ELSEVIER

MCN Molecular and Cellular Neuroscience

www.elsevier.com/locate/ymcne
Mol. Cell. Neurosci. 37 (2008) 781–793

Developmental regulation of GABAergic interneuron branching and synaptic development in the prefrontal cortex by soluble neural cell adhesion molecule

Leann Hinkle Brennaman and Patricia F. Maness *

Department of Biochemistry and Biophysics, and Silvio Conte Center for Schizophrenia Research, University of North Carolina School of Medicine, Chapel Hill, NC 27599, USA

Received 17 October 2007; revised 17 December 2007; accepted 4 January 2008
Available online 17 January 2008

Neural cell adhesion molecule, NCAM, is an important regulator of neuronal process outgrowth and synaptic plasticity. Transgenic mice that overexpress the soluble NCAM extracellular domain (NCAM-EC) have reduced GABAergic inhibitory and excitatory synapses, and altered behavioral phenotypes. Here, we examined the role of dysregulated NCAM shedding, modeled by overexpression of NCAM-EC, on development of GABAergic basket interneurons in the prefrontal cortex. NCAM-EC overexpression disrupted arborization of basket cells during the major period of axon/dendrite growth, resulting in decreased numbers of GAD65- and synaptophysin-positive perisomatic synapses. NCAM-EC transgenic protein interfered with interneuron branching during early postnatal stages when endogenous polysialylated (PSA) NCAM was converted to non-PSA isoforms. In cortical neuron cultures, soluble NCAM-EC acted as a dominant inhibitor of NCAM-dependent neurite branching and outgrowth. These findings suggested that excess soluble NCAM-EC reduces perisomatic innervation of cortical neurons by perturbing axonal/dendritic branching during cortical development.

Keywords: NCAM; Development; Branching; Interneuron; Synapse

Introduction

The neural cell adhesion molecule (NCAM) is a member of the immunoglobulin (Ig)-like superfamily of cell adhesion molecules. NCAM participates in homophilic and heterophilic interactions that induce signal transduction, axon/dendrite outgrowth, and synaptic plasticity (reviewed in (Hinkle and Maness, 2006; Maness and Schachner, 2007)). Through alternative splicing, three major NCAM isoforms of 120, 140 and 180 kDa are generated. NCAM140 and -180 are transmembrane isoforms that differ in the length of their cytoplasmic domain and have roles in axon/dendrite growth and synaptic plasticity, respectively. NCAM120 is linked to the membrane through a GPI anchor and is predominantly found in glia. NCAM-null mutant mice exhibit deficient hippocampal long-term potentiation (LTP, (Cremer et al., 1998; Bukalo et al., 2004; Stoenica et al., 2006)), spatial learning (Cremer et al., 1994; Bukalo et al., 2004), and emotional memory (Stork et al., 1997; Stork et al., 1999), and they display synaptic abnormalities in the hippocampus (Cremer et al., 1997) and at the neuromuscular junction (Polo-Parada et al., 2001). NCAM can be modified by addition of α-2,8 neuraminic acid or polysialic acid (PSA, (Bruses and Rutishauser, 2001)) to the fifth Ig domain by the polysialyltransferases PST and STX (Nakayama et al., 1998; Angata and Fukuda, 2003). Polysialylation of NCAM is most prominent in brain during development (Edelman and Chuong, 1982), and serves to decrease NCAM homophilic binding affinity (Rutishauser et al., 1988; Johnson et al., 2005) to promote axonal/dendritic growth (Ulfig and Chan, 2004; Brocco and Frasch, 2006) and motility (Conchonaud et al., 2007). However, the mechanisms that regulate NCAM during synaptic development are poorly understood.

Recently, NCAM was found to be subject to ectodomain shedding by proteolytic cleavage to release the entire NCAM extracellular region (NCAM-EC) as a soluble fragment (Vawter et al., 2001; Diestel et al., 2005; Hubschmann et al., 2005; Hinkle et al., 2006; Kalus et al., 2006). This cleavage is mediated by proteases with characteristics of ADAM (*a d*isintegrin *a*nd *m*etalloprotease)

Abbreviations: NCAM, neural cell adhesion molecule; NCAM-EC, NCAM extracellular domain; NCAM-EC^{tg}, NCAM-EC transgenic protein; LTP, long-term potentiation; WT, wild-type; SNP, single nucleotide polymorphism; PSA, polysialic acid; PFC, prefrontal cortex; PPI, prepulse inhibition of acoustic startle; EGFP, enhanced green fluorescent protein; PMSF, phenylmethylsulfonylfluoride; HA, hemagglutinin; FBS. fetal bovine serum; ADAM, a disintegrin and metalloprotease.

