-based cultures grown on chips with and without a hole in the underlying semiporous insert membrane.

4.2. Immunocytochemical staining for MAP2 and GFAP

Cultures grown directly on the semiporous membrane and on the microelectrode arrays with silicon nitride and platinum surfaces displayed the same pattern of MAP2 and GFAP staining at tissue levels above and around the platinum-coated tips of the microelectrodes. This pattern is similar to that described before in hippocampal slice cultures for both MAP2 [20,21] and GFAP [7,13] and it is also similar to the general patterns of MAP2 [16] and GFAP [24] staining in brain sections from rats, suggesting that both silicon nitride and platinum are biocompatible as supporting materials for brain slice cultures.

At the tissue levels penetrated by the electrodes and at the bottom surface of the culture facing the holes in the chip a number of interesting observations were made. Most importantly, in relation to the electrophysiological functionality of the microelectrodes (signal-to-noise ratio), there was no glia limitans or glial scar in terms of accumulation of glial processes around the upper platinum-coated tips of the microelectrodes. Normally spaced and sized MAP2- and GFAP-immunoreactive processes were found equally distributed right next to the position of the platinum electrode tips. This corresponds to the description

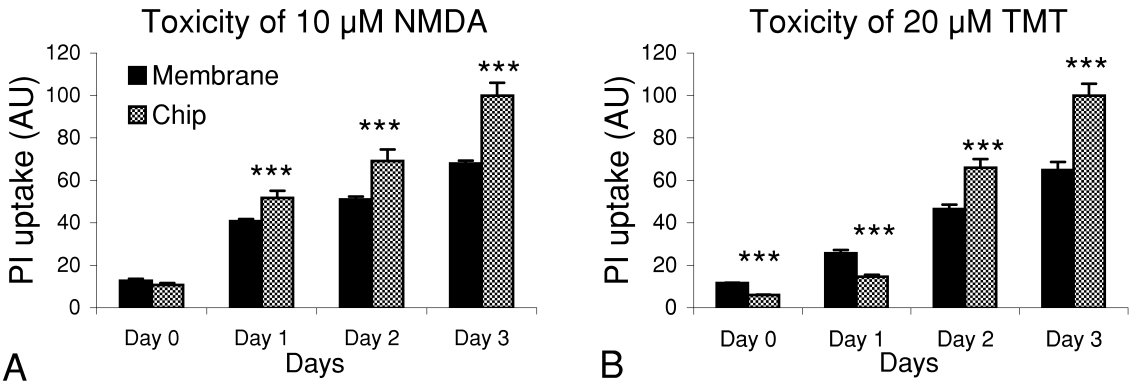

Fig. 16. Comparison of the arbitrary values of the densitometric measurements of entire PI uptake induced by NMDA (A) and TMT (B) in hippocampal slice cultures grown for 3.5–4 weeks on membrane or chip (with a silicon nitride surface and without microelectrodes) placed over a hole in the membrane. The values were significantly higher for the chip-based cultures than for the membrane-based cultures, although the PI uptake in percentage of total uptake after treatment with 50 mM of glutamate was not (see Figs. 13 and 15). Data are shown as means+S.E.M., with $n=18$. ***$P<0.001$, using t-test for comparison of the two groups of cultures.

of platinum as being neutral to the surrounding biological tissue when implanted into the cerebral cortex [29]. Tips of platinum are also used in a 3-dimensional electrode array, which has been developed over the last decade for acute and chronic intracortical implantation [22].

Around the basal electrically isolated silicon nitride-coated parts of the electrodes, there was a narrow, GFAP-rich, capsule-like zone devoid of MAP2-immunoreactive processes. This might be a reaction of the tissue to silicon nitride, but rather it may have been induced by the initial injury, when the tissue slices were cut and the electrodes penetrated into the tissue as the slices were placed on the chips. Since neurons are more susceptible to trauma than astroglial cells, the preparation of the brain tissue slices are likely to result in a surface with loss of neurons and neuronal elements leaving a glial-rich zone next to the slice surface and around the basal parts of the electrodes. Also according to this, there will only be minor injury deeper inside the tissue slices around the tips of the electrodes. In this relation, it is of interest that a corresponding gradient in the gliotic reaction was found after chronic implantation of an array with 1.5-mm-long silicon electrodes into cat cerebral cortex [27] with a decrease in gliosis from the base of the electrodes at the surface of the cortex towards the tip of the electrodes inside the tissue, consistent with both a reduction in the cross-sectional area of the electrodes and an increasing distance from the cortical surface. The difference in material along the electrode with platinum covering the upper part of the electrodes, while the lower part was covered by silicon nitride, might also add to the difference in glial reaction around the electrodes, but it should be noted that the width of the GFAP-rich zone (glia limitans) at the bottom surface of the slice cultures was the same (~20 μm) in cultures grown on chips with silicon nitride and platinum surfaces and on conventional insert membranes. Silicon nitride is also covering the surface layer in planar or microwell arrays used for hippocampal slice cultures [6] and dispersed cell cultures [14,15], respectively, and these cultures have been described to have the same viability as cultures grown by conventional techniques.

Regarding the holes in the microelectrode array, GFAP-immunoreactive processes appeared to be located closer to the inner surface of the holes than the MAP2-immunoreactive processes, thereby forming a ‘coating’ arrangement similar to the arrangement around the basal part of the electrodes. The extension of these neuronal and astroglial processes into the holes did not appear to interfere with the nutrition of the cultures. To what extent the processes directly facilitated access to the medium is not known.

4.3. Differential susceptibility of hippocampal subfields to NMDA and TMT

The density and distribution of neuronal degeneration induced in the slice cultures by NMDA and TMT exposures according to established protocols [20,38] were evaluated by densitometric analysis of the cellular uptake of PI in the slice cultures. PI has over the past few years been used increasingly as a quantifiable marker for neuronal degeneration in hippocampal slice cultures exposed to a variety of insults, e.g. excitotoxins [2,28,34,38], hypoxia and hypoglycemia alone or in combination [23,25,35], nitric oxide [1] and trimethyltin [19,20].

The high susceptibility of hippocampal CA1 pyramidal cells to NMDA in both conventional slice cultures and chip-based cultures is in agreement with earlier reports [4,9,10], suggesting that the high density of NMDA receptors in CA1 in vivo [17,18] is preserved and the same in both conventional and chip-based array cultures, and that the same excitotoxic mechanisms of neuronal degeneration take place in the two culture situations.

The conventional and the chip-based slice cultures exposed to TMT also displayed the same subfield differences in vulnerability as reported in earlier studies, with the dentate granule cells being the most susceptible and CA3ab the least susceptible subfield [19,20]. This again suggests that a given toxin activates the same mechanisms for neuronal degeneration in the two culture situations.

NMDA induced a slightly higher PI uptake in CA1 and TMT a slightly higher PI uptake in all subfields in cultures grown on insert membranes compared to cultures grown on silicon chips. This may be explained by longer diffusion distances for NMDA and TMT in the chip-based slice cultures or by the presence of tissue ‘plugs’ in the holes of the chips. In contrast to this, we found that the arbitrary values for PI uptake were higher for both NMDA and TMT in chip-based cultures than for the membrane-based cultures, presumably due to reflection of light by the chip, enhancing the fluorescence signal. In spite of these minor differences between membrane- and chip-based cultures, it is evident that PI can be used as a quantifiable marker for cellular degeneration in chip cultures with the same easiness and advantages as in conventional slice cultures. With PI as a marker the development in neuronal degeneration can be followed over time and directly compared with electrophysiological measurements.

5. Conclusion

The histological and toxicological studies of hippocampal slices grown on silicon-based chips with electrodes have demonstrated the biocompatibility of these three-dimensional microelectrode arrays. The coupling between slices of developing brain tissue and the silicon-based chips had only little effect on the slice cultures, when compared with conventionally grown slice cultures. The histological stains showed that cultures grown on the microelectrode arrays were vital and displayed a distinct organotypic organization with development and preserva-

tion of the main hippocampal cell and neuropil layers and intrinsic afferent fiber connections. There were no signs of astrogliosis or neurodegeneration around the upper, recording part of the electrodes, favoring a high electrophysiological signal-to-noise ratio. The neurons also displayed in vivo-like differential susceptibility to a well-known toxin (TMT) and excitotoxin (NMDA). Based on these results, we expect that the three-dimensional microelectrode array can be developed to a new and feasible tool for in vitro acute and chronic screening for neurotoxic agents as well as for stable long-term recordings of developmental and reorganizational cellular and connective changes in brain slice cultures.

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