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Protocol for culturing low density pure rat hippocampal neurons supported by mature mixed neuron cultures

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HIGHLIGHTS

- A method for culturing very low density rat hippocampal neurons is described.
- Low density culture depend on neurotrophic support from mature hippocampal culture.
- This method yields neurons for good morphology and electrophysiology analysis.

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ABSTRACT

Background: primary hippocampal neuron cultures allow for subcellular morphological dissection, easy access to drug treatment and electrophysiology analysis of individual neurons, and is therefore an ideal model for the study of neuron physiology. While neuron and glia mixed cultures are relatively easy to prepare, pure neurons are particular hard to culture at low densities which are suitable for morphology studies. This may be due to a lack of neurotrophic factors such as brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and Glial cell line-derived neurotrophic factor (GDNF).
New method: In this study we used a two step protocol in which neuron-glia mixed cultures were initially prepared for maturation to support the growth of young neurons plated at very low densities.
Comparison with existing methods: Our protocol showed that neurotrophic support resulted in physiologically functional hippocampal neurons with larger cell body, increased neurite length and decreased branching and complexity compared to cultures prepared using a conventional method.
Conclusion: Our protocol provides a novel way to culture highly uniformed hippocampal neurons for acquiring high quality, neuron based data.

1. Introduction

Cultured neurons provide easy access to direct observation of protein localization, and drug treatment. Protocols in preparing neurons of different origins such as cortex, hippocampus, striatum, dorsal root ganglia and retina have been established (Banker and Goslin, 1998). Among them, cultured hippocampal neurons are widely used in the morphology analysis of neurites and spines which are important aspects of neurite outgrowth, synaptogenesis and synaptic plasticity.

Neuronal development depends on a variety of secreted factors such as BDNF, NT-3 and nerve growth factor (NGF) that act on autocrine or paracrine signaling (Lewin and Carter, 2014). Hence the density of neurons is important for the outcome of the culture, with high density cultures being fast growing and morphologically healthier than low density cultures. In addition, neurotrophic factors secreted by glia cells also contribute significantly to the development and growth of neurons (Parpura and Zorec, 2010; Martineau, 2013; Karki et al., 2014). The neuron culture density should be based on the primary purpose of the experiment. While most drug treatment experiments require moderate density cultures, some activity-dependent physiological changes are only significant in high density cultures. Low density pure neuron cultures on the other hand, are optimal for imaging studies aimed to analyze subcellular morphology and organelle dynamics. However, preparation of such cultures using traditional protocols is usually unsatisfactory because the neurons are often misshaped or underdeveloped possibly due to a lack of paracrine neurotrophic factors. This hampers further study of individual neurons because fine growth and morphology implicates robust physiological

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functions, and obtaining good quality photos is necessary for reliable data analysis.

Neuron health can be improved by plating them onto a confluent layer of glia cells (Forsythe and Westbrook, 1988). However, glia cells can cause misinterpretation of data from experiments involving staining or tracking proteins that are present in both cell populations. Novel protocols have also been used for culturing low density pure neurons with the basic idea of enhancing neurotrophic support from physically separated cell populations. Inverting a coverslip plated with low density neurons over a layer of glia cells has provided a way to culture high quality pure neurons suitable for morphological analysis and studying paracrine interaction between glia cells and neurons (Kaech and Banker, 2006; Shimizu et al., 2011). Neuronal development in this culture system may primarily benefit from the neurotrophic effect of glia cells. In addition, the chamber between the inverted coverslip and culture dish surface allows concentration of neurotrophic factors (Chen et al., 2011) and lowers oxygen levels which may be beneficial for neuronal growth (Brewer and Cotman, 1989). Low density hippocampal or dopamine neurons can also be cultured inside a ring of cortex or striatum neurons respectively for neurotrophic support (Fath et al., 2009).

Here, we describe a protocol in which very-low density neurons plated right side up on coverslips are supported by a mixed culture of DIV 14 (14 days in vitro) glia and neurons. Preparation of the feeder cells (mixed culture) is based on simplified canonical neuron culture protocols (Kaech and Banker, 2006; Fath et al., 2009; Beaudoin et al., 2012), and can be used for culturing hippocampal or cortical neurons for biochemical studies. The procedure is simple and easy to perform and is suitable for continuous production of high quality, highly uniformed hippocampal neurons optimized for imaging studies.

2. Materials and methods

2.1. Antibodies and reagents

Anti-PSD95 (Abcam, ab2723), Anti-GluN2A (Abcam, ab133265), anti-GluN2 B (self-made mice monoclonal antibody), TRITC phalloidin (YEASON, 40774ES03, Shanghai, China)

2.2. Immunocytochemistry

For staining of endogenous GluN2A, GluN2B, and PSD95, hippocampal neurons (DIV 14–16) were fixed for 15 min with 4% paraformaldehyde. After fixation, neurons were washed three times with PBS and incubated with blocking buffer (0.2% Triton X-100, 5% BSA in PBS) at room temperature for 30 min. Primary antibodies were then applied in blocking buffer for 1 h at room temperature. Neurons were then washed three times with PBS. Secondary antibodies were applied in blocking buffer for 1 h at room temperature (For staining of actin, TRITC Phalloidin was added to the diluted secondary antibody). After washing three times with PBS, coverslips were mounted on slides. The images were acquired with a confocal microscope (Fluoview FV1000; Olympus).