* Corresponding author. Department of Biochemistry and Biophysics, CB#7260, 505 Mary Ellen Jones Building, University of North Carolina, Chapel Hill, NC 27599, USA.
E-mail address: srclab@med.unc.edu (P.F. Maness).
Available online on ScienceDirect (www.sciencedirect.com).

1044-7431/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.mcn.2008.01.006

family metalloproteases. Cleavage of NCAM decreases NCAM-dependent neurite growth and branching of primary cortical neurons (Hinkle et al., 2006), but promotes neurite growth in hippocampal neuron cultures (Hubschmann et al., 2005; Kalus et al., 2006). An important question is whether the shed NCAM-EC cleavage fragment modulates these neuronal responses *in vivo*.

Abnormally high levels of the soluble cleavage fragment consisting of the entire extracellular region of NCAM (NCAM-EC) have been reported in affected brain regions of individuals with schizophrenia (van Kammen et al., 1998; Vawter, 2000; Vawter et al., 2001), correlating with disease severity (Lyons et al., 1988). Furthermore, PSA-NCAM is decreased in schizophrenic brain (Barbeau et al., 1995), and single nucleotide polymorphisms (SNPs) in the STX gene are associated with schizophrenia in Japanese and Chinese Han subpopulations (Arai et al., 2006; Tao et al., 2007). Recently SNPs in the NCAM gene were found to be associated with neurocognitive impairments such as working memory in a large schizophrenia population (Sullivan et al., 2007). Thus, abnormalities of NCAM expression or regulation could contribute to synaptic alterations that impair cortical function.

A transgenic mouse that overexpresses NCAM-EC from the onset of neuronal differentiation provides a model for investigating the effect of excessive NCAM shedding on synaptic development of cortical neurons. NCAM-EC mice display reduced numbers of GABAergic inhibitory synapses in the adult prefrontal cortex (PFC), and exhibit abnormal behaviors such as reduced prepulse inhibition of acoustic startle (PPI), impaired fear conditioning, and hyperactivity (Pillai-Nair et al., 2005). One type of interneuron affected by NCAM-EC overexpression in the PFC is the GABAergic basket cell, which expresses parvalbumin and forms inhibitory perisomatic synapses at the soma of cortical pyramidal cells (Markram et al., 2004; Lewis et al., 2005). Perisomatic innervation by interneurons modulates output and synchronizes pyramidal neuron groups, which is thought to underlie working memory (Spencer et al., 2003; Wang et al., 2004; Lewis et al., 2005; Huang et al., 2007). The phenotype of NCAM-EC transgenic mice raises the possibility that soluble NCAM-EC may act as a dominant inhibitor of normal NCAM function; however it is not known whether NCAM-EC perturbs developmental NCAM functions, such as axonal/dendritic outgrowth and branching or only affects its mature synaptic role.

To investigate whether interneuron development in the PFC is affected by perturbing NCAM function through overexpression of the soluble NCAM-EC fragment, NCAM-EC mice were intercrossed to a fluorescent reporter strain in which a subclass of basket interneurons express enhanced green fluorescent protein (EGFP) from the GAD67 promoter (GAD67-EGFP) throughout the soma, axons and dendrites (Chattopadhyaya et al., 2004). By analyzing development of perisomatic innervation by basket cell interneurons, we found that overexpression of NCAM-EC perturbed basket cell arborization during early postnatal development of the PFC, consistent with a mechanism in which soluble NCAM-EC inhibits developmental NCAM interactions that regulate outgrowth and branching of interneuronal processes.

