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Development of dissociated cryopreserved rat cortical neurons *in vitro*

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ABSTRACT

Dissociated neuronal cultures of various brain regions are commonly used to study physiological and pathophysiological processes *in vitro*. The data derived from these studies are often viewed to have relevance to processes taking place in the mature brain. However, due to the practical challenges associated with lengthy neuronal culture, neurons are often kept for 14 days *in vitro* (DIV), or less, before being subject to experimentation. Non-proliferative cultures such as primary neuronal cultures can be maintained for more than 42 DIV if water evaporation from culture media is monitored and corrected. To determine appropriate time points corresponding to the stages of cortical development, we compared characteristics of cryopreserved cortical neurons in cultures at various DIV using immunofluorescence, biochemical measurements and multielectrode array recordings. Compared to 21 and 35 DIV, at 14 DIV, cultures are still undergoing developmental changes and are not representative of adult *in vivo* brain tissue. Specifically, we noted significant lack in immunoreactivity for synaptic markers such as synapsin, vesicular GABA transporter and vesicular glutamate transporter at 14 DIV, relative to 21 and 35 DIV. Moreover, multielectrode array analysis indicated an increase in network firing up to 46 DIV with patterned firing peaking at 35 DIV. Our results provide specific evidence of the maturational stages of neurons in culture that can be used to more successfully plan various types of *in vitro* experimentation.

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1. Introduction

Dissociated neuronal tissue culture allows for investigation of neurobiological processes in a controlled environment that provides easy access to individual cells (Sohya et al., 2007; Yudowski et al., 2007). Neurons are most often harvested from the brain at embryonic developmental time points to optimally isolate neurons before extensive synaptic adhesion. Therefore, major developmental processes including some neurogenesis takes place *in vitro* and must recapitulate late embryonic and early postnatal development. These developmental events include neurite elongation, dendritic arborization, synaptogenesis and expression of neurotransmitter receptors and ion channels (Lu et al., 1999; Luhmann et al., 2003; Wallis et al., 2008). Many of these changes take place during a critical period of postnatal development until day 14 in the mouse (Rauch and Hitzemann, 1986; Bi et al., 1997; Thompson and Potter, 2000; Benitez-Diaz et al., 2003; Zhang and D'Ercole, 2004; Nosyreva and Huber, 2005; Burnstock, 2007; Brill and Huguenard, 2008).

In the current literature, cortical neurons are typically cultured for 7–14 days *in vitro* (DIV) before being used in experiments (Fig. 1). During *in vivo* development, neurons at this time period are still undergoing acute developmental changes (Kato-Negishi et al., 2004; Van Pelt et al., 2004; Sohya et al., 2007; Takayama et al., 2009) and the same is true *in vitro*.

Lack of recognition of the immature state of neurons at 7–14 DIV could likely lead to errors of differentiation between early developing and mature neurons in neuronal networks.

It is thought that neurons are difficult to maintain in culture past 14 DIV. Reasons for this include cell death due to infection and hyperosmolarity due to medium evaporation (Potter and DeMarse, 2001). Hyperosmolarity is easily corrected for, by adding water to the media during changes, although Potter and DeMarse used semipermeable covers to maintain osmolarity. By taking account of this dehydration neuronal cells have been cultured on multielectrode arrays for more than 72 DIV, even up to one year (Potter and DeMarse, 2001; Otto et al., 2003). It remains then, to find out how long neurons should be maintained in culture, before being

Abbreviations: DIV, days *in vitro*; MEA, multielectrode array; NID, naturally induced cell death; PB, population burst; PFR, population firing rate; RSME, root mean square error; vGAT, vesicular GABA transporter; VGLUT-1, vesicular glutamate transporter 1.

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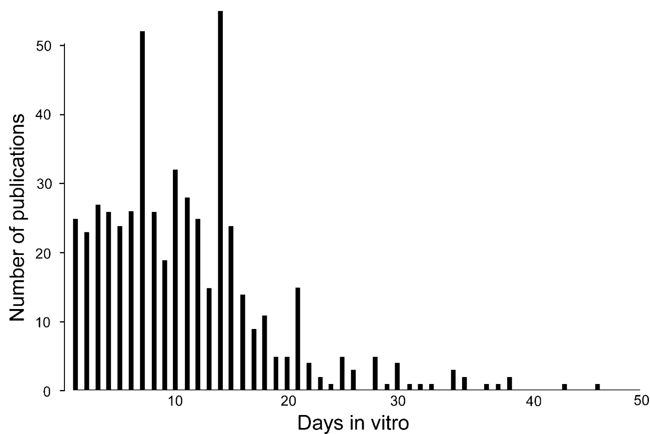

Fig. 1. This graph illustrates the results of a Google Scholar search of the past 10 years for the terms ['cortical culture' – 'weeks in culture' '*n* DIV'] where *n* = culture age in days. The culture age *in vitro* at which the experiments were assumed to be performed is plotted against the number of references found.

considered mature, or at least arriving at a developmental steady state.

With this study, we sought to determine the time line for the maturation of dissociated cortical cell cultures by observing their development up to 42 days *in vitro* (DIV). We aimed to reveal the developmental transition points and the times at which there is a stable mature value for a given parameter. The ultimate goal of this study was to determine the length of time neurons need to be kept in culture before their molecular and electrophysiological characteristics are representative of mature brain.