2.3. Immunocytochemistry analysis

All the immunocytochemistry images were quantified using MetaMorph analysis software (Universal Imaging). In brief, images were thresholded to distinguish synaptic puncta (PSD95 positive postsynaptic puncta). Dendrite length and puncta number were quantified as cluster count per 100 μm and average intensities were quantified as average cluster size. More than three independent experimental repeats were used for each quantification, and 10–20 neurons were analyzed in each independent experiment.

GraphPad prism 5 software was used for data analyzing. All data were represented as mean ± SEM. Student's *t*-test was used to measure significance of differences between data from two different groups. Differences with $P < 0.05$ were considered significant.

2.4. Whole-cell electrophysiology recording of sEPSC (spontaneous excitatory postsynaptic current)

Coverslips containing DIV14 cultured hippocampal neurons were transferred to a recording chamber containing a saline solution (138 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 1 μM TTX, 20 μM bicuculline, pH7.4). sEPSCs were recorded at a holding potential of −70 mV using a micro pipette of 3–6 MΩ resistance filled with internal solution (136.5 mM potassium-gluconate, 17.5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2 \cdot 6H_2O$, 4 mM Mg-ATP, 0.3 mM Na-GTP, 0.2 mM EGTA, 9 mM NaCl, pH7.2-7.3). The micro pipette was made in the lab using a vertical pipette puller.

2.5. Reagents for cell culture

Neurobasal (Gibco, 21103-049), Fetal bovine serum (FBS; Gibco, 10099-141), B27 supplement (Gibco, 17504-044), Glutamax (Gibco, 35050-061), 0.25% Trypsin (Gibco, 25200-056), Poly-L-lysine (Sigma, P2636), Hank's balanced salt solution (Gibco, 14025-092), MilliQ water, Penicillin/Streptomycin (10378-016), 35 mm and 60 mm petri dish (Corning, 430166, 430165), 12 mm Glass Coverslips (Deckglaser, 01 11520).

2.6. Coating of culture dish and frozen HBSS preparation

For culture dish coating, add 2.5 ml of 0.1 mg/ml poly-L-lysine (PLL) into each 60 mm culture dish. Make sure the PLL solution covers the surface area of each dish. Incubate at 4 °C overnight or at room temperature of 30 min.

Add 4 ml of HBSS into three 60 mm culture dish. Stack the culture dish and place them in a tilted position at −20 °C overnight for the HBSS to freeze on one side of the culture dish.

Before embryo dissection, remove all PLL solution from the incubated culture dish and collect it in a 50 ml centrifugation tube. Allow the culture dish to dry and add 3 ml of plating medium (10% FBS in Neurobasal), then precondition the dish in the cell culture incubator. Freshly prepared PLL (0.1 mg/ml) solution can be reused for 3–5 times.

2.7. Preparation of neuron cell suspension

The required animal and instruments include: 1. A P17-P19 pregnant rat. 2.scissors and tweezers for dissection. 3.Frozen HBSS in 60 mm culture dish. 4. 0.25%Trypsin and Neurobasal supplemented with 2% B27.

Sterilize instruments in 70% ethanol. Dry under ultraviolet light radiation.

Add enough HBSS to the culture dish with frozen HBSS prepared earlier to submerge the brains, roughly 4–6 ml. This allows the whole dissection process to be carried out at near 0 °C in partially frozen HBSS.

Euthanize the pregnant rat by an approved protocol and remove the embryos from the uterus and place them in the 100 mm dish with partially frozen HBSS. Fig. 1

Cut open the embryo sac to release the fetus. Decapitate the fetus and transfer the head to a new 60 mm dish with partially frozen HBSS.

To remove the brain (Fig. 1A), pin down the head by impaling the eyes with one pair of forceps, and make a sagittal laceration with another pair of fine tipped forceps starting from lambda to the foramina magnum. Squeeze the brain out using the side of the

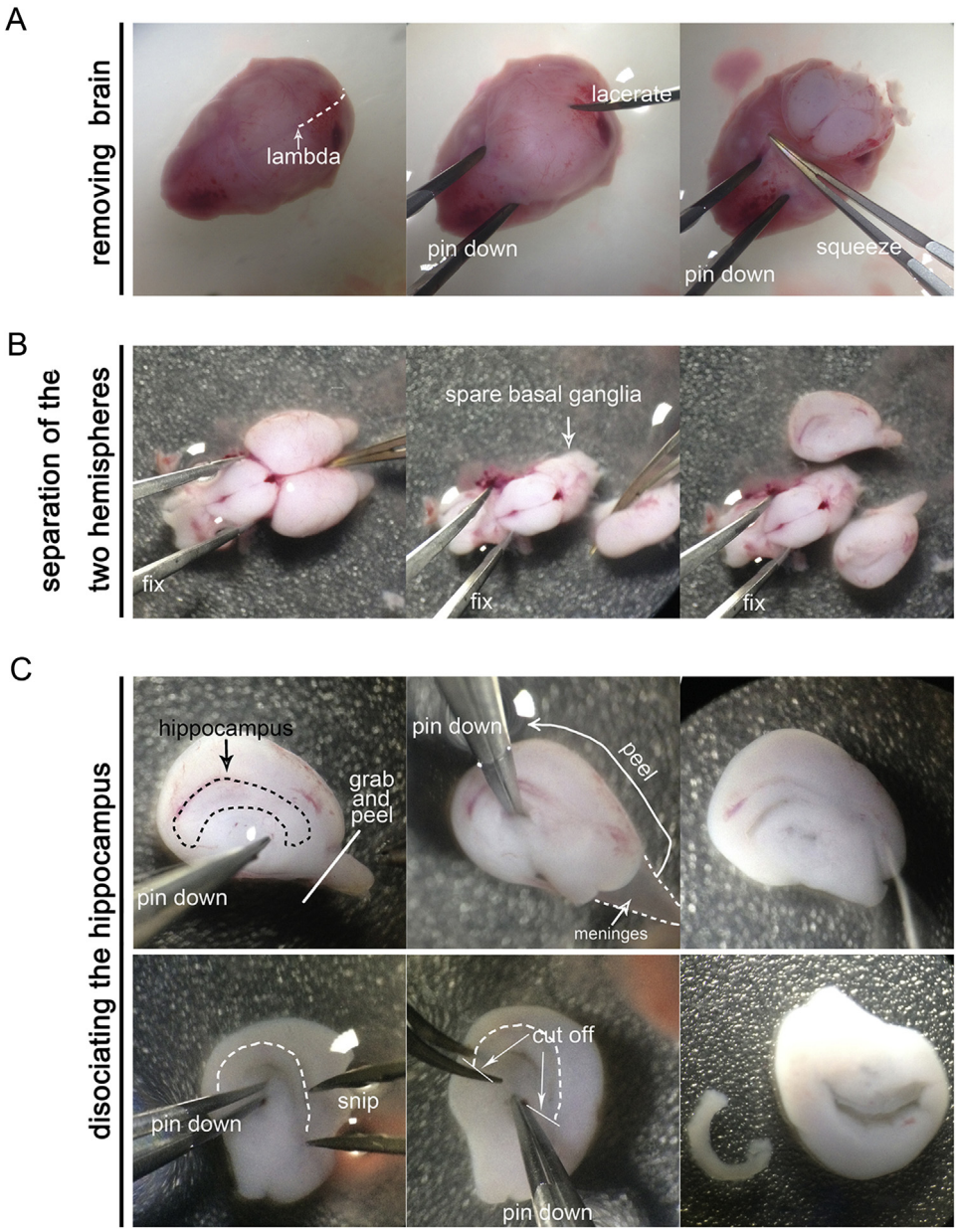

Fig. 1. Isolating the hippocampus. (A) After decapitation of the fetus, tear through the skull along the midline using a pair of fine tipped tweezer, and sqeeze out the brain. Older animals (over P2) may require partial removal of the skull for successful brain removal. (B) The right tweezer is clamped tight to work its way around the basal ganglia and separate the two hemispheres. (C) The hippocampus is a crecent shaped brain tissue located at the border of the in-folded cortex with slightly different texture. With the left tweezer clamped tight, pin down the cortex without hurting the hippocampus. Use the right tweezer to grab the olfactory bulb or a piece of meninges and peel to remove all the meninges. Then use the right tweezer to snip along and isolate the hippocampus.

forceps, and transfer the isolated brains to another 60 mm dish with partially frozen HBSS. A successfully isolated brain should include the cerebellum and olfactory bulbs. Ensure a clean laceration or the brain might be damaged while being squeezed out.

For dissection of the hippocampus, place the dish containing the isolated brain under a Stereoscopic microscope with top and bottom illumination (Olympus, SZX7). face the dorsal back of the brain upwards and pin the brain at the junction between the cerebellum and the cerebrum using one pair of forceps, and isolate the two hemispheres while sparing the basal ganglia(Fig. 1B).

Turn the inner side of the brain hemisphere upwards to expose the hippocampus.

Pin down the brain at the temporal lobe using one pair of forceps and pinch the olfactory bulb with another to peel off the meninges. Be sure not to damage the hippocampus.

Snip along the interface between the hippocampus and the cortex with another pair of forceps to isolate the hippocampus (Fig. 1C).

Transfer the hippocampus to the third 60 mm dish with paritially frozen HBSS, for rinsing.

Pool hippocampi from 1 to 8 fetuses and transfer them to a 15 ml tube with 1 ml of 0.25% trypsin and digest in a cell culture incubator at 37 °C, 5% CO2 humidified with saturated copper sulfate solution for 15–20 min. Add 0.5 ml of trypsin for every additional 4 pairs of hippocampi. Note that a hippocampus from a younger fetus (DIV15 to DIV17) is easier to digest (15 min) while an older hippocampus (DIV18 to DIV21) takes longer to digest (18–20 min) and may require mincing with scissors before digestion.

Prepare two 15 ml centrifuge tubes with 3 ml and one with 1 ml of plating medium (neurobasal containing 10% FBS). Aspirate the digested hippocampus and transfer to the first tube using a 1 ml

