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Postsynaptic neuroligin enhances presynaptic inputs at neuronal nicotinic synapses

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Abstract

Neuroligins are cell adhesion molecules that interact with neurexins on adjacent cells to promote glutamatergic and GABAergic synapse formation in culture. We show here that neuroligin enhances nicotinic synapses on neurons in culture, increasing synaptic input. When neuroligin is overexpressed in neurons, the extracellular domain induces presynaptic specializations in adjacent cholinergic neurons as visualized by SV2 puncta. The intracellular domain is required to translate the SV2 puncta into synaptic input as reflected by increases in the frequency of spontaneous mini-synaptic currents. The PDZ-binding motif of neuroligin is not needed for these effects. Together, the extracellular and proximal intracellular domains of neuroligin are sufficient to induce presynaptic specializations, align them over postsynaptic receptor clusters, and increase synaptic function. Manipulation of endogenous neuroligin with β-neurexin-expressing cells confirms its presence; repressing function with dominant negative constructs and inhibitory shRNA shows that endogenous neuroligin helps confer functionality on existing nicotinic synaptic contacts. Endogenous neuroligin does not appear to be required, however, for initial formation of the contacts, suggesting that other components under these conditions can also initiate synapse formation. The results indicate that postsynaptic neuroligin is important for functional nicotinic synapses on neurons and that the effects achieved will likely depend on neuroligin levels.

Keywords: Neuroligin; Neurexin; Nicotinic; Receptor; Synapse formation; Ciliary ganglion; PDZ

Introduction

The formation and maturation of synapses are elaborate processes requiring assembly, alignment, and stabilization of pre- and postsynaptic elements. The neuroligin (NL) family of cell adhesion molecules plays a key organizing role. The cytoplasmic domain of NL localizes with glutamate receptors in the postsynaptic cell while the extracellular acetylcholinesterase-like domain of NL interacts with neurexins (NRXs) on the presynaptic axon to form an intercellular link (Ichtchenko et al., 1995; Scheiffele et al., 2000; Dean et al., 2003; Boucard et al., 2005; Craig and Kang, 2007). NLs also have a PDZ-binding motif, enabling them to interact with PSD-95 and further facilitate assembly of postsynaptic components (Irie et al., 1997; Graf et al., 2004; Nam and Chen, 2005).

NL-1 and -3 enhance glutamatergic synapse formation while NL-2 can promote GABAergic synapse formation (Graf et al., 2004; Chih et al., 2005; Chubykin et al., 2005; Levinson et al., 2005). Recent evidence shows that interactions between NL and PSD-95 can influence the ratio of glutamatergic to GABAergic synapses formed. While the PDZ-binding domain of NL recruits PSD-95 to the synapse, it also allows PSD-95 to recruit NL. Increased PSD-95 levels may shift the distribution of neuroligin away from GABAergic and towards glutamatergic contacts, thereby influencing the ratio of excitatory to inhibitory input a cell receives (Graf et al., 2004; Prange et al., 2004; Chih et al., 2005; Levinson et al., 2005; Gerrow et al., 2006). Recent evidence, however, has challenged the view that NL actually initiates synapse formation, and instead suggests that it may act primarily to enhance subsequent maturation of glutamatergic and GABAergic synapses (Varoqueaux et al., 2006).

Nicotinic synapse formation at the vertebrate neuromuscular junction follows different rules from those operative between neurons. Agrin acting through MuSK guides postsynaptic development (Sanes and Lichtman, 2001). No role has been identified for NL. Nicotinic synapses are also found throughout the vertebrate central and autonomic nervous systems where

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they mediate excitatory transmission and influence calcium-dependent events. The roles of cell adhesion molecules at neuronal nicotinic synapses have yet to be defined.

The chick ciliary ganglion (CG) offers an attractive system for examining nicotinic synapse formation on neurons (Dryer, 1994). CG neurons have abundant nicotinic acetylcholine receptors homomeric for α7 subunits (α7-nAChRs) and localize them on somatic spines; they have heteromeric α3-containing receptors (α3*-nAChRs) concentrated in postsynaptic densities (Conroy and Berg, 1995; Williams et al., 1998; Shoop et al., 1999, 2002). Neuronal nAChRs are associated with members of the PSD-95 family (Conroy et al., 2003; Parker et al., 2004), and α7-nAChRs undergo rapid agonist-induced trafficking (Liu et al., 2005). These features recall elements of glutamatergic synapses.

We show here that the CG expresses NL-1 and that NL interactions determine the levels of nicotinic synaptic activity the neurons receive. Overexpression of NL-1 aligns presynaptic elements over nAChR clusters on the neurons and increases synaptic input to them in culture. Both the NL extracellular domain and a short cytoplasmic domain are essential, the former for recognizing NRXs in adjacent neurites and the latter for increasing synaptic function. The PDZ-binding motif is unnecessary for this enhancement. High levels of NL can enhance synapse formation and nAChR clustering even in the absence of PDZ-scaffolds. The lower levels of endogenous NL normally found in the neurons are necessary for normal nicotinic synaptic activity but are not required for the basal number of synaptic contacts seen. The results indicate that NLs can play both organizing and regulatory roles at neuronal nicotinic synapses.

Materials and methods

CG cultures

Dissociated embryonic day (E) 8 CG neurons were maintained in cell culture for 7–10 days on glass coverslips coated with poly-D-lysine, fibronectin, and lysed fibroblasts (Nishi and Berg, 1981; Zhang et al., 1994). Co-cultures of E8 CG neurons and HEK293 cells were prepared by transfecting HEK293 cells and adding them to CG cultures prepared 48 h earlier. The co-cultures were taken for analysis after an additional 48 h. Co-cultures of QT-6 fibroblasts and E8 CG neurons were prepared by transfecting QT-6 cells, fixing with 0.5% paraformaldehyde (PFA) after 48 h to prevent subsequent transfection by GFP, and then adding CG neurons and transfecting the neurons with GFP or GFP–CRIPT using Effectene (Qiagen, Valencia, CA) and culturing an additional 5 days. The cultures were then fixed with 2% PFA and stained for nAChRs or PSD-95 and Flag-tagged proteins.

