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Comparison of NMDA and AMPA Channel Expression and Function between Embryonic and Adult Neurons Utilizing Microelectrode Array Systems

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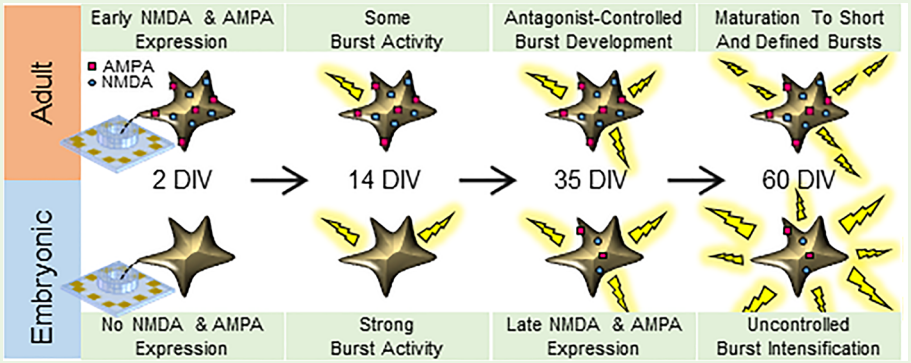

ABSTRACT: Microelectrode arrays (MEAs) are innovative tools used to perform electrophysiological experiments for the study of electrical activity and connectivity in populations of neurons from dissociated cultures. Reliance upon neurons derived from embryonic tissue is a common limitation of neuronal/MEA hybrid systems and perhaps of neuroscience research in general, and the use of adult neurons could model fully functional in vivo parameters more closely. Spontaneous network activity was concurrently recorded from both embryonic and adult rat neurons cultured on MEAs for up to 10 weeks in vitro to characterize the synaptic connections between cell types. The cultures were exposed to synaptic transmission antagonists against NMDA and AMPA channels, which revealed significantly different receptor profiles of adult and embryonic networks in vitro. In addition, both embryonic and adult neurons were evaluated for NMDA and AMPA channel subunit expression over five weeks in vitro. The results established that neurons derived from embryonic tissue did not express mature synaptic channels for several weeks in vitro under defined conditions. Consequently, the embryonic response to synaptic antagonists was significantly different than that of neurons derived from adult tissue sources. These results are especially significant because most studies reported with embryonic hippocampal neurons do not begin at two to four weeks in culture. In addition, the utilization of MEAs in lieu of patch-clamp electrophysiology avoided a large-scale, labor-intensive study. These results establish the utility of this unique hybrid system derived from adult hippocampal tissue in combination with MEAs and offer a more appropriate representation of in vivo function for drug discovery. It has application for neuronal development and regeneration as well as for investigations into neurodegenerative disease, traumatic brain injury, and stroke.

KEYWORDS: *hippocampal neurons, adult neurons, embryonic neurons, microelectrode array, electrophysiology, in vitro*

INTRODUCTION

Electrophysiological studies into learning and memory drug discovery as well as for neurodegenerative diseases most commonly rely upon patch-clamp electrophysiology for functional evaluation of neurons.[1−3] While this method provides detailed information, it is very labor-intensive, technically complicated, and has limited long-term capability in relation to noninvasive techniques for measuring electrical activity as compared to microelectrode arrays (MEAs). MEAs are innovative tools used to perform electrophysiological neuronal activity and connectivity in populations of neurons from dissociated cultures.[4−6]

A common limitation of neuronal MEA systems and neuroscience research in general has been the reliance upon neurons derived from embryonic tissue.[4,7−9] While neurons derived from embryonic sources are fully differentiated, they are developmentally immature, with transcriptional profiling

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indicating two-thirds of genes are expressed only postnatally and >95% of the expressed genes showing highly significant changes during postnatal development.[10,11] When evaluating the machinery responsible for synaptic transmission, AMPA channel expression is limited at birth, increasing only postnatally.[12] The gene expression for the NMDA channel subunits NR2A and NR2B is not detected until near birth at E21−22, with expression not peaking until P20.[13] In vitro, NR2A/B channels are detected at only very low levels until after two weeks in embryonically derived neurons.[14] Gene expression for the axonal sodium transporter subunit 1 begins around P15 and then increases until P30.[15,16]

In vitro expression patterns for axonal and synaptic channels responsible for signal transmission are similar to those seen in vivo, with gene expression indicating significant changes over the course of weeks.[13,14,16−19] While embryonic rat tissue is relatively easy to obtain, culture, and maintain, the usefulness of these developmentally immature neurons for studies of neuronal electrical activity and synaptic transmission is restricted by the limited or absent expression of certain channels responsible for synaptic communication in the adult brain. In addition, using these developmentally immature neurons in studies of neurodegenerative diseases or drug discovery can yield results that are difficult to correlate with the function or the action of mature neurons in adult brain tissue. In fact, because of this lack of NMDA expression, embryonic or neonatal neurons are promising candidates for their use in in vitro epilepsy models.[20,21]

We developed a long-term hybrid in vitro system utilizing mature, terminally differentiated neurons derived from adult rat hippocampal tissue in dissociated culture[22,23] on MEAs. Whereas patch-clamp electrophysiology is labor-intensive, requires specialized training, and cannot be used to monitor neuronal function over more than a few hours at a time, MEAs can perform electrophysiological experiments for both neuronal activity and connectivity in populations of neurons[4−6] for extended time periods (days to months).[24−28]