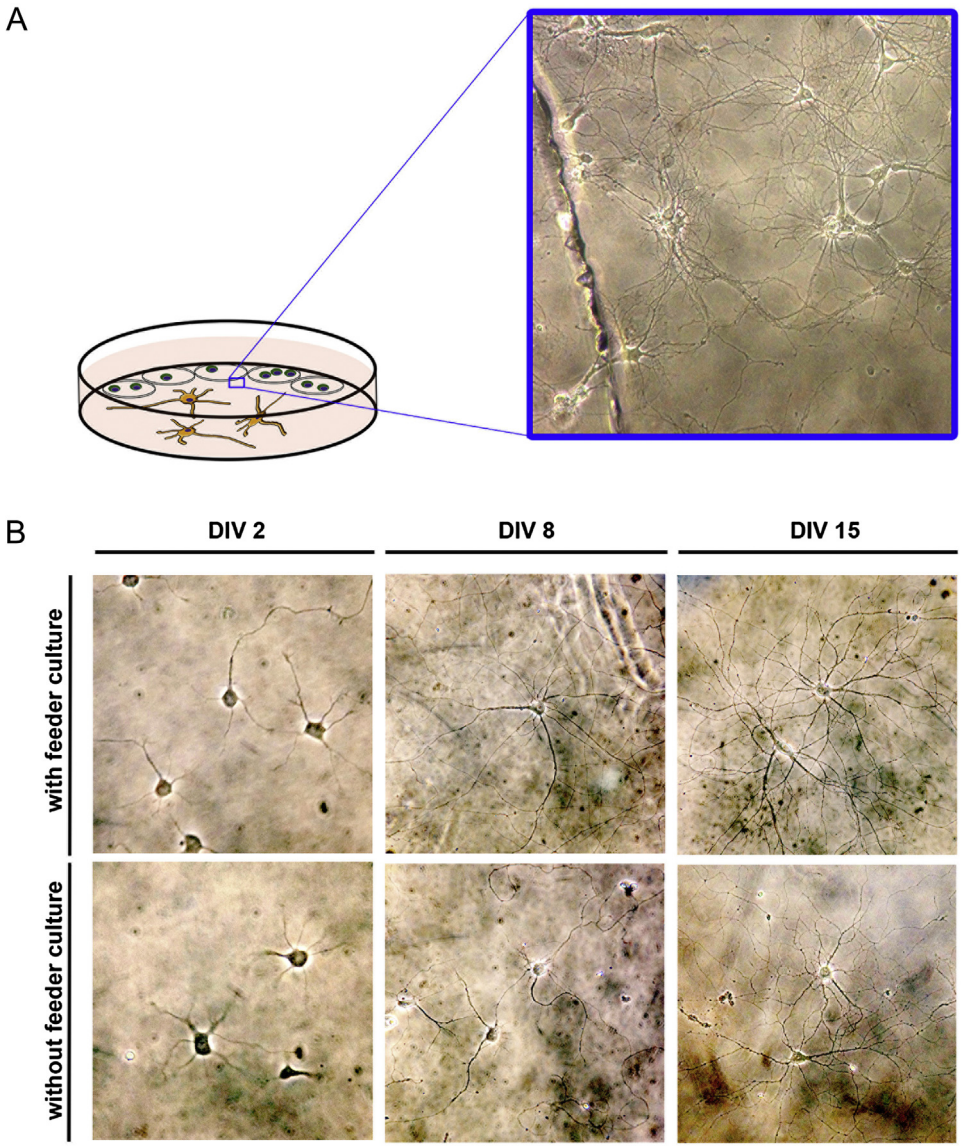

Fig. 2. Comparison of hippocampal neuron culture with or without feeder culture support. (A) The feeder culture contains a mixture of neurons and glia cells supporting growth of newly seeded neurons on coverslips. (B) Hippocampal neurons with feeder culture support show faster neurite outgrowth at DIV2, and better morphology at DIV8 and DIV15 compared to cultures without support.

pipette. Invert the tube gently 6–8 times for rinsing and neutralization of the trypsin. Allow the tissue to sediment and transfer to the second tube and repeat inverting gently for rinsing. Allow the tissue to settle and transfer to the third tube.

Gently triturate the digested tissue 15–20 times with a 1 ml pipette to obtain a cell suspension. (We use 1 ml plating medium for every 3 pairs of hippocampi, but importantly, trituration should always be done in 1 ml for better results, additional medium can be added afterwards to recover more suspension.)

Allow the cell suspension to settle for 5 min for visible undigested tissue to sink to the bottom. A thorough digestion may leave no visible tissue.

Transfer the cell suspension to a new 15 ml tube with a 1 ml pipette (Fig. 3F). While aspirating the suspension, be sure to keep a distance from the bottom of the tube to avoid taking in chunks of undigested tissue.

Centrifuge the cell suspension at 1000 g for 3 min.

Discard the supernatant which contains debris and resuspend the cells in 1 ml of neurobasal with 10% FBS, by gently triturating 18 times.

Dilute a sample of the resuspended cells 10 times for cell count on a Hemacytometer.

Generally, each pair of hippocampi can yield 400,000 ~ 600,000 cells based on the gestational age of the embryo.

2.8. Preparation of feeder glia neuron mixed cultures

Plate 300,000–400,000 cells for each 60 mm dish. Low density cultures survive longer in vitro.

4 h after plating, change plating medium to complete medium (fresh neurobasal containing 2% B27 supplement, 0.6% Glutamax and 0.2% Penicillin/Streptomycin). Since the feeder culture is to be maintained for over 4 weeks, the 60 mm dish should be placed in a 20 cm dish containing a lid-less dish filled with ddH2O for humidity to minimize evaporation and condensation of the medium.

On the fourth day after plating, add 0.5 μl of 10 μM AraC to inhibit glia growth

Refresh 1 ml of complete medium twice a week and maintain the culture in the incubator for 2 weeks until maturation.

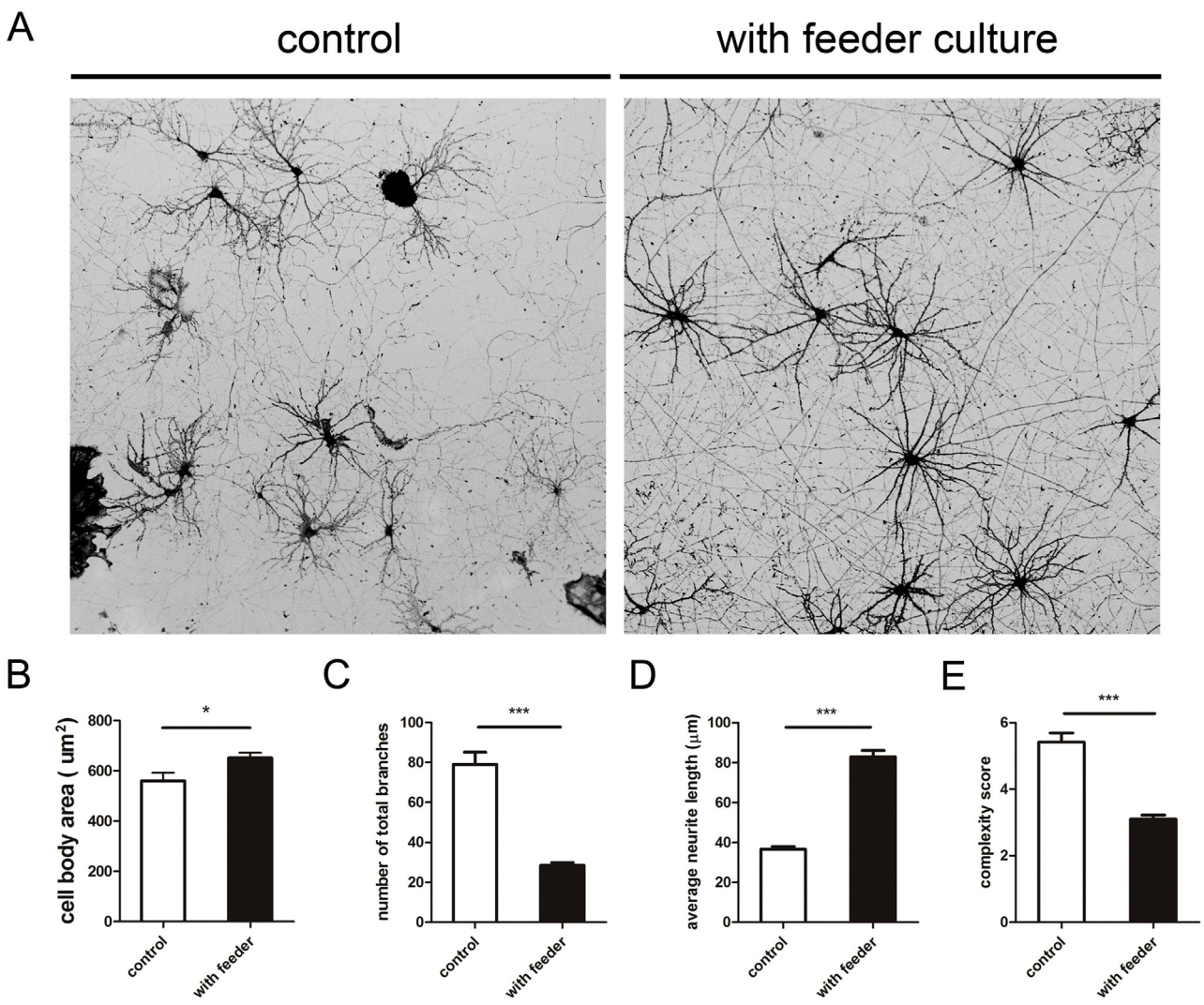

Fig. 3. Comparison of neuron morphology with or without feeder culture. (A) Staining of actin by phalloidin. Cultured hippocampal neurons with neurotrophic support from the feeder culture show robust growth with stronger staining of axons and dendrites (scale bar, 50 μm). (B and D) The cell body area and average dendrite length is significantly lower in neurons without neurotrophic support ($P < 0.05$ and $P < 0.0001$ respectively). (C and E) The number of total dendrite branches and complexity score of the average neuron is significantly higher in the control group ($P < 0.0001$ and $P < 0.0001$ respectively). Data displayed by SEM, by Student *t*-test with Welch's correction.

2.9. Plating low density pure neuron cultures (2 weeks after preparing feeder cells)

1 day before dissection, place 12 mm coverslips in ceramic racks. Place racks in a glass beaker covered with aluminum foil and sterilize in an oven at 225 °C for 6 h. (The temperature also makes our coverslips hydrophilic).

Pour the coverslips into a 60 mm dish containing freshly prepared coating PLL solution for storage.

Arrange 4 12 mm coverslips in a 35 mm dish. PLL does not need to be completely dried.

Add 2 ml of plating medium to the 35 mm dish and equilibrate in the cell culture incubator.

Plate 10,000–20,000 cells for each 35 mm dish.

4 h after plating, transfer up to 8 coverslips to the 60 mm dish containing 2-week-old cultures with the DIV0 neurons facing upwards (Fig. 2A). The coverslips will kill the mature neurons on which it settles, but it does not affect the growth of DIV0 neurons.

Maintain culture as mentioned in 2.9.4

3. Results

3.1. Comparison of neuron development with or without feeder cells

Our culture density is approximately 10002000/cm^2. At this density, neurotrophic support is poor, especially when glia growth is inhibited by AraC. We compared the development of neurons cultured with or without feeder cells. The feeder cells significantly increases neurite growth speed indicated by longer neurite extension on day 2. On, day 8, neuron morphology is much healthier with neurotrophic support and the dendrites showed a more stretching and spreading appearance. On day 15, neurons cultured with feeder cells are physically strong and robust, while those without feeder cells have inter-winding neurites with more fragmention, which is a sign of degeneration (Fig. 2B).

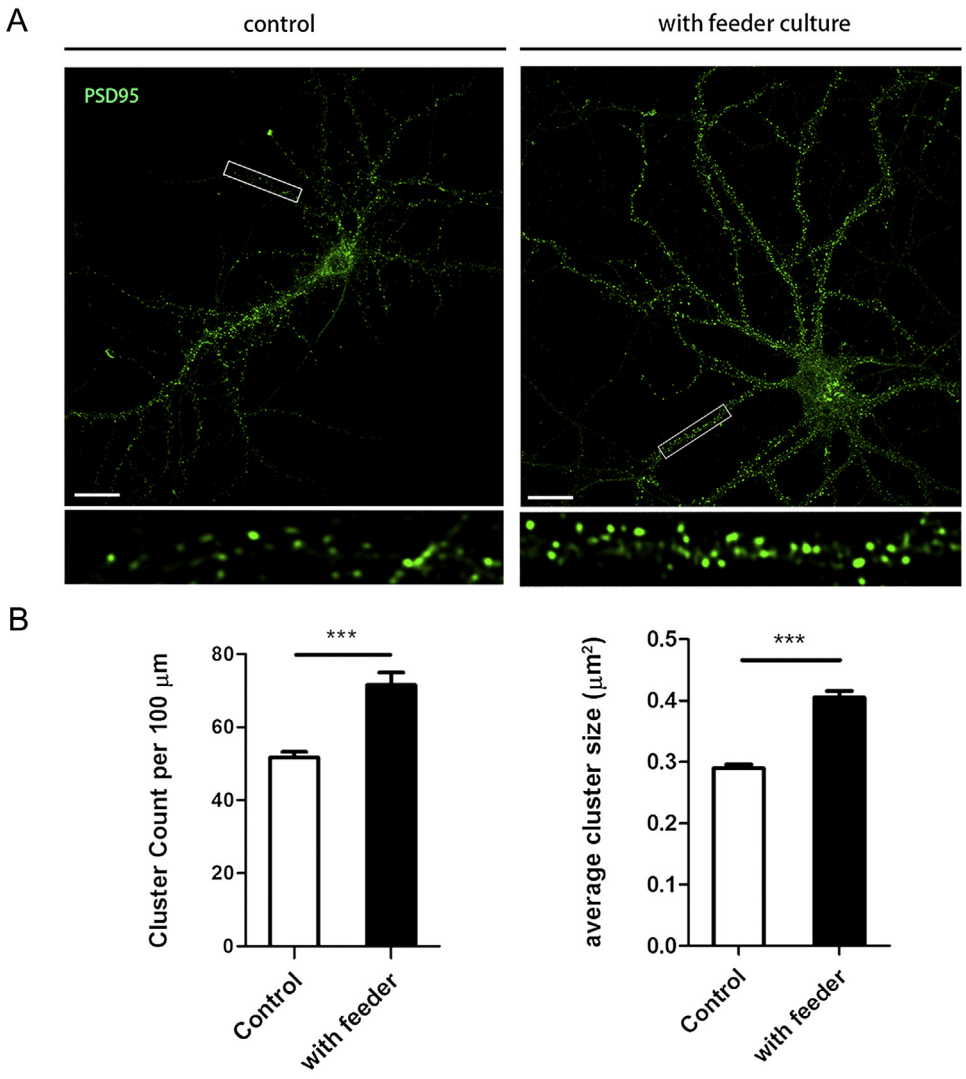

Fig. 4. Comparison of the PSD95 positive synapses with or without feeder culture. (A) Staining of PSD95 in DIV15 hippocampal neurons with feeder culture shows larger and more intensely stained PSD95 clusters (scale bar, 20 μm). (B) PSD95 positive cluster density and size are increased in neurons with neurotrophic support in comparison to control neurons. Data displayed by SEM, by Student *t*-test with Welch's correction,***P < 0.0001.

3.2. Comparison of neuron morphology with or without feeder cells

As we have shown that neurotrophic support boosts neuron growth, we made general comparison of DIV15 neurons cultured with or without feeder cells. To improve the health status of the control group for better analysis, we did not add AraC, and significant fraction of the coverslips were covered with astrocytes and oligodendrocytes (not shown). We visually compared neurons from the two groups (Fig. 3A). Acquiring photos from the neurotrophic group was very easy because the culture medium in the feeder group already contains AraC, so inhibition of glia growth was sufficient and a clean view of a single neuron could be easily selected. We stained coverslips from the two groups with TRITC Phalloidin for fluorescence imaging of neuron morphology. Neurons supported by feeder cells showed relatively uniformed morphology, and robust growth indicated by strong staining of TRITC Phalloidin on the cell body and dendrites. Also, a neat meshwork of axons could be observed between each neuron. Neurons in the control group are smaller with more complex dendritic morphology, and axons seemed to grow in a more complex and interwinding manner. Consistently, statistical analysis show that with neurotrophic support, the cell body size was significantly increased (cell body area; control, $560.6 \pm 31.70\ \mu m^2$, n = 22; with feeder, $651.4 \pm 21.12\ \mu m^2$, n = 26; P < 0.05) (Fig. 3B), while the number of total branching (number of branching; control, 79.00 ± 6.12, n = 21; with feeder, 28.48 ± 1.40, n = 26; P < 0.0001) and complexity score (complexity score; control, 5.42 ± 0.27, n = 21; with feeder, 3.10 ± 0.12, n = 29; P < 0.0001) for each neuron was decreased (Fig. 3C and D). Despite reduced branching, the average length of each dendrite branch was significantly increased (branch length; control, $36.64 \pm 1.44\ \mu m$, n = 21; with feeder, $82.98 \pm 3.125\ \mu m$, n = 29; P < 0.05) (Fig. 3E).

To compare the synaptic density, we stained both groups with PSD95, a postsynaptic marker. Obviously, both the number and size of PSD95 clusters in the feeder group were significantly increased when compared to control group (cluster count per 100 μm; control, 51.67 ± 1.503,n = 60; with feeder, 71.51 ± 3.426, n = 55; P < 0.0001); average cluster size; control, $0.2894 \pm 0.006534\ \mu m^2$,n = 60; with feeder, $0.4051 \pm 0.01065\ \mu m^2$, n = 55; P < 0.0001 (Fig. 4A and B).

3.3. Validity of low density neurons

As we have successfully cultured hippocampal neurons at very low densities with simple dendritic morphology, we further

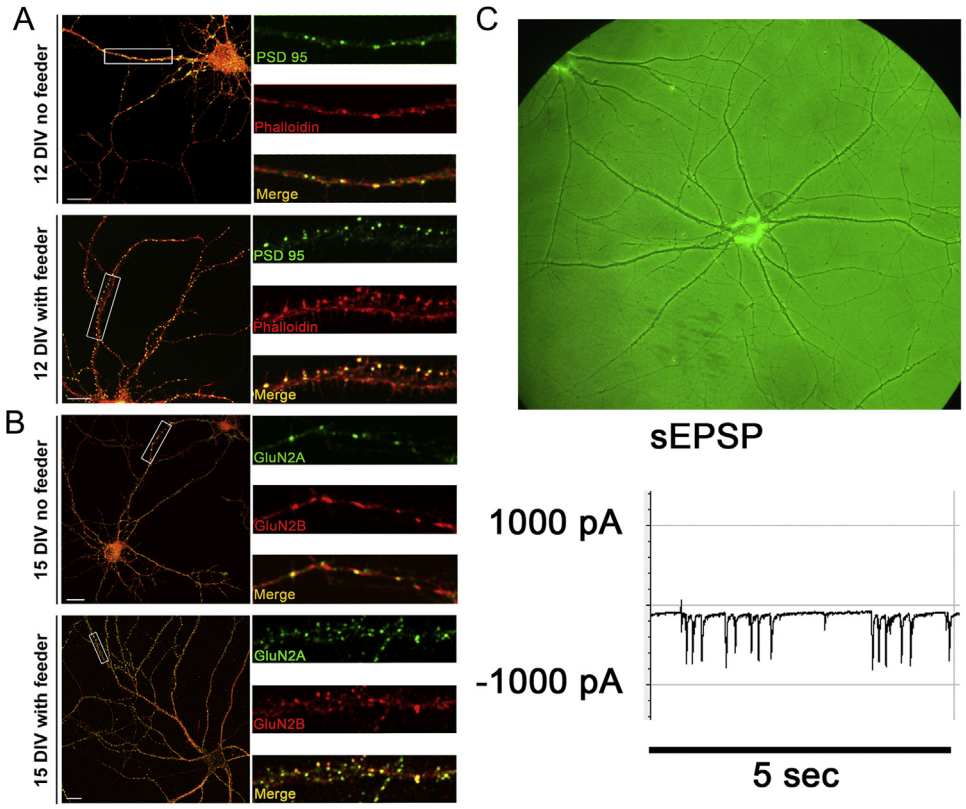

Fig. 5. Validity of cultured hippocampal neurons with neurotrophic support. (A) Staining of actin and PSD95 in DIV 12 hippocampal neurons shows colocalization of PSD95 with actin clusters located in dendritic spines. (B) Staining of the NMDAR subunits GluN2A and GluN2 B clusters in DIV 15 hippocampal neuron showing strong colocalization along the dendritic shaft. (C) sEPSC sweep from the representative DIV 14 hippocampal neuron.

determined whether these neurons are functional at the cellular level. So we co-stained actin with PSD95, a marker for excitatory postsynaptic compartments. Results showed that in DIV12 neurons with neurotrophic support, the dendritic shaft and spine heads are enriched in actin. PSD95 colocalized perfectly with the spine heads suggesting the presence of excitatory synapses. Filipodia which are dendritic protrusions negative for PSD95 can also be observed. The control group exhibit significantly less developed spines (Fig. 5A). In DIV 15 neurons, NMDA receptors, an important ion channel implicated in synaptic plasticity can be detected by staining of its GluN2A and GluN2 B subunits which were colocalized in presumably synaptic structures. (Fig. 5B), consistent with the localization of NMDARs in the post-synaptic membrane. (Wenthold et al., 2003; Sheng and Hoogenraad, 2007). NMDAR subunits can also be detected in the control group but at much lower density. Finally, we recorded sEPSC using whole cell patch clamping. A coverslip containing DIV 14 neurons cultured at low density with neurotrophic support was placed in a recording chamber containing Mg^{2+} free extracellular saline (ECS). sEPSC could be recorded at −70 mV holding potential, indicating that these neurons are functionally connected with intact synaptic transmission (Fig. 5C). The electrophysiology data could not be obtained from neurons in the control group. The membrane/pipette seal was unsuccessful, which was probably the result of poor membrane properties.

