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Synaptic adhesion-like molecules (SALMs) promote neurite outgrowth

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ABSTRACT

SALMs are a family of five adhesion molecules whose expression is largely restricted to the CNS. Initial reports showed that SALM1 functions in neurite outgrowth while SALM2 is involved in synapse formation. To investigate the function of SALMs in detail, we asked if all five are involved in neurite outgrowth. Expression of epitope-tagged proteins in cultured hippocampal neurons showed that SALMs are distributed throughout neurons, including axons, dendrites, and growth cones. Over-expression of each SALM resulted in enhanced neurite outgrowth, but with different phenotypes. Neurite outgrowth could be reduced by applying antibodies targeting the extracellular leucine rich regions of SALMs and with RNAi. Through over-expression of deletion constructs, we found that the C-terminal PDZ binding domains of SALMs 1–3 are required for most aspects of neurite outgrowth. In addition, by using a chimera of SALMs 2 and 4, we found that the N-terminus is also involved in neurite outgrowth.

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Introduction

Neurite outgrowth is a fundamental event in the development and maintenance of synaptic connections in the nervous system. Through highly regulated mechanisms, young neurons undergo axonal/dendritic polarization, and subsequent outgrowth of these neurites is essential to the establishment of synaptic connections that lead to brain function (da Silva and Dotti, 2002). Cell adhesion molecules (CAMs) are a diverse class of proteins that function in neurite outgrowth, synaptic development and maintenance, and cell adhesion at synaptic and non-synaptic sites (Craig and Banker, 1994; Dalva et al., 2007). Several CAMs are enriched at growth cones and are required for normal neurite outgrowth. For example, neural cell adhesion molecule (NCAM), N-cadherin, and L1-CAMs have been shown to regulate neurite outgrowth through various mechanisms, including changes in intracellular calcium levels, associations with cytoskeletal proteins at growth cones, and the activation of FGFR and MAPK signaling cascades (Doherty et al., 2000; Francavilla et al., 2007; Meiri et al., 1998; Utton et al., 2001). In humans, mutations in L1-CAMs lead to various neurological disorders, including hydrocephalus and MASA (mental retardation, aphasia, shuffling gait, and adducted thumbs) syndrome, and expression of constructs encoding L1 with these known mutations leads to deficits in neurite outgrowth (Moulding et al., 2000). While a wealth of information implicates CAMs in neurite outgrowth, the mechanism is highly complex and not completely understood.

Synaptic adhesion-like molecules (SALMs) are a family of CAMs that is largely restricted to the CNS and is involved in neurite outgrowth and synapse formation (Ko et al., 2006; Morimura et al., 2006; Wang et al., 2006). SALMs are also present in the adult where they may play a role in synaptic maintenance and other cellular interactions. Five family members have been identified: SALMs 1–5 (Ko et al., 2006; Morimura et al., 2006; Wang et al., 2006). The domain structure of SALMs includes extracellular leucine-rich repeats (LRR), an immunoglobulin C2-like domain (IgC2), a fibronectin type III (FN3) domain, a transmembrane (TM) region, and a PDZ-BD (PSD-95, Discs-large, ZO-1, binding domain; absent in SALMs 4 and 5). This domain structure is homologous with that of various related CAMs that function in outgrowth, including AMIGO, LINGO, NGL-1, and FLRT proteins (Chen et al., 2006).

Over-expression of SALM1 in young (4 days in vitro, DIV4) primary hippocampal cultures promotes an increase in neurite outgrowth (Wang et al., 2006), while alterations in SALM2 expression affects synapse formation and may play a role in regulating the balance of excitatory and inhibitory synapses (Ko et al., 2006). Therefore, individual SALMs may have a range of different functions. Alternatively, all SALMs may have multiple roles and function in neurite outgrowth and synapse formation in developing animals, as well as maintenance of synapses in adults. To investigate these possibilities, we have studied the role of all SALMs in neurite outgrowth by using a combination of over-expression, RNAi-mediated knock-down of expression, and blocking of function with antibodies to extracellular domains. Our results show that all SALMs promote neurite outgrowth, but with various phenotypes.

Results

Distribution of SALMs in neurons

SALM1 and SALM2 localize to both axons and dendrites (Ko et al., 2006; Wang et al., 2006). Additionally, SALM1 co-localizes with NMDA

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receptors (Wang et al., 2006), while SALM2 co-localizes with both pre- and post-synaptic proteins at excitatory synapses in mature neurons (Ko et al., 2006). To understand the roles of SALMs in neurite outgrowth, we began by characterizing the cellular localization and morphological effects of overexpressed SALMs early in neuronal development. Young primary hippocampal neurons (DIV4) were co-transfected with GFP and myc-SALM1, myc-SALM2, untagged SALM3, myc-SALM4, or HA-SALM5 cDNA constructs. Neurons transfected with GFP and pcDNA 3.1^+ empty vector were used as a control, and immunocytochemistry was performed 48 h after transfection. Transfected SALM constructs over-expressed their respective proteins by about 300%, as compared to endogenous SALM levels (data not shown). Over-expressed SALMs are localized throughout the cell in the soma, axons, dendrites, and growth cones (Fig. 1) with a largely diffuse pattern. However, punctate staining is present and is particularly apparent when staining is restricted to SALMs present on the cell surface (supplemental Fig. 1). Therefore, SALMs are present in intracellular pools represented by the diffuse staining as well as on the surface where they appear more clustered.

The various SALMs qualitatively appear to have distinct effects on cell morphology. For example, SALM4-transfected neurons often had a dramatic increase in the number of shorter primary neurites protruding from the cell body (Fig. 1D, arrows). These shorter neurites appeared to be dendritic, as MAP2 immunostaining localized at these neurites (data not shown). Many SALM5-transfected neurons showed another unique phenotype. The primary neurites often overlapped and appeared to adhere to one another at regions proximal to the cell body. SALM5 accumulated at these points of neurite adhesion (Fig. 1E, arrows). These various changes in cell morphology of SALM-transfected neurons imply that individual SALMs may have different functions in the CNS.

SALMs promote neurite outgrowth

To further extend our initial qualitative observations indicating that SALMs may affect neurite outgrowth, we performed a detailed computer-assisted analysis of neuronal morphology using Metamorph Neurite Outgrowth Module (v7.0r3). The parameters tested included total neurite outgrowth (defined as the total skeletonized pixel area in μm), mean process length, number of primary neurites extending from the cell body, and number of total neurite branches. Primary hippocampal cultures were transfected at DIV4, a time when significant neurite outgrowth takes place (Dotti et al., 1988). Cultures were co-transfected with GFP (to visualize the entirety of each cell) and myc-SALM1, myc-SALM2, SALM3, myc-SALM4, or HA-SALM5 cDNA. Neurons were fixed and immunostained for GFP and SALM proteins 48 h after transfection (DIV6); GFP staining was used to quantify neurite outgrowth and SALM staining served to verify SALM expression.

Our results showed that SALMs promote neurite outgrowth with various phenotypes. SALMs 1–5 all promoted significant increases in total outgrowth and number of branches, as compared to the control (Figs. 2B and D, respectively). Additionally, SALM4 promoted more branching than other SALMs (Fig. 2D). SALM2 promoted an increase in the number of primary processes, as compared to the control, SALM1, and SALM5 (Fig. 2E). Consistent with the dramatic increase in short primary neurites described earlier, the number of processes in SALM4-transfected cells more than doubled, as compared to control and other SALM-transfected neurons, including those transfected with SALM2 (Fig. 2E). The mean process length of SALM4-transfected neurons was significantly less than that of SALM1, SALM3, and SALM5-transfected neurons (Fig. 2C). Together, this increase in short primary neurites in SALM4 is a visually distinctive phenotype as compared to the other SALMs. Thus, SALMs promote neurite outgrowth with various distinct phenotypes, and the major outgrowth parameters that SALMs 1–5 modify are total outgrowth and neurite branching.