2. Methods

2.1. Cortical neuronal cultures

Vials of cortical neurons harvested from E18.5 rat embryos (QBM Cell Science, Ottawa, Canada) were stored in liquid nitrogen. Rat cells were thawed for 2.5 min at 37 °C and resuspended in prewarmed Neurobasal supplemented with B27 (2% final volume, Invitrogen) plus 100 units/ml penicillin/streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (media is replaced and serum removed after cells have been plated for 4 h to obtain serum free cultures). Neurons were plated in dishes precoated with 50 μg/ml poly-D-lysine (Sigma–Aldrich, P6407) in water at a density of 1×10^5 cells per well (96 well plates) or 2×10^6 cells per 60 mm dishes (BD BioCoat cellware, VWR). Cells were grown in a Thermo Scientific Forma series II water jacket incubator in a humidified atmosphere (5% CO_2/95% air) at 37 °C in Neurobasal medium with B27 supplement (Gibco) without serum.

Cells were plated at a volume of 200 μl of which 62.5% (125 μl) was removed and substituted for fresh media 4 h later. From then on 62.5% (125 μl) of media was removed and replaced every 3–4 days (*i.e.*, on DIV 4, 7, 11, 14, 17, 19, 21, 24, 26, 28, 31, 33, 35 38, 40). On the DIV 17, 24, 31 and 38, forty two percent (84 μl) of media was removed and substituted by 50% (100 μl) of fresh media plus 8% (16 μl) of water. The amount of water added to the cultures was determined by previous unpublished experiments measuring the osmolarity of the cultures over time. Cells were fixed at 42 DIV. Media and water additions were scaled up on pro rata basis for cultures plated on 60 mm dishes. No mitotic inhibitors were used.

2.2. Immunofluorescence

Cultures were fixed in 4% paraformaldehyde containing 7% (v/v) saturated picric acid for 20 min at room temperature, rinsed three times with 10 mM phosphate buffered saline (PBS) and stored at 4 °C until assessed. Cultures were incubated with primary antibodies (see Table 1) diluted in 10 mM PBS containing 0.3% triton X-100 over night at 4 °C, then rinsed with 10 mM PBS for 5 min. Appropriate secondary antibodies (Invitrogen) were used at a 1:400 dilution in 10 mM PBS containing 0.3% triton X-100. Secondary antibodies were incubated for 35 min at 37 °C, rinsed again for 5 min and studied under the microscope (Zeiss Axiovert 200 M or a Zeiss Axioplan fluorescence microscope).

2.3. Western blot

Protein levels were determined by Western blotting using antibodies listed in Table 1 at the concentrations indicated. To harvest the cells, plates were washed twice with cold PBS, and lysed in radioimmunoprecipitation assay buffer (RIPA) containing protein inhibitors (Sigma–Aldrich) for 30 min on ice. Spinning by vortex was performed every 5 min. Cell lysates were centrifuged at 10,000 × *g* at 4 °C for 2 min. The protein concentration was determined by the Bradford method (BioRad). Cell lysates containing 20 μg of protein were added to 4× reducing sample buffer (100 mM Tris–Cl pH 6.8, 200 mM dithiothreitol, 4% SDS, 20% glycerol and 0.2% bromophenol blue) and heated at 100 °C for 5 min. The samples were electrophoresed on an 8–12% polyacrylamide gel under constant current (400 mA). Separated proteins were transferred onto a polyvinylidene fluoride membrane (Perkin-Elmer). The immunoblots were blocked with 1% non-fat dried milk solution in TBST (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature and then incubated overnight at 4 °C with primary antibody at the indicated concentrations (see Table 1). Following a wash (three times with TBST) the immunoblots were incubated (1–2 h) with horse-radish peroxidase conjugated second antibody (Santa Cruz Biotechnology). The immunoblots were washed three times with TBST and visualized on X-ray films (Interscience) after exposure to enhanced chemiluminescence reagent (Amersham). Actin bands were monitored on the same blot to verify consistency of protein loading. Briefly, the immunoblots were stripped with Reblot Plus solution (Chemicon) for 20 min at room temperature. The blots were probed with anti-actin primary antibody and second antibody (anti-mouse, Santa Cruz) as described above. The molecular size of the protein was determined by running prestained protein markers (Biorad) in an adjacent lane.

2.4. Counting and statistics

Cell counting was performed using public domain ImageJ software (NIH Image). Cells were labeled with anti-NeuN or Hoechst and a consistent pattern of five pictures at a resolution of 950 pixels/mm were taken to represent the whole area of the well. These pictures were opened in ImageJ and the successive processing steps were (i) linear adjustment of the threshold so that only the NeuN or Hoechst positive cells were highlighted and (ii) analysis of the particles with a size >20 pixels^2-infinity (one pixel = 1.11 μm^2). For glial cell numbers Hoechst's positive but NeuN negative cells were counted. Counts were made on two wells from three independent experiments. Several independent observers determined that the counts were reliable. Statistical significance was determined by a one-way ANOVA. The range is indicated by standard error of the mean. Pearson's coefficient of correlation (r) was calculated to provide evidence for correlation between variables.

2.5. Total RNA isolation

Dissociated cultures were lysed in Trizol reagent (Invitrogen) and total RNA was extracted following the manufacturer's

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