Fluorescence microscopy

To label surface nAChRs and Flag-tagged proteins, neurons were incubated with the anti-α1/α3/α5 monoclonal antibody (mAb) 35 (Conroy and Berg, 1998) or goat anti-DDDDK (AbCAM, Cambridge, MA) for 15–30 min at 37 °C. After rinsing in Grey's Balanced Salt Solution, the cells were fixed with 2% PFA in 0.1 M sodium phosphate, pH 7.4, for 20 min at room temperature. To label intracellular antigens, appropriate dilutions of rabbit anti-NL (generous gift from Peter Scheiffele, Columbia University), anti-SV2 primary antibody (Developmental Studies Hybridoma Bank, University of Iowa) or anti-PSD-95 (clone K28/43; Upstate Biotechnology, Lake Placid, New York) were incubated overnight at 4 °C in PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) containing 5% normal donkey serum and 0.05% Triton X-100. After washing in PBS, the cells were incubated with appropriate donkey Cy3-, Cy5- or FITC-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). After rinsing, the cells were viewed with a 63×, 1.4 NA objective on a Zeiss Axiovert equipped with CCD camera and digital imaging with Slidebook software (Intelligent Imaging Innovations, Santa Monica, CA). Typically, fields containing both a transfected and an untransfected neuron not contacting other neurons were randomly selected and imaged. The experimenter was blinded to the transfection. For visualization, reconstructed images were generated from *z*-axis stacks of 0.3 μm deconvolved optical sections. Control and experimental images were taken with the same exposure settings and displayed with the same dynamic range of pixel intensities for direct comparison. For quantification of receptor, SV2, or PSD-95 clusters a 2D-projection image of 3 focal planes was constructed from deconvolved images. Image intensities were thresholded, and areas of contact or cell surface were masked for analysis by highlighting the linear contact or perimeter of the cell surface with a drawing tool having a width of 12 pixels. Clusters within the masked area were defined as having at least 5 contiguous pixels with staining intensities at least twice background. Clusters of SV2 were counted manually as being aligned with nAChR clusters if they were within 2 pixels of each other. On average, 18% of the masked cell surface area on both GFP- and NL-transfected neurons was occupied by nAChR clusters (with the cluster size increased to included a 2-pixel annulus to account for the 2 pixel allowance used for determining alignment). Only 1–3% of the same surface area was occupied by SV2 clusters from adjoining neurons. Greater than 60% of the SV2 clusters, however, were aligned with nAChR clusters, indicating an association 3-fold greater than expected from random placement. Transfected HEK293 cells co-cultured with CG neurons were considered positive for multiple SV2 clusters if 3 puncta having more than 5 pixels each were found at the cell surface. Statistical significance was assessed by Student's *t*-test or ANOVA and Dunnett's post test.

RT–PCR

For detection of NL-1, cDNA was reverse-transcribed from E14-15 CG, whole brain, or trunk blood RNA (RNAeasy; Qiagen, Valencia, CA) using a Thermoscript RT–PCR System (Invitrogen Corp., Carlsbad, CA) with oligo $d(T)_{20}$ primers. The cDNAs were then used in PCR with the primers 5′-caaatgaagcacactgacttgg and 5′-ctggttgttgagtgtgaatgg corresponding to sequences in the cytoplasmic domain of the chick *NL-1* gene. The blood cDNA was competent for PCR in that several homer isoforms could be amplified. For RT–PCR cloning of full-length chick NL-1, primers 5′-caaaaattggatgataccaacc-cagtggtg and 5′-ctataccctggttgttgagtgtgaatgg were used; for full-length β-NRX-1 the primers 5′-ataagaatgcggccgcggcctccagcctgggcgctcaccaca and 5′-gctctaga-gatgttgagatcagacatagtactccttatcctt were used. PCR products of the appropriate sizes were subcloned into a pGEM-T easy vector (Promega Corp., Madison, WI) and sequenced. The full-length chick NL-1, cloned by RT–PCR from CG, was identical in sequence to that of rat and mouse NL-1, and contained the two inserts in the extracellular domain found in the mouse variant (Scheiffele et al., 2000). The β-NRX sequence used in these studies lacked the insert in splice site 4 in the LNS domain.

NL and chimeric constructs

All truncated NL and chimeric constructs were prepared by PCR and were sequenced. NL constructs were obtained from a rat NL-1 construct (N-FLAG-NL-1; Comoletti et al., 2003) having a FLAG epitope at the mature amino terminus, a 10-residue linker peptide, and the NL-1 sequence starting at Gln-46. This construct contains the two alternately spliced inserts found in the extracellular domain (Ichtchenko et al., 1996). NL-pdz, NLc54, and NLc14 include residues 46–838, 46–759, and 46–719, respectively. ExNLgpi is the same as construct NLGGPI described by Scheiffele et al. (2000), with gpi designating a glycosylphosphatidylinositol linker site. ExFmsCyNL replaces the entire extracellular and transmembrane sequences of NL with the extracellular and transmembrane domains of the mouse proto-oncogene *fms-c* (M-CSF-1 receptor). ExFmsCyNL-pdz was made by introducing a stop codon five residues before the authentic stop codon in ExFmsCyNL. The full-length chick NL-1 cDNA containing the two alternately spliced inserts in the extracellular domain