SALMs promote axonal and dendritic outgrowth

The increase in the number of MAP2-positive processes extending from the cell body, particularly evident with SALM4, indicates a change in dendrite growth. In order to determine if the SALM-mediated increases in number of processes were axonal as well, we co-transfected DIV4 neurons with SALM2 and GFP, immunostained for MAP2, and imaged the cells as described earlier. Since more than 90% of all GFP-transfected cells also expressed SALM2 (data not shown), we used GFP expression as a positive indicator of SALM2 transfection in these experiments. Along with MAP2 staining, the transfected cells were analyzed through the morphological criteria described in the methods to distinguish axons from dendrites (Figs. 3A and B, top panels). Once axons and dendrites were identified, the dendrites were digitally removed from the cell and the resulting axon-only image was analyzed for neurite outgrowth (Figs. 3A and B: bottom panels). Our results showed a 75% increase in total axonal outgrowth (3543 ± 371.42 μm for SALM2, 2029± 419.03 for control) (Fig. 3C), 108% increase in axon number (2.43 ± 0.37 axons for SALM2, 1.17 ± 0.17 for control) (Fig. 3F), and an 82% increase of axonal branches for SALM2 (119 ± 17.57 branches for SALM2, 65 ± 18.37 for control) (Fig. 3E), as compared to control axons, showing that axon growth is also affected by SALM expression. Analysis of the non-axonal processes also showed increases in outgrowth with SALM2 expression. These results showed that the SALMs affect both axon and dendrite outgrowth, although the effects may vary with individual SALMs.

Application of antibodies directed to the extracellular LRR region of SALMs inhibits neurite outgrowth

While over-expression experiments suggest a role for SALMs in neurite outgrowth, they do not address a role for endogenous SALMs. Previous studies have demonstrated the efficacy of applying antibodies directed to extracellular domains of endogenous transmembrane proteins, including cell adhesion molecules like cadherin, L1-CAM, and NCAM, to block their activity (Garcia-Castro et al., 2000; Lindner et al., 1983; Muller et al., 1996; Tang et al., 1998). The extracellular domains of SALMs contain several functional regions of potential protein–protein interaction, including six highly conserved LRR regions, a FN3 domain, and an IgC2-like domain. Recently, Seabold et al. (2008) showed that application of polyclonal antibodies that bind to the LRR of SALMs (anti-LRR) inhibited SALM4 *trans* interactions in transfected heterologous cells. Directed to amino acids 38–297 of SALM2 (a region of high similarity among the SALMs), anti-LRR has been shown to interact with the extracellular domains of SALMs expressed in heterologous cells (Seabold et al., 2008). To test the ability of anti-LRR application to inhibit SALM-mediated outgrowth, DIV4 primary hippocampal neurons were transfected with GFP and treated by bath application with anti-LRR. To control for variability due to the antibody application technique, parallel cultures were treated with antibodies directed to the intracellular C-terminus of SALM4 (anti-S4CT). Neurons were fixed and analyzed for neurite outgrowth 48 h after antibody application. Representative examples of treated neurons are shown in Fig. 4A. Anti-LRR-treated neurons showed a significant reduction in total outgrowth, mean process length, and neurite branches (Figs. 4B–D). There was no significant change in the number of processes (Fig. 4E). While these results are in accordance with increases in neurite outgrowth and branches promoted by the over-expression of SALMs, we cannot rule out the possibility that anti-LRR antibodies interact with a variety of other endogenous proteins present on the surface of the neurons which have LRR domains, and that these interactions contribute to the changes in neurite outgrowth.

RNAi knock-down of SALM expression reduces neurite outgrowth

To further examine the function of SALMs in mediating neurite outgrowth, individual RNAi plasmid constructs were generated for