Results

Developmental regulation of NCAM expression, polysialylation, and ectodomain shedding

To analyze the developmental regulation of NCAM and its modification by polysialylation, the expression of NCAM isoforms and their polysialylation status were examined in mouse brain at developmental stages that corresponded to major periods of interneuronal differentiation (E18.5-P1), axon/dendrite outgrowth (P1-P10), synaptogenesis/remodeling (P20), and maturation (P40) (Lauder et al., 1986; Vincent et al., 1995; Flames et al., 2004). Immunoblotting with an antibody specific for the polysialylic modification of NCAM (Fig. 1A) showed PSA-NCAM as a broad band of approximately 250 kDa that was present at highest levels in developing brain from E18.5 to P10, decreasing abruptly at P20 and almost undetectable at P40. Immunoblotting with antibodies directed against common determinants in the NCAM intracellular cytoplasmic domain (NCAM-ICD) of both NCAM140 and NCAM180 showed non-polysialylated NCAM isoforms appearing at P5-P10, and persisting from P20 to adult (P40) (Fig. 1A, second panel). Immunoblotting with antibodies directed against the NCAM extracellular domain (NCAM-ECD) revealed two NCAM ectodomain fragments of ~105 and 110 kDa that were present from E18.5 to P10, but undetectable at P20-40 (third panel, Fig. 1A). These fragments were not recognized by PSA antibodies (not shown). The ectodomain fragments were of similar sizes reported for the cleaved NCAM extracellular region, and may result from separate cleavage events (Bock et al., 1987; Nybroe et al., 1989; Krog et al., 1992; Todaro et al., 2004). The mobility of the NCAM-ECD protein fragments further suggested that they represented non-polysialylated fragments of the NCAM ectodomain. The corresponding NCAM-ICD fragments resulting from cleavage of NCAM180 (~85 kDa) and NCAM140 (~30 kDa) were not apparent on immunoblots, and thus may be degraded intracellularly. Glial-associated NCAM120 was seen upon longer exposure times of the blots, and first appeared at P20 with higher levels in adult brain (Fig. 1A, bottom panel). This pattern was consistent with previous reports demonstrating that NCAM120 expression in the cortex begins at P14 and is highest in adult brain (Edelman and Chuong, 1982).

NCAM-EC transgenic mice secrete the transgenic NCAM-EC protein (NCAM-EC^{tg}) at 50% of the total NCAM level in the brain (Pillai-Nair et al., 2005). Previous studies have demonstrated that the strength of the NCAM-NCAM homophilic interaction (K_d ~60 nM) is reduced approximately 5 fold by the addition of PSA (Johnson et al., 2005; Kiselyov et al., 2005). This facilitates axonal and dendritic growth and arborization (Ulfig and Chan, 2004; Brocco and Frasch, 2006) and prevents inappropriate synaptic connections (di Cristo et al., 2007). As development progresses, PSA is down-regulated to allow for synaptogenesis (di Cristo et al., 2007; Gascon et al., 2007). Thus, the inhibitory effects of NCAM-EC^{tg} on inhibitory synapse number (Pillai-Nair et al., 2005) could, in principle, occur if the transgenic fragment was polysialylated. To evaluate this possibility, the expression (left set of panels, Fig. 1B) and polysialylation (right set of panels, Fig. 1B) of NCAM-EC^{tg} was analyzed in developing brain from early postnatal stages to adult. NCAM-EC^{tg} protein contains a hemagglutinin (HA) epitope tag at its C-terminus, which can be detected by anti-HA antibodies. In NCAM-EC brain, NCAM-EC^{tg} protein was present at roughly equivalent levels from P10 to adult as shown by immunoblotting of brain lysates using HA antibodies (Fig. 1B, top left panel). HA-tagged NCAM-EC protein was not detected in WT brain, as expected. To determine if NCAM-EC^{tg} was polysialylated, brain lysates were immunoprecipitated with antibodies to the HA tag, followed by immunoblotting with antibodies to PSA (right set of panels, Fig. 1B). Although the HA antibodies successfully precipitated NCAM-EC^{tg} at all stages (Fig. 1B, top right panel), NCAM-EC^{tg} was not recognized by PSA antibodies at any stage, even with long exposure times (right middle panel). In contrast, full-length PSA-NCAM was present at equivalent levels in WT and NCAM-EC transgenic brain at P10,