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Research Report

Increased expression of Slit2 and its receptors Robo1 and Robo4 in reactive astrocytes of the rat hippocampus after transient forebrain ischemia

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ABSTRACT

Slit2 is a secreted glycoprotein that was originally identified as a chemorepulsive factor in the developing brain; however, it was recently reported that Slit2 is associated with adult neuronal function including a variety of pathophysiological processes. To elucidate whether Slit2 is implicated in the pathophysiology of ischemic injury, we investigated the temporal changes and cellular localization of Slit2 and its predominant receptors, Robo1 and Robo4, for 28 days after transient forebrain ischemia. Slit2 and its receptors had similar overall expression patterns in the control and ischemic hippocampi. The ligand and receptors were constitutively expressed in hippocampal neurons in control animals; however, in animals with ischemic injury, their upregulation was detected in reactive astrocytes, but not in neurons or activated microglia, in the CA1 region. Astroglial induction of Slit2 and its receptors occurred by day 3 after reperfusion, and appeared to increase progressively until the final time point on day 28. Their temporal expression patterns overlapped with the time period in which reactive astrocytes undergo dynamic structural changes and appear hypertrophic in the ischemic hippocampus. The immunohistochemical data were consistent with the results of the immunoblot analyses, indicating that the expression of Slit2 and Robo increased progressively over the relatively long period of 28 days examined here. Collectively, these results suggest that Slit2/Robo signaling may be involved in regulating the astroglial reaction via autocrine or paracrine mechanisms in post-ischemic processes. Moreover, this may contribute to the dynamic morphological changes that occur in astrocytes in response to ischemic injury.

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1. Introduction

Slit proteins are secreted glycoproteins that were originally identified as chemorepulsive factors and as key regulators of midline crossing and axonal fasciculation (Battye et al., 1999; Brose et al., 1999; Rajagopalan et al., 2000a,2000b; Simpson et al., 2000a,2000b). Moreover, Slit proteins regulate neuronal migration and axonal branching (Brose et al., 1999; Brose and Tessier-Lavigne, 2000; Andrews et al., 2007; Chedotal, 2007; Cayre et al., 2009; Cariboni et al., 2012). The functions of Slit are modulated by the Roundabout (Robo) family of transmembrane receptors, which transmit the signal downstream (Kidd et al., 1998; Brose et al., 1999; Li et al., 1999). Three Slit proteins (Slit1–3) and four Robo proteins (Robo1–4) have been identified in mammals (Itoh et al., 1998; Kidd et al., 1998; Huminiecki et al., 2002). Although most studies on the Slit/Robo system have been performed in the developing nervous system, the persistent and broad expression of Slit and Robo in mature neurons in the adult brain and spinal cord suggests that they are involved in biologic functions that extend beyond development, i.e., adult neuronal function (Marillat et al., 2002; Wehrle et al., 2005).

The Slit/Robo system has also been investigated in various models of central nervous system diseases. For instance, it was found that Slit2 modulates cerebrovascular inflammation by suppressing leukocyte recruitment in the brain. Moreover, Slit2 mediates neuroprotection in the ischemic brain, possibly due to the direct effects of Slit on neurons and/or on neuron–glia interactions (Altay et al., 2007). A recent study using an in vitro model of ischemia revealed that the neuroprotective effects of isoflurane could be mediated via the upregulation of Slit2/Robo1, which may stabilize the cytoskeleton, thereby suppressing neuronal death (Zhao et al., 2013). Furthermore, Slit2 is upregulated in spinal cord neurons after spinal cord injury (Liu et al., 2011).

In addition to its possible role in neurons, Slit2 mRNA was upregulated in the reactive astrocytes that surrounded the necrotic tissue in a cryo-injured brain (Hagino et al., 2003) and was upregulated in temporal lobe epileptic foci (Fang et al., 2010). Reactive astrogliosis is the universal astrocytic response to all central nervous system injuries, including ischemia. Furthermore, reactive astrogliosis is known to play a multifaceted and complex role in the response to ischemia and has the potential to both enhance and impair neuronal survival and regeneration (Anderson et al., 2003; Takano et al., 2007; Zhao and Rempe, 2010; Barreto et al., 2011). Thus, the aforementioned data suggest that Slit2 is involved in the pathophysiology of ischemic injury including neuronal damage and the glial response. However, little is known about the selective expression and regulation of Slit2 and its Robo receptors in *in vivo* models of transient forebrain ischemia.

In the present study, we investigated the temporal changes and cellular localization of Slit2 and its receptors, Robo1 and Robo4, both of which have been shown to interact with Slit2 (Park et al., 2003; Liu et al., 2011; Zhao et al., 2013) during the initial 28 days after transient forebrain ischemia. Interestingly, our results revealed selective and long-lasting expression of Slit2/Robo in reactive astrocytes with a reactive hypertrophic phenotype and elevated levels of glial fibrillary acidic protein (GFAP). These findings suggest that the Slit2/Robo system may be involved in the reactive astrogliosis process that occurs in response to ischemic insults.

2. Results

2.1. *Slit2 expression was selectively induced in reactive astrocytes in the ischemic hippocampus*

To establish whether the expression of Slit2 and its Robo receptors is modulated in the ischemic hippocampus, the expression and cellular localization of Slit2, Robo1, and Robo4 were examined using a murine model of transient forebrain ischemia. Consistent with a previous study (Pulsinelli and Brierley, 1979), routine histological staining with Cresyl violet revealed characteristic neuronal cell loss in the CA1 region of the hippocampus following 10 min of ischemia (data not shown). Immunoreactivity for Slit2 was localized to the pyramidal (Fig. 1A) and granule cell layers (Supplementary Fig. 1) in the hippocampus of sham-operated rats. To define the phenotype of Slit2-positive cells in the hippocampus of control animals, we performed double labeling with Slit2 and other cell-type-specific markers including the neuronal-specific marker NeuN, and three glial markers, namely GFAP, OX42, and ionized calcium-binding adapter molecule 1 (Iba1). The cells that expressed Slit2 in the control hippocampus were NeuN-positive neurons (Fig. 1A–D, and Supplementary Fig. 1A–C), while negligible immunoreactivity for Slit2 was observed in GFAP-positive astrocytes (Fig. 1E and F, and Supplementary Fig. 1D–F) or Iba1-positive microglia (Fig. 1G and H, and Supplementary Fig. 1G–I). Beginning 3 days after reperfusion (data not shown), Slit2 immunoreactivity was observed in glial-like cells localized in the strata of the CA1 hippocampal region, and this activity had increased further by day 7 (Fig. 1I–L); the cells were confirmed to be astrocytes by double labeling with Slit2 and GFAP. At 14 days after reperfusion, Slit2 expression was more evident in the CA1 region, and nearly all of the Slit2-positive cells were GFAP-positive astrocytes (Fig. 1M–P). At 28 days after reperfusion, additional increases in Slit2 immunoreactivity were noted in the CA1 region, wherein Slit2-positive astrocytes appeared to be more hypertrophic with increased GFAP labeling compared to the astrocytes observed at day 14 (Fig. 1Q–T). These reactive astrocytes revealed both nuclear and cytoplasmic Slit2 immunoreactivity. To verify that the astrocytes secrete Slit2 in response to ischemic insults, enzyme-linked immunosorbent assay (ELISA) was conducted using rat primary cortical astrocytes subjected to ischemia-like oxygen–glucose deprivation (OGD). As shown in Supplementary Fig. 2, ELISA demonstrated that OGD-treated astrocytes secreted a 2.5-fold higher level of Silt2 compared with that found in normally cultured media. The level of Slit2 in cultured media at 12 h after reoxygenation was also higher (1.5-fold) than that in the control.

Next, we clarified whether Slit2 expression was induced in microglia using double labeling with Slit2 and Iba1. Almost no colocalization of Slit2 and Iba1 was detected in the CA1 region of animals at 14 (Fig. 4A–D) and 28 days (Fig. 4E–H) after reperfusion, although Iba1-positive cells revealed large cell bodies with retracted and short processes, which are characteristics of the reactive form of microglia.