Concurrent spontaneous network activity from both embryonic and adult neurons on MEAs in the presence/absence of synaptic transmission antagonists against NMDA and AMPA channels was measured. In addition, NMDA and AMPA channel subunit expression was evaluated over 36 days in vitro (div) for both embryonic and adult neurons. Adult neurons cultured in defined medium in vitro displayed expression patterns consistent with mature neurons in vivo, were electrically active, and formed functional synaptic connections. Our results indicate that neurons derived from embryonic tissue did not express mature synaptic channels for several weeks, and consequently, their response to synaptic antagonists was significantly different than that of neurons derived from adult tissue sources. These results establish the usefulness of this unique hybrid system derived from adult hippocampal tissue for drug discovery and fundamental research. Moving toward using a high-content hybrid in vitro system as a phenotypic model system will expand and improve drug testing and basic research by providing a viable, easily manipulatable alternative to expensive, resource intensive in vivo testing. Applications for these systems include studies into the mechanisms of learning and memory formation,[29] investigations into drug discovery, the study of epilepsy, neurodegenerative diseases such as Alzheimer's and other dementias, and biosensor applications.[8,24,27,30]

MATERIALS AND METHODS

Substrates and Surface Modification. Glass coverslips (Thomas Scientific 6661F52, 22 × 22 mm no. 1) were cleaned by acid washing using a 50/50 mixture of concentrated hydrochloric acid and methanol. The coverslips were washed 3 times, 30 min per wash, and were rinsed in distilled deionized water between each washing. The DETA (*N*-1-[3-(trimethoxysilyl) propyl]-diethylenetriamine, United Chemical Technologies Inc., Bristol, PA, T2910KG) monolayer was formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904).[31] The DETA-coated coverslips were heated to just below the boiling point of toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven-dried. The DETA formed a reaction site limited monolayer on the surface of the coverslip.[31] The DETA coverslips were characterized to authenticate the monolayer formation. First, contact angle measurements were taken using an optical contact angle goniometer (KSV Instruments, Monroe, CT, Cam 200). The contact angle for the DETA-coated coverslips was 54.2 ± 0.2, which was previously shown to be acceptable for neuronal hippocampal culture.[31] Second, X-ray photoelectron spectroscopy (XPS) (FISONS ESCALab 220i-XL) was used to characterize the elemental and chemical state of the DETA-coated coverslip surfaces. The XPS survey scans as well as high-resolution N 1s and C 1s scans using monochromatic Al Kα excitation were obtained, similar to previously reported results.[31,32]

Embryonic Rat Hippocampal Dissociated Cell Culture Methodology. Embryonic hippocampal neurons were cultured using a protocol described previously.[33,34] Pregnant rats, 18 days in gestation, obtained from Charles River were euthanized with carbon dioxide, and the fetuses were collected in dissecting medium made with ice-cold Hibernate E (BrainBits) supplemented with B27 (2%), Glutamax (2 mM), and antibiotic−antimycotic (1%) (Invitrogen). Each fetus was decapitated, and the whole brain was transferred to fresh ice cold dissecting medium. After isolation, the hippocampi were enzymatically digested at 37 °C for 10 min with papain (2 mg/mL, Worthington 3119), Hibernate-E without calcium (BrainBits) and 2 mM Glutamax (Invitrogen). Hippocampi were quickly transferred to fresh dissection medium to halt enzymatic digestion. Hippocampal neurons were dissociated by triturating the tissue using a fire-polished Pasteur pipet. After centrifugation (300*g*, 4 °C), cells were resuspended in culture medium (Neurobasal Medium (Gibco)/B27/Glutamax/antibiotic−antimycotic) and plated on MEAs (see below). All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines.

Adult Rat Hippocampal Dissociated Cell Culture Methodology. Adult neurons were extracted, dissociated, cultured, and maintained using a protocol and medium similar to a protocol described previously.[22] Briefly, the hippocampi of adult rats (Charles River, age 6−12 months) were dissected and homogenized into small tissue fragments in cold medium (∼4 °C) consisting of Hibernate-A (HA, BrainBits) supplemented with B27 (2%), Glutamax (2 mM), and an antibiotic−antimycotic (1%). The tissue was digested for 30 min at 37 °C in calcium-free Hibernate-A (BrainBits) containing papain (2 mg/mL). Following digestion, the tissue was washed three times with cold HA media to remove any active enzyme. Next, the tissue was suspended in dissociation medium, (Hibernate-A Brain Bits (HA) supplemented with B27 (2%, Gibco 17504-044), Glutamax (2 mM, Gibco 35050-061), antibiotic−antimycotic (1%, Gibco 15240-096), Z-Asp(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone (4 mM, Sigma C0480), *Z*-Val-Ala-Asp fluoromethyl ketone (5 mM, Sigma C2105), dextrose-coated cerium oxide nanoparticles (100 nM, a gift from Dr. Manuel Perez), and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 70 nM, Sigma 238813)) and broken apart into individual cells through mechanical dissociation with fire-polished Pasteur pipettes. The dissociated cells were suspended in plating medium; Neurobasal-A Medium with osmolarity adjusted to 295 mOsm (Gibco, 10888-022), B27 (2%), Glutamax (2 mM), antibiotic−antimycotic (1%), recombinant human brain-derived neurotrophic factor (20 ng/mL, Cell Sciences CRB600B), NT-3 (20 ng/mL, Cell

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Sciences CRN500B), bFGF (5 ng/mL, Invitrogen 13256−029), Insulin-like Growth Factor (20 ng/mL, Sigma I2656), dextrose-coated Cerium Oxide Nanoparticles (100 nM), Trolox (70 nM, Sigma 238813), and then deposited onto DETA coated glass coverslips, according to published protocols.[31,32] After 30−45 min, the coverslips were washed with warm HA by gently swirling the medium to remove tissue debris. Following this washing step fresh plating medium was applied and remained for the first 3 div. On 3 div, the medium was removed and replaced by maintenance medium (composed of all components of plating medium except Trolox and dextrose-coated cerium oxide nanoparticles) supplemented with 5 μM Roscovitine (Rosc, Sigma, R7772). After 4 div, the adult hippocampal neurons on the DETA coverslips were passaged to MEAs. Briefly, neurons were dislodged from the DETA with trypsin (0.05% trypsin/EDTA in HBSS, Gibco, 25200). Trypsin inhibitor (trypsin inhibitor, soybean, Gibco, 17075-029) in dissociation medium at 0.5 mg/mL deactivated the trypsin. The dislodged neurons were collected and spun at 500g for 5 min. The supernatant of deactivated trypsin in HBSS was discarded, and the neuronal cell pellet was suspended in 1 mL plating medium for culturing. All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines.

