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Research Report

A low-density culture method of cerebellar granule neurons with paracrine support applicable for the study of neuronal morphogenesis

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ABSTRACT

Cerebellar granule neuronal cultures have been used to study the molecular mechanisms underlying neuronal functions, including neuronal morphogenesis. However, a limitation of this system is the difficulty to analyze isolated neurons because these are required to be maintained at a high density. Therefore, in the present study, we aimed to develop a simple and cost-effective method for culturing low-density cerebellar granule neurons. Cerebellar granule cells at two different densities (low- and high-density) were co-cultivated in order for the low-density culture to be supported by the paracrine signals from the high-density culture. This method enabled morphology analysis of isolated cerebellar granule neurons without astrocytic feeder cultures or supplements such as B27. Using this method, we investigated the function of a polarity factor. Studies using hippocampal neurons suggested that glycogen synthase kinase-3 (GSK-3) is an essential regulator of neuronal polarity, and inhibition of GSK-3 results in the formation of multiple axons. Pharmacological inhibitors for GSK-3 (6-bromoindirubin-3′-oxime and lithium chloride) did not cause the formation of multiple axons of cerebellar granule neurons but significantly reduced their length. Consistent results were obtained by introducing kinase-dead form of GSK-3 beta (K85A). These results indicated that GSK-3 is not directly involved in the control of neuronal polarity in cerebellar granule neurons. Overall, this study provides a simple method for culturing low-density cerebellar granule neurons and insights in to the neuronal-type dependent function of GSK-3 in neuronal morphogenesis.

Abbreviations: CGN, cerebellar granule neurons; GSK-3, glycogen synthase kinase-3; div, days in vitro; CS, calf serum; BIO, 6-bromoindirubin-3′-oxime; LiCl, lithium chloride; DMSO, dimethyl sulfoxide; BME, basal medium Eagle; GFP, green fluorescent protein; PBS, phosphate-buffered saline

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1. Introduction

Cerebellar granule neurons (CGNs) are the most abundant type of neurons in the mammalian brain. A homogeneous population of these cells can be cultured from postnatal animals Messer, 1977; Thangnipon et al., 1983; Kingsbury et al., 1985). Cultured CGNs are polarized and establish a mature morphology by 7 days in vitro (div) (Gallo et al., 1987; Balazs et al., 1988). In addition, they can be transfected with the plasmid DNAs. Therefore, CGNs are used to study the molecular systems governing fundamental neuronal functions, including cellular survival (D'Mello et al., 1993; Galli et al., 1995; Datta et al., 1997; Bonni et al., 1999; Padmanabhan et al., 1999; Konishi et al., 2002), migration (Borghesani et al., 2002, Hatten 2002) and synaptogenesis (Kingsbury et al., 1988; Schramm et al., 1990; Nakanishi and Okazawa, 2006). Recently, the CGN culture has been used to study its cell-intrinsic mechanisms underlying neuronal morphogenesis (Bito et al., 2000; Konishi et al., 2004; Wayman et al., 2004; Kim et al., 2009) by expressing genes of interest (via plasmid transfection) in neurons, together with fluorescent proteins, to only visualize gene-expressing neurons. With subsequent morphological observations, their axons and dendrites can be distinguished, and thus molecules that specifically regulate axonal or dendritic maturation can be identified (Gaudilliere et al., 2004; Konishi et al., 2004; Stegmuller et al., 2006). However, a limitation associated with this culture system is that CGNs require paracrine support from neighboring cells for their survival, and thus need to be maintained at a high density.

The hippocampal pyramidal neuronal culture has also been frequently used to study the mechanisms of neuronal morphogenesis. Pioneering studies by Banker et al. established the isolated culture of hippocampal neurons with an astroglial monolayer as a support (Dotti et al., 1988; Kaech and Banker,2006). In this culture, hippocampal neurons extend short immature neurites, which become polarized by establishing axonal and dendritic compartments (Dotti et al., 1988; Wiggin et al., 2005; Arimura and Kaibuchi, 2007). This system has therefore enabled single cell analysis of neuronal morphogenesis, and consequently the discovery of molecular mechanisms involved in neuronal polarity (Shi et al., 2003; Jacobson et al., 2006; Shelly et al., 2007; Konishi and Setou, 2009). However, a key issue that remains is whether the polarity factors found in this paradigm are also involved in the formation of axonal and dendritic compartments of other neurons.

Low-density cultures of CGNs exhibit neuronal polarization by first extending a single long axon from their cell body, followed by the extension of several short dendrites (Powell et al., 1997; Zmuda and Rivas, 1998). Therefore, the mechanism of establishing neuronal polarity may be different between CGNs and hippocampal neurons. However, low-density CGN cultures require glia-conditioned media, and/or remain viable for only a few days (Powell et al., 1997; Zmuda and Rivas, 1998; Gupta et al., 2010). Therefore, in the present study, we aimed to establish a simple and cost-effective method for the maintenance of low-density CGNs. Owing to a large yield of cells that can be generated from this culture system, we cultured low- and high-density CGNs, with both densities facing each other via a narrow space. With support from the high-density culture, low-density CGNs can be maintained in normal serum containing media or in serum-free media. In this culture system, extension of CGN axons and dendrites enable the establishment of a matured morphology like the high-density culture system.

Using this method, we also investigated the function of a polarity factor in CGNs. Previous studies suggested that glycogen synthase kinase-3 (GSK-3) (Doble and Woodgett, 2003) is an essential regulator of neuronal polarity (Yoshimura et al., 2005; Jiang et al., 2005). In hippocampal neurons, when neurons begin to polarize, GSK-3 beta is inactivated at the tip of future axon. It is suggested that local inactivation of GSK-3 beta is crucial for polarization (Hur and Zhou, 2010). Indeed, global inhibition of GSK-3 results in the formation of multiple axons (Yoshimura et al., 2005; Jiang et al., 2005). Whether GSK-3 controls neuronal polarity in other type of neurons remain to be elucidated. In this study, we aimed to determine the involvement of GSK-3 in polarity control of CGNs by using our low-density culture method.

2. Results

2.1. *Effect of high-density CGN culture on the cellular survival of the low-density CGN culture*

To test if CGNs cultured at low-density were indeed supported by paracrine signals from the high-density culture, the survival of these neurons was analyzed. In the absence of the high-density culture, cell death occurred to most low-density neurons by 3 div (Fig. 1B and C). However, more than half of low-density neurons remained at this time-point in the presence of either the high-density culture or the glial feeder sheet (Fig. 1B and C). These neurons exhibited extended neurites, which were maintained for more than 7 div (data not shown). Therefore, the low-density culture may be maintained by the paracrine support from the high-density culture, without feeder glial cultures for support.

2.2. *CGNs at low-density exhibit normal neuronal morphology*

Next, we explored whether CGNs at low-density could establish neuronal polarity by extending their axon and dendrites like those at high-density. At 5 div, low-density cells were positive for MAP2 and Tau-1 (Fig. 2A), indicating normal neuronal polarity. Furthermore, axonal length of low-density neurons was comparable to that of high-density neurons, whereas dendritic length tended to be shorter (Fig. 2B). These results indicated that low-density CGNs had extended axons and dendrites, thus establishing neuronal polarity like high-density neurons. A low-density culture was also successfully maintained with KCl-deprived media (5 mM KCl), serum free media (contain insulin), or the glial feeder sheet (Fig. 2C). Under these conditions, neurons exhibited long axons and several short dendrites (Fig. 2C and D). Notably, we found that the number of axonal branches was decreased in the absence of KCl (Fig. 2E, 33% of control; $p<0.05$), indicating that neuronal