4. Discussion

Isolation and culture of hippocampal neurons allows for specific functional and morphological dissection studies in vitro. It is well known in the literature that pure neuron cultures are harder to prepare and maintain than mixed cultures, especially at low densities. A number of studies suggest that neurtrophic support from neurons or astrocytes can increase health and viability of cultured neurons (Kaech and Banker, 2006; Shimizu et al., 2011). In an experimental approach, we added conditioned medium from DIV17 mature cultures to newly prepared cultures and found that the growth of the young neurons was strongly boosted (data not shown). So we speculate that young neurons would benefit from a continuous supply of neurotrophic factors from mature mixed feeder culture. Our results showed that the mixed culture could indeed support healthy neuron growth at a very low density.

The feeder cells were in fact, a mixed culture of primary neurons and astrocytes. Oligodendrocytes and microglia were absent because AraC was added at DIV3-4, before the generation of these glia cells. This also ensured that the glia cells mixed in the cell suspension for low density cultures would be under the effect of AraC inhibition from DIV0 resulting in minimum glia contamination. Simultaneously, the newly seeded neurons benefit from a conditioned medium rich in neurotrophic factors. It was surprising that mature neurons from our protocol had less branching than control since neurotrophic factors have been reported to promote dendrite or axon branching (Jeanneteau et al., 2010; Lazo et al., 2013). We cannot rule out the possibility that increased branching may be mediated by overgrowth of different types of glia cells in the control group, without which the neurons would hardly survive at the given density. Above all, in comparison to the control group, neurons prepared using our method exhibit larger cell body with longer dendrites and less dendritic branching which allows us to easily isolate a single dendrite for better analysis of dendritic clusters or for live imaging of protein and organelle transport. We have also verified that our neurons contain synapses, NMDA receptors and spontaneous synaptic transmission, suggesting that they are properly wired, and suitable for cellular studies.

Several protocols have been established for culturing low density neurons with neurotrophic support from neurons or astrocytes

(Kaech and Banker, 2006; Shimizu et al., 2011; Beaudoin et al., 2012; Lu et al., 2016). Neurotrophic factors include neurotrophins and different kinds of growth factors such as heparin-binding EGF-like growth factor (HBEGF), insulin-like growth factor (IGF) and fibroblast growth factor (FGF) (Lewin and Carter, 2014). Neurotrophins are a class of secreted peptides that regulates neurite outgrowth and axon guidance. NGF is the first identified neurotrophin, followed by BDNF, NT3 and NT4, which make up the neurotrophin family(Barde et al., 1982; Lewin and Barde, 1996). Among them, BDNF has been extensively studied in many aspects of neuron function and brain development. BDNF is synthesized in both neurons and astrocytes(Miklic et al., 2004; Kolarow et al., 2007). Previous studies have shown that glutamate stimulates brain-derived neurotrophic factor (BDNF) synthesis in glial cells through metabolic glutamate receptors(Taylor et al., 2003; Wu et al., 2004). Moreover, neuronal activity induces synaptic-like microvesicle release of glia-transmitters through calcium signaling in glia cells (Ben Achour et al., 2010). Since neurotrophic factors such as BDNF is sorted into vesicles for activity dependent release (Lessmann and Brigadski, 2009), it is likely that Neuron and astrocyte interaction may enhance neurotrophic factor release through glutamate mediated neuronal activity. While GDNF (glial cell line-derived neurotrophic factor) and Activity-dependent neurotrophic factor (ADNF) are produced primarily by glia cells (Smith-Swintosky et al., 2005), and are both strong stimulators for neurite outgrowth, not much efforts has been made in classifying neurotrophic factors by exclusive neuron or glia origin. However, since pure high density hippocampal neurons are capable of supporting ultra-low density neurons (Lu et al., 2016), it is safe to say that neurons also secret neurotrophic factors in a paracrine manner. We believe that neurotrophic factors produced by neurons and astrocytes differ in types and abundance, and a mixture of these factors would be a step closer to physiological conditions where the two cell populations co-exist. An additional advantage of our protocol over astrocyte feeder based protocols is that the mixed feeder culture and the low density neurons can be prepared in a single procedure. The drawback on the other hand, is the need to prepare healthy feeder cells, which matures over two weeks and is therefore time consuming for a single round of culture. Time cost could be reduced when chaining multiple rounds. Above all, we propose that a mixed culture would be a better source of neurotrophin support compared to pure neurons or glia cultures. However, further experiments must be conducted to justify this statement.

Finally, our method does not work for cortical neuron cultures. Cortical neuron growth seems to rely heavily on physical contact with glia cells. The addition of AraC shows severe toxicity towards cortical neurons, presumably by inhibiting the growth of glia cells. Cortical neuron cultures can be prepared in the same way we prepare our feeder culture, but should be plated at a much higher density 300,000 to 700,000 per 35 mm dish.

5. Conclusion

In conclusion, Our method provides a novel way to culture highly uniformed hippocampal neurons at densities in which single neurons could be clearly isolated for acquiring high quality morphology or electrophysiology analysis.

Ethical approval

All experiments involving animals were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory

Animals and the ethical guidelines of the Zhejiang University Animal Experimentation Committee.

Conflict of interest statement

All authors declare no actual or potential conflicts of interest.

Acknowledgements

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