Immunocytochemistry and Laser Scanning Confocal Microscopy. To prepare cells for immunocytochemical characterization, coverslips were rinsed twice with phosphate buffered saline (PBS). Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and subsequently rinsed three times with PBS. Cells were permeabilized for 5 min with 0.5% Triton X-100 in PBS, and were then blocked for 2 h in 5% normal goat serum (Gibco) in PBS. Anti-neurofilament-M (Chemicon, AB5735, 1:500), anti-synaptophysin (Chemicon, MAB368, 1:300), and either anti-NMDAR2A (Chemicon, AB1555P, 1:200), anti-NMDAR2B (Chemicon, AB15557P, 1:200), or antiglutamate receptor 2/3 (Chemicon, AB1506, 1:50) were added in blocking solution for 12 h at 4 °C. After 3 washes with PBS, fluorescently labeled secondary antibodies (Invitrogen, A11011 (594 nm), A21449 (647 nm), and A11029 (488 nm) in blocking buffer were applied (1:200) for 2 h. Vectashield mounting medium with DAPI (H1200, Vector Laboratories, Burlingame, CA) was used to mount the coverslips onto slides. Fluorescent images were acquired with the UltraView spinning disc confocal system (PerkinElmer) with AxioObserver.Z1 (Carl Zeiss) stand, and a Plan-Apochromat 40×/1.4 Oil DIC plan-apochromat objective with 26 μm resolution. Z-stack projections of the scanned images were generated and modified within the Volocity image processing program (PerkinElmer).

MEA Plating and Culture Maintenance. The MEA chips (Axion Biosystems) contained 64 platinum-black coated gold-electrodes with a diameter of 30 μm, organized in an 8 by 8 array with 200 μm pitch. Clean MEAs were sterilized with 70% alcohol and then incubated with 1 mL of poly-L-lysine (100 μg/mL) for 30 min. An area just large enough to cover all electrodes was additionally coated with 3 μL laminin (2 μg/mL) overnight.

Embryonic rat hippocampal neurons were plated directly on MEAs at a density of 500 cells/mm^2. Adult rat hippocampal neurons were first cultured on DETA coated coverslips for initial recovery and removal of debris. Embryonic tissue did not require this preplating step as there is very little debris generated from the culturing process. After 4 days on DETA coverslips, the adult neurons were passaged onto MEAs at a density of 500 cells/mm^2 (Figure 1). Every 2−3 days, half the medium was replaced with fresh maintenance medium supplemented with 2 μM Roscovitine. Supplementing the adult neurons with 25 μM glutamate (*N*-acetyl-L-glutamic acid, Aldrich, 855642) at 2 div increased the electrical activity of the adult neurons.[22] MEAs were incubated with their covers off to allow gas exchange but covered upon removal from the incubator to reduce contamination, media evaporation, and gas exchange. The head stage of the recording system (Axion Biosystems) was preheated to 37 °C before MEAs with adult or embryonic hippocampal cultures were investigated.

Experimental Procedure. The activity of neuronal networks was recorded at 25 kHz using the software Axion's Integrated Studio (AxIS). Signal amplitudes six times larger than the standard deviation

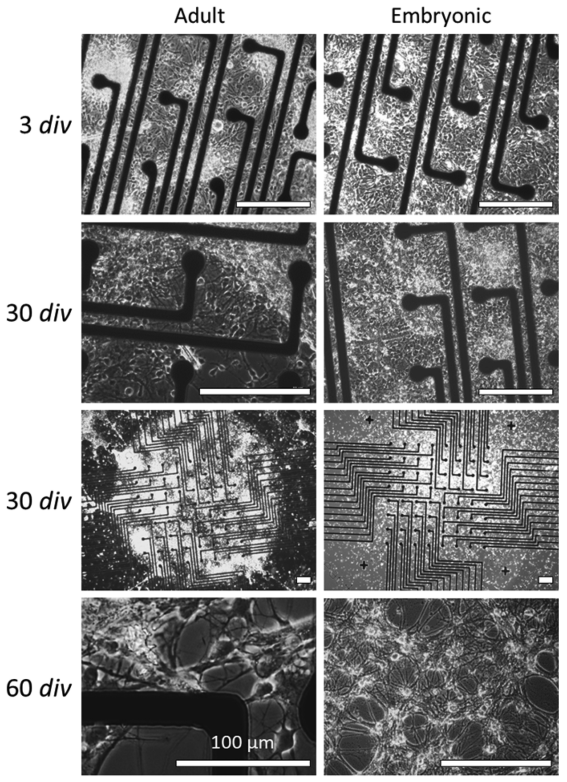

Figure 1. Phase contrast images of cultures on MEAs after 3, 30, and 60 div. Neurons were deposited at levels between 500 and 1000 cells/mm^2. Cells attached and regenerated on the PDL/laminin surface covering the MEAs can be seen in the dense collection of cells covering the electrodes. Each 64-channel MEA is arranged in an 8 × 8 array of electrodes, each 30 μM in diameter and spaced 200 μM apart. The MEA was sampled 25 000 times per second at 16 bits of depth. Scale bar = 200 μm (unless indicated otherwise).

of the baseline were detected as action-potential (AP) spikes. The spike data were then imported into Matlab 2010b (The MathWorks) for further processing. Baseline spontaneous activity in adult and embryonic neurons was recorded for 3 min on 5 consecutive days per week, starting at 7 div. The synaptic antagonists D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5, 25 μM, Tocris Bioscience, 0106) and 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX, 25 μM, Tocris Bioscience, 1045) were separately administered to both adult and embryonic neurons on 14 div and at various time points between 30 and 60 div. Three minute recordings were made to quantify the effect from these antagonists on spontaneous activity. If necessary, additional baselines were recorded prior to the administration of antagonists. After MEA measurements were recorded, the antagonists were washed from the neurons with an entire media change; 24 h later, the activity of the neurons had returned to baseline levels.

Evaluation and Statistics. Data analysis was performed off-line using unpublished Matlab functions to evaluate spike files created during each MEA recording. In brief, each 3 min data set was processed in 3 passes while leveraging information from previous passes (for example, third pass output of embryonic data as shown in Figure 2). This method allowed inactive (less than 0.125 Hz) or noisy channels (maximum detectable AP frequency sustained for 50 ms or longer) to be excluded before the well-wide firing behavior was determined. Eventually, a multitude of parameters was extracted: "active channels" and their average firing frequencies (number of APs divided by recording length); "AP frequencies" (reciprocal interspike intervals); "bursts", defined as 2 or more APs within 1 ms on a particular channel; "burst duration" time-interval from the first AP in a burst to the last AP in a burst; "average burst frequency", the reciprocal time-difference between the onsets of two subsequent bursts; "in-burst

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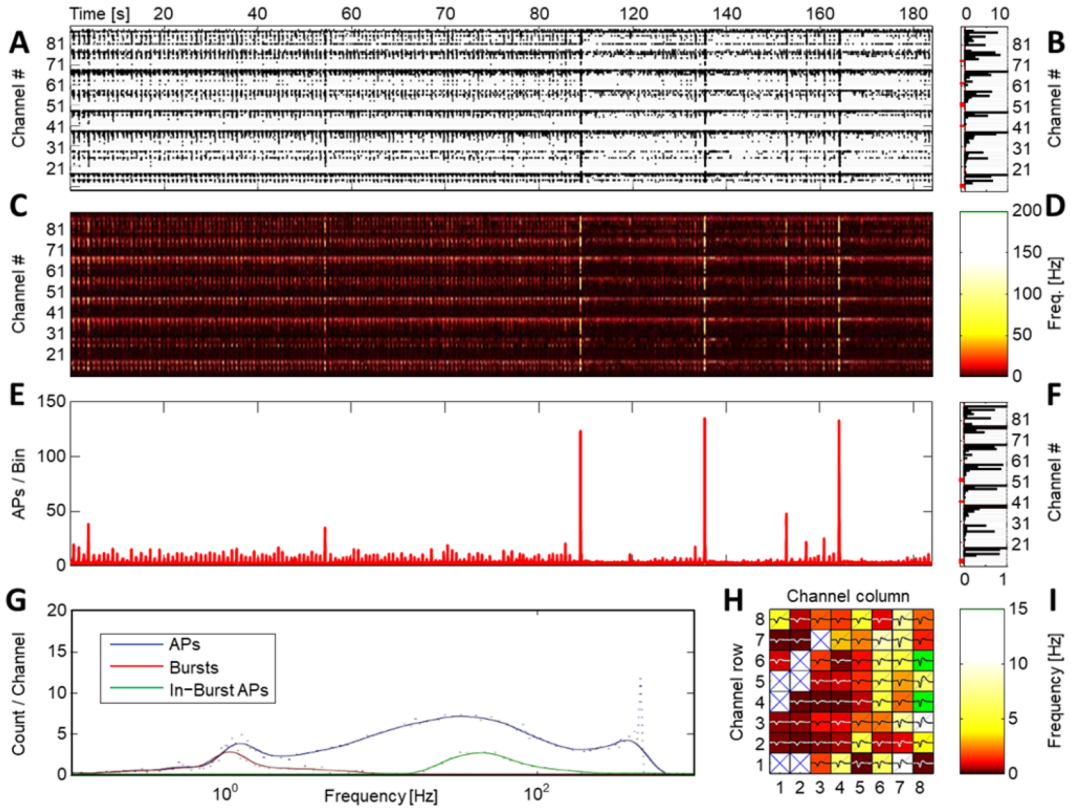

Figure 2. MEA data processing results: Each data set was processed in a three-pass method (third and final pass shown here). (A) Event map: a detailed dot-plot to indicate individual firing events on each channel over time. (B) Average firing frequency (*x*-axis capped at 10 Hz), red dots indicate channels that were determined to be inactive or noisy during previous passes. (C) Activity map: a heat map to better visualize AP density per channel (100 μs binning). (D) Color bar coding firing frequencies for panel C. (E) Array-wide burst event plot: sum of bursts on individual channels in 10 ms bins. (F) Channel burst frequencies (*x*-axis caped at 1 Hz), red dots indicate channels that were determined to be inactive or noisy during previous passes. (G) Frequency distribution: histogram of overall AP frequencies, burst frequencies, and in-burst frequencies (*x*-axis is logarithmic). (H) Channel map: showing the 8 × 8 electrode grid with average AP shapes; color indicates average firing rate, and X indicates ignored channels (inactive or noisy). (I) Color bar for average firing rate in H.

frequency", the number of APs within a detected burst divided by the duration of that burst; and "non-burst frequency", the reciprocal interspike intervals between APs that were not associated with bursts. "Array-bursts" were defined as synchronous bursting in 10% or more of the active channels. Array-bursts were then characterized similarly to bursts on individual channels. To accommodate the different numbers of active channels between MEAs, all values were normalized to the amount of active channels on an MEA. For each of the 3 min recordings, a representative results chart was produced containing the graphs shown in Figure 2. Effects from synaptic antagonists were in addition normalized with the baseline activity of the same culture recorded before administration of the antagonist.

RESULTS

Dissociated neuronal cultures from adult and embryonic sources recovered on MEAs and formed networks at a density of 500 cells/mm^2 (Figure 1). Three-minute recordings of spontaneous activity were taken of each MEA, 5 times per week, for up to 60 div. Phase-contrast pictures of the MEAs were taken after each recording for a daily assessment into the condition of the cells as well as verification of physical contact between cells and electrodes (Figure 1). Neurons, both adult and embryonic, were stable and electrically active on MEAs for up to 90 div (data not shown).

Spontaneous Activity of Adult and Embryonic Neurons. Spontaneous firing activity started in both the adult and embryonic neurons between 7−10 div. Movement of MEAs from the incubator to the heated recording stage and the subsequent 3 min recording period did not significantly affect the pH or temperature of the medium, represented by consistent baseline activity. However, brief increases in baseline neuronal activity were observed as a result of medium changes. Over the more than 2 month experimental period, embryonic MEAs (n = 6) consistently displayed a higher number of active channels with an average of 37 ± 8 channels active per MEA versus 15 ± 5 channels in adult MEA cultures (n = 9). Action potential (AP) firing frequencies in embryonic cultures with approximately 2−4 Hz were higher than in adult cultures with AP firing frequencies of 1−2 Hz. Spontaneous bursting occurred about 1 week earlier in adult cultures as opposed to embryonic cultures. The burst development over the first six weeks was consistent between the two types of neuronal networks (Figure 3A). After the sixth week, however, bursts in the adult cultures appeared less often, whereas the burst frequency in the embryonic cultures increased further. For both embryonic and adult cultures, the duration of bursts decreased over the time span of 10 weeks (Figure 3B). The bursts of embryonic cultures were on average 3−5 times longer as opposed to bursts in adult cultures. While burst durations in the embryonic cultures were consistently variable (about ±1 s) over the 10 weeks of experimentation, bursts in the adult cultures were more uniform and decreased in length. This focusing of bursts in adult cultures was accompanied by a

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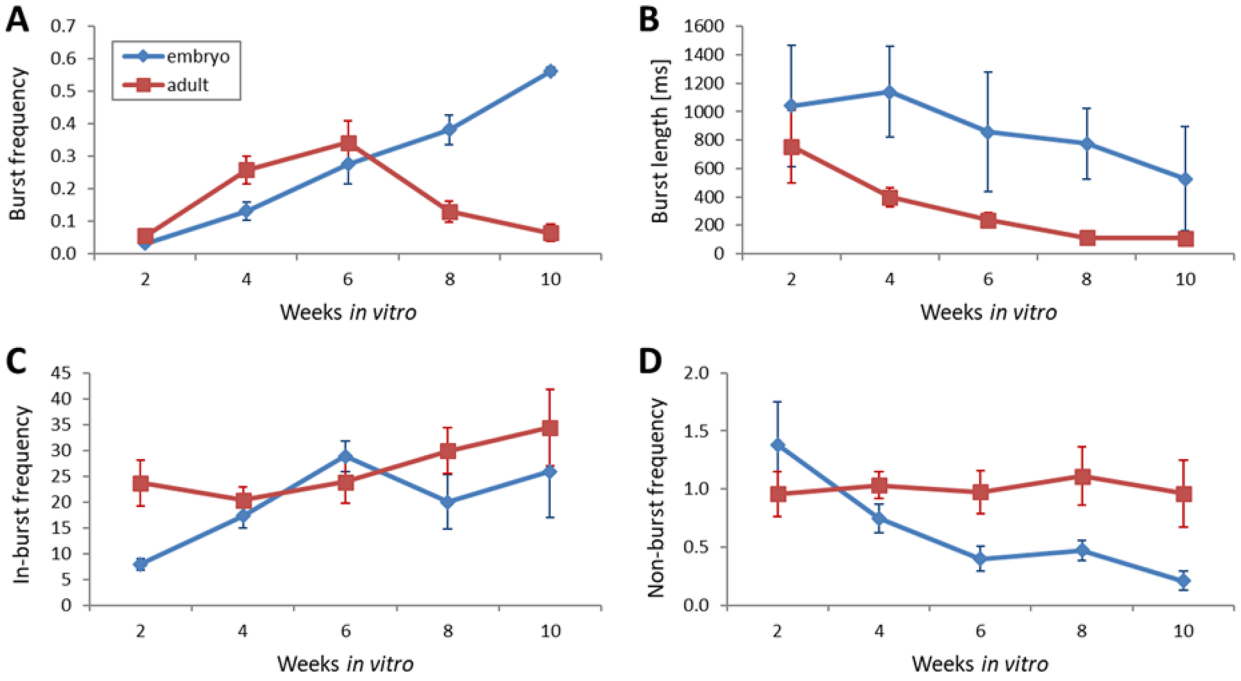

Figure 3. Basic firing patterns of embryonic and adult hippocampal neurons on MEAs over time. Weekly averages of spontaneous activity in embryonic (n = 6) and adult neurons (n = 9). The activity data for each day was processed to filter out inactive channels and noise before averaging. (A) Average burst frequency. (B) Average burst length. (C) Average firing frequency within a burst. (D) Average firing frequency outside of bursts.

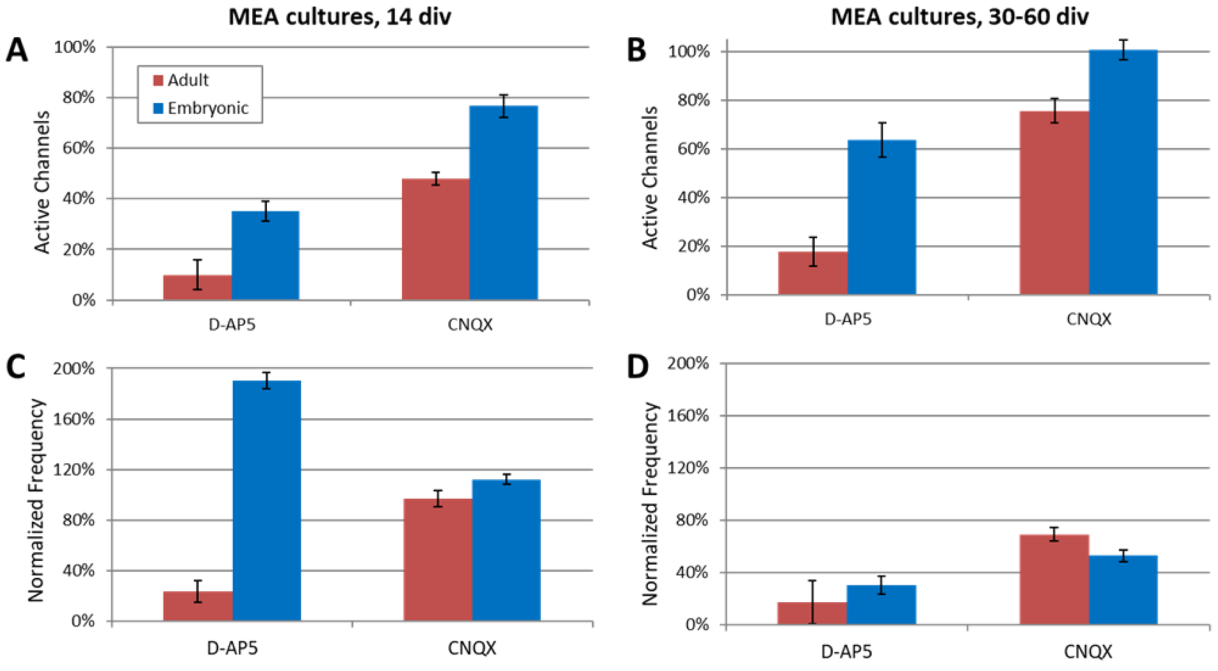

Figure 4. Comparison of the impact on adult or embryonic spontaneous activity from addition of synaptic antagonists. Remaining active channels (A and B) and AP frequency (C and D) were evaluated in adult or embryonic hippocampal neuron MEA systems on either 14 or 30−60 div in the presence of D-AP5 (25 μM) or CNQX (25 μM) in culture medium.

steady increase of firing frequencies within bursts over time (Figure 3C). The in-burst frequencies of adult cultures recovered within the first two weeks, whereas embryonic cultures recovered slower and reached mature in-burst levels after about six weeks. The non-burst firing frequencies (APs not associated with bursts) were consistent over the entire time for the adult cultures but decreased steadily in the embryonic cultures (Figure 3D). Overall, the burst activity focused and matured earlier and more consistently in adult cultures on MEAs, whereas embryonic cultures showed a slower, more chaotic maturation.

NMDA and AMPA Channel Activity and Expression in Adult and Embryonic Neurons. Antagonists were administered to embryonic and adult cultures on MEAs in separate experiments: 25 μM D-AP5 (NMDA channel antagonist) and 25 μM CNQX (AMPA channel antagonist). D-AP5 (25 μM)

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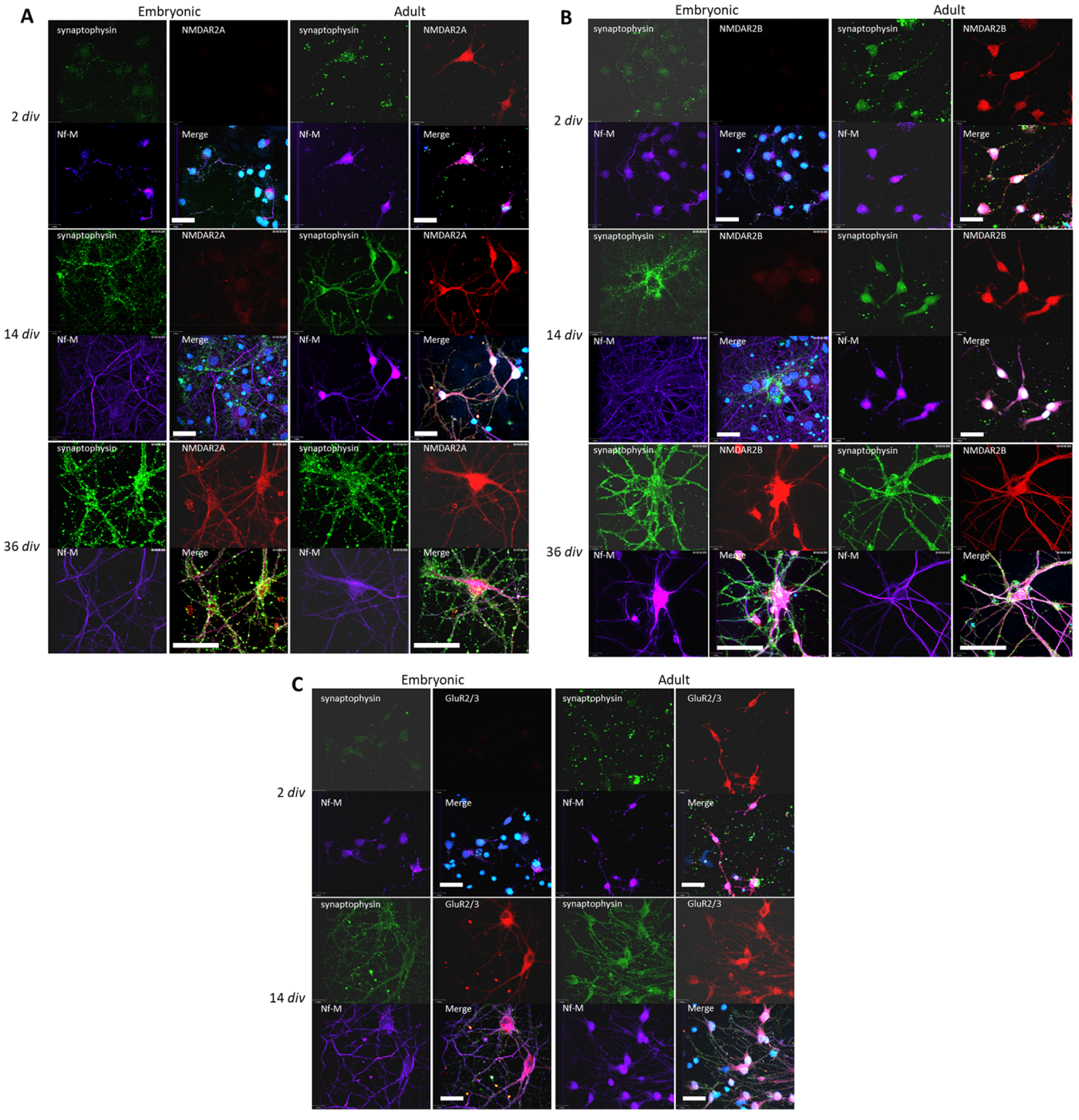

Figure 5. Expression of presynaptic proteins and postsynaptic channel subunits in embryonic and adult neurons in vitro: immunohistochemistry for (A) NR2A, (B) NR2B, and (C) GluR2/3. NMDA and AMPA channel proteins, respectively (red); synaptophysin (green); neurofilament-M (far-red); and DAPI (blue) expression after 2, 14, and 36 div. Scale bars 25 μm.

caused a significant decrease in the number of active channels in both adult and embryonic MEA cultures (Figures 4A and B). Due to the addition of inhibitors, adult cultures lost a greater percentage of previously active channels (90 ± 6% at 14 div, 82 ± 6% on 30−60 div) versus embryonic cultures (65 ± 4% at 14 div, 36 ± 7% on 30−60 div). Changes in the action potential frequency also varied with lower frequencies in adult cultures (76 ± 8% at 14 div, 82 ± 17% on 30−60 div), which were significantly different from the measured effect on embryonic cultures (Figures 4C and D). At 14 div, the firing rate of embryonic neurons increased 90 ± 6%, while in 30−60 day-old cultures, the firing rate decreased 70 ± 7%. While NMDA channels were expressed at low levels in embryonic neurons on 14 div, the level of expression was not nearly as high as in adult neurons at 14 div (Figures 5A and B). The difference in NMDA channel expression (NR2A and NR2B subunits) in adult and embryonic neurons in vitro likely caused the contrasting reaction to D-AP5 in the two populations of neurons. Because adult neurons expressed a greater number of NMDA channels, their reaction to the competitive NMDA antagonist D-AP5 was much more pronounced.

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Adult neurons showed a significantly decreased percentage of active channels and AP frequency due to D-AP5 in both early 14 div cultures as well as older 30−60 div cultures. This drop in activity was significantly different from embryonic 14 div, where a lower percentage of active channels was lost and activity increased in the remaining channels. The AMPA-channel antagonist CNQX also caused a decrease in spontaneous activity. The drop in activity between adult and embryonic cultures was, however, only reflected in the loss of more active channels in the adult system. AP frequency declines due to the addition of inhibitors were consistent between the two culture systems.

Addition of CNQX caused the activity of far fewer channels to be lost in both adult and embryonic MEA cultures as compared to D-AP5 (Figure 4 A and B). Adult cultures lost a greater percentage of channel activity (52 ± 3% at 14 div, 24 ± 5% on 30−60 div) versus embryonic cultures (23 ± 5% at 14 div, 0 ± 7% on 30−60 div). Changes in the AP frequency in both the adult and embryonic cultures were not affected by CNQX after 14 div (Figure 4D). The activity of neurons was only slightly decreased in both embryonic and adult cultures between 30 to 60 div (31 ± 6% drop in adult MEAs, 47 ± 5% drop in embryonic MEAs).

The channel proteins NMDAR2A and NMDAR2B (Figures 5A and B, respectively) were not expressed in embryonic neurons on 2 div and were not strongly expressed on 14 div when compared to channel expression in adult neurons. After 36 div, the NMDA channels were expressed at similar levels by both the embryonic as well as the adult neurons. While expression of AMPA channel subunits GluR2/3 was not observed in embryonic neurons on 2 div, expression had increased to mirror adult levels by 14 div (Figure 5C). These postsynaptic channel subunits were all found in adult neurons from 2−36 div. Synaptophysin and neurofilament-M expression grew stronger as both the embryonic and adult neurons recovered and regenerated in vitro.

DISCUSSION

The results demonstrated that adult neurons cultured from the hippocampus of rats recovered functionally and had the capacity to fire spontaneously on MEAs over 60 div. Additional culture techniques were used to allow adult neurons to recover from initial plating trauma and reduce debris before being densely deposited on the MEAs and to improve recovery of electrical activity in vitro. Adult cells were first precultured at a low density and then were passaged and deposited onto MEAs at 500 cells/mm^2, a density high enough to allow the formation of multiple synaptic connections. This step was for the removal of debris from the cultures and allowed recovery after dissociation to enable high survival in MEA cultures. The application of a preplating step along with the supplementation of glutamate in the culture medium promoted elevated electrical activity in the adult neurons.[22] After 7 to 10 div, sporadic spontaneous firing activity was detected, and stable reliable recordings were possible after this point. MEAs with adult neurons yielded significantly less active channels than the MEAs with embryonic neurons. Future experiments with dissociated adult neurons may require higher seeding densities to compensate for the difference in electrically active cells. As an alternative, devices with higher electrode densities may be able to yield more active channels for adult neurons, although the yield with embryonic neurons should scale similarly.[35]

Because neuronal MEA systems have typically relied upon neurons derived from embryonic tissue,[3,4,7,8,24,26,27] we compared our adult hippocampal MEA system to traditional embryonic MEA systems. Recordings from both adult and embryonic MEAs were made daily through 90 div (data not shown) and fully characterized out to 60 div. Embryonic MEAs consistently displayed higher numbers of active channels with an average of 40 channels active versus 20 active on adult MEAs. To minimize a possible influence of active channels during comparison of embryonic and adult cultures, all experiments were normalized by the number of active channels, and experiments with inhibitors were normalized with preceding control recordings on the same MEA. Accordingly, the majority of other parameters, including AP frequency, activity, average burst frequency, and average in-burst frequency, all displayed similar characteristics between the adult and embryonic MEA systems. This new adult neuronal hybrid MEA system, at its core, exhibited similar firing characteristics to the more traditional embryonic MEA systems. However, the adult cultures developed the capability of synchronized bursting about 1 week earlier than the embryonic cultures. Bursting in neuronal networks is usually seen as an indicator for maturity.[36−38] While embryonic neurons regenerate, fire APs and eventually develop synchronized bursts on MEAs, the developmental maturity of these neurons had not been robustly established. In the hippocampus of embryonic rats, neural progenitors differentiate into neurons between E15−E18.[10,17,39] While these cells obtained from embryonic rats are fully differentiated, they are developmentally immature, with transcriptional profiles indicating two-thirds of genes are only expressed postnatally, and >95% of expressed genes show highly significant changes during postnatal development.[10]

Expression patterns from rat mRNA indicate the NR2A and NR2B subunits of the NMDA channel do not peak until P20[13] and normally are not detected in vitro with embryonic tissue until after two weeks.[14] This trend is reflected in the embryonic channel expression in Figures 5A and B where a progressive increase in NR2A and NR2B subunits is visible. During this developmental period, NR2B is predominantly expressed in the early postnatal brain, while NR2A expression increases to eventually outnumber NR2B with each subunit lending different kinetics of excitotoxicity, neurotoxicity, and plasticity.[40] Figure 5C demonstrates that AMPA channel expression increases only postnatally and, similar to NMDA channel expression, is limited for in vitro systems. The importance of these dynamics of neuronal maturation highlights the need for utilizing gene and protein expression in a study of populations of neurons to mirror that of mature adult neurons in vivo. If embryonic neurons do not express the same machinery responsible for electrical activity or signal propagation in adult neurons, then their response to neurotoxic agents or drug therapies may not be correlative to responses in the mature adult brain.

Our method of culturing adult neurons resulted in a system where NMDA and AMPA channel subunits were expressed throughout the lifespan of the culture (see Figure 5). NMDA channel subunits NR2A and NR2B as well as AMPA channel subunits GluR2/3 were expressed on and after 2 div. This contrasted greatly from neurons derived from embryonic tissue with delayed NMDA and AMPA channel expression until 14 div and later. As shown in Figure 4, the responses of neurons to NMDA and AMPA channel antagonists were found to be significantly different in embryonic neurons as compared to

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adult neurons with each antagonist decreasing activity in adult neurons to a greater degree than in embryonic neurons. Our results indicate that embryonic neurons in culture develop a mature profile of ion channel subunits after 3−4 weeks. Therefore, embryonic neurons should not be employed for some experiments until they have fully matured in culture, especially in studying neurodegenerative diseases such as Alzheimer's where synaptic protein profiles may play a critical role in the process of synaptic failure.[41−46]

In comparison to embryonic MEA systems, this method of using adult hippocampal neurons on MEAs is more appropriate to acquiring data that mimics the adult brain. While preparation of these MEAs was slightly more complicated than embryonic neuronal MEAs, the end result yielded a long-term screen methodology that is more biomimetic of the synaptic machinery responsible for critical synaptic function required in learning and memory.[38,47−50] Additionally, due to the earlier development of bursts and general maturation, this system can facilitate quicker, more reliable, and more correlative investigations into drug discovery, neurotoxic agents, and neurodegeneration. Finally, this method can be used in the future to allow for adult human neuronal networks cultured on MEAs.

CONCLUSIONS

In conclusion, we demonstrated critical differences between adult and embryonic neurons and their respective synaptic connections which could be highly relevant in neurodegenerative disease research. By demonstrating the similarities and differences between adult and embryonic neurons and the response of each to synaptic antagonists, the value of this adult neuron culture system has been established for application in neuronal regeneration and drug discovery studies. The significance of this finding is that most in vitro neuronal research utilizes embryonic hippocampal neurons for periods of less than 7−11 weeks. By incorporating adult neurons into an MEA system, a long-term system has been created to enable the screening of a large number of cells and the study of pathogen and drug effects on the same population of cells over an extended period of time. This screen could find important applications in pharmaceutical drug development by providing an in vitro test platform for investigations into neurodegenerative disease such as Alzheimer's, traumatic brain injury, stroke, drug discovery, and fundamental research.

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Notes

The authors declare the following competing financial interest(s): The authors confirm that there has been no financial support for this research that could have influenced its outcome. However, J.J.H. has interest in a company that potentially could utilize the results.

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