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Developmental Regulation of Neural Cell Adhesion Molecule in Human Prefrontal Cortex

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Abstract

Neural cell adhesion molecule (NCAM) is a membrane-bound cell recognition molecule that exerts important functions in normal neurodevelopment including cell migration, neurite outgrowth, axon fasciculation, and synaptic plasticity. Alternative splicing of NCAM mRNA generates three main protein isoforms: NCAM-180, -140, and -120. Ectodomain shedding of NCAM isoforms can produce an extracellular 105–115 kDa soluble NCAM fragment (NCAM-EC) and a smaller intracellular cytoplasmic fragment (NCAM-IC). NCAM also undergoes a unique post-translational modification in brain by the addition of polysialic acid (PSA)-NCAM. Interestingly, both PSA-NCAM and NCAM-EC have been implicated in the pathophysiology of schizophrenia. The developmental expression patterns of the main NCAM isoforms and PSA-NCAM have been described in rodent brain, but no studies have examined NCAM expression across human cortical development. Western blotting was used to quantify NCAM in human postmortem prefrontal cortex in 42 individuals ranging in age from mid-gestation to early adulthood. Each NCAM isoform (NCAM-180, -140, and -120), post-translational modification (PSA-NCAM) and cleavage fragment (NCAM-EC and NCAM-IC) demonstrated developmental regulation in frontal cortex. NCAM-180, -140, and -120, as well as PSA-NCAM, and NCAM-IC all showed strong developmental regulation during fetal and early postnatal ages, consistent with their identified roles in axon growth and plasticity. NCAM-EC demonstrated a more gradual increase from the early postnatal period to reach a plateau by early adolescence, potentially implicating involvement in later developmental processes. In summary, this study implicates the major NCAM isoforms, PSA- NCAM and proteolytically cleaved NCAM in

pre- and postnatal development of the human prefrontal cortex. These data provide new insights on human cortical development and also provide a basis for how altered NCAM signaling during specific developmental intervals could affect synaptic connectivity and circuit formation, and thereby contribute to neurodevelopmental disorders.

Keywords

NCAM; adhesion molecules; synaptic plasticity; neurite outgrowth; adolescence; schizophrenia

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Neural cell adhesion molecule (NCAM) is a membrane-bound cell recognition molecule of the immunoglobulin superfamily. NCAM contributes to normal brain development, with pleiotropic functions that include cell adhesion, cell migration, neurite outgrowth, axon fasciculation and guidance, and synaptic plasticity, as well as learning and memory (Maness and Schachner, 2007). Post-transcriptional modifications of NCAM generate three main isoforms. NCAM-180 and NCAM-140 are transmembrane forms that have long and short cytoplasmic domains, respectively. NCAM-180 is found in postsynaptic densities of mature neurons (Persohn et al., 1989; Fux et al., 2003), while NCAM-140 is expressed in growth cones and axon shafts of developing neurons (Stork et al., 2000). NCAM-120 is anchored to the cell membrane via a glycosylphosphatidylinositol (GPI) linkage and expression is primarily in glia (Noble et al., 1985). However, all NCAM isoforms may support synaptogenesis as synapses preferentially formed on cells expressing any of the 3 isoforms in culture (Dityatev et al., 2004). NCAM-180 and NCAM-140 are both highly expressed in the rodent neocortex during fetal and early postnatal development, and persist into adulthood at lower levels (Chuong and Edelman, 1984; Gennarini et al., 1986; Oltmann-Norden et al., 2007). NCAM-120 is found at low levels in fetal rodent brain but increases in early postnatal development and is expressed at stable levels into adulthood (Gennarini et al., 1986; Brennaman and Maness, 2008).

During embryonic development, each main isoform of NCAM can also undergo post-translational polysialylation by the addition of α-2,8-linked sialic acid residues to the fifth Ig-like domain of NCAM. Polysialic acid (PSA)-NCAM has important functions in axon growth, synaptic plasticity, and synaptogenesis (Bonfanti, 2006). Expression of PSA-NCAM is developmentally regulated in the rodent cortex, with high fetal levels and rapid postnatal downregulation (Seki and Arai, 1991; Bonfanti et al., 1992; Oltmann-Norden et al., 2007; Brennaman and Maness, 2008). PSA-NCAM may also contribute to the pathophysiology of certain neurodevelopmental disorders. For example, PSA-NCAM immunoreactivity is reduced in postmortem hippocampal neurons in schizophrenia, potentially contributing to altered synaptic connectivity (Barbeau et al., 1995). Furthermore, polymorphisms in the promoter of the major sialyltransferase gene, ST8SiaII/STX, essential for NCAM polysialylation, have been associated with schizophrenia (Arai et al., 2006).

Transmembrane NCAM isoforms can undergo ectodomain shedding via a disintegrin and metalloprotease (ADAM) family of proteases, resulting in a 105–115 kDa soluble extracellular domain fragment (NCAM-EC) and a smaller intracellular domain fragment (NCAM-IC) (Vawter et al., 2001; Diestel et al., 2005; Hubschmann et al., 2005; Hinkle et al., 2006; Secher, 2008). Transgenic mice that overexpress NCAM-EC in addition to full-length NCAM demonstrate marked reductions in synaptic terminals in gamma-aminobutyric acid (GABA) ergic interneurons and pyramidal neurons and have deficits in sensory gating and amphetamine-induced locomotor activity (Pillai-Nair et al., 2005). The NCAM-EC mouse has been advanced as a potential model of GABAergic and behavioral deficits in schizophrenia, and elevated levels of NCAM-EC have been reported in the hippocampus and prefrontal cortex in schizophrenia (Vawter et al., 1998).

The pleotropic functions of NCAM are further demonstrated by recent evidence that NCAM mimetic peptides have been found to enhance synaptic plasticity and produce neuroprotective effects in animal models (Berezin and Bock, 2008). For example, FGL – an NCAM-derived fibroblast growth factor receptor agonist – can produce reversal of working memory deficits in phencyclidine (PCP)-treated rat pups, a model of cognitive deficits with relevance to schizophrenia (Secher et al., 2009).

Although the developmental expression patterns of the major NCAM isoforms and PSA-NCAM have been described in rodent brain, we are aware of only one report in human brain in a study of PSA-NCAM in dentate gyrus (Ni Dhuill et al., 1999). The current study measured the main isoforms of NCAM (NCAM-180, -140, -120), as well as PSA-NCAM, NCAM-EC and NCAM-IC proteins in human prefrontal cortex from mid-gestation into early adulthood. Insight into the expression patterns of NCAM protein in human cortex across the first 3 decades of life can inform on diverse developmental processes in human brain including neuronal migration, synaptogenesis, and synaptic plasticity. Identifying the patterns of NCAM protein expression can help identify windows during which NCAM signaling may be vulnerable to developmental perturbation and provide a basis for understanding the pathophysiology of neurodevelopmental disorders such as schizophrenia.

EXPERIMENTAL PROCEDURES

Postmortem samples and tissue preparation

This study was approved by the University of North Carolina School of Medicine Institutional Review Board. Human tissue was obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland. Coronally cut slabs of prefrontal cortex (areas 9/46) from 42 human postmortem brains were frozen at autopsy and stored at −80°C until use. Gross and microscopic examination by a neuropathologist revealed no evidence of central nervous system pathology. Subjects had no known history of neurological or psychiatric illness, including substance abuse. Subjects ranged in age from 18 gestational weeks to 25 years. *A priori*, the subjects were divided into seven age groups: fetal (n=6), 0–12 months (n=4), 1–5 years (n=6), 6–10 years (n=6), 11–15 years (n=9), 16–20 years (n=4), and 21–25 years (n=7). For subject demographics, see Table 1.

Tissue was prepared as described previously (Jarskog and Gilmore, 2000). Briefly, the tissue was homogenized (1:10, wt:vol) for 30 seconds on ice in homogenization buffer (50 mM Tris-HCl buffer (pH 7.4), 0.6 M NaCl, 0.2% Triton X-100, 1 mM benzamidine, 0.1 mM benzethonium chloride, and 0.1 mM PMSF) and then sonicated at 10mV for 10 seconds. This method measures both soluble and membrane-bound NCAM isoforms. Samples were centrifuged for 15 minutes at $15,000 \times g$ and at 4°C and supernatants were assayed for total protein using the bicinchronic acid method (Micro BCA Protein Assay Kit, Pierce Chemical, Rockford, IL).

Western blotting

Samples were separated on 4% (NCAM-180, -140, -120, PSA-NCAM) or 12% (NCAM-IC) 10-well Tris-glycine polyacrylamide gels (NOVEX, San Diego, CA) or 7.5% (NCAM-EC) 15-well Tris-HCl polyacrylamide gels (BioRad, Hercules, CA) by adapting previously established methods (Hochstrasser et al., 1988; Vawter et al., 1998; Jarskog and Gilmore, 2000). Equal amounts of total protein (10 μg for main isoforms and NCAM-EC, 100 μg for PSA-NCAM, and 55μg for NCAM-IC) were loaded on gels with a molecular weight marker (MagicMark XP, Invitrogen) and run at 25°C for 90 minutes at 125V, except for NCAM-EC gels that were run on ice for 35 minutes at 300V. All samples were run in triplicate on separate gels. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes

(Immunobilon-P, Millipore, MA) at 25V for 90 minutes. Complete transfers were verified by membrane staining with Ponceau S (data not shown). Nonspecific protein binding was blocked for 1 hour at room temperature in 5% dry milk. Membranes were incubated with primary antibody overnight at 4°C as follows: rabbit anti-human polyclonal NCAM (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA) for main isoforms and NCAM-EC, mouse anti-meningococcal monoclonal PSA-NCAM (1:200, Chemicon, Temecula, CA), and mouse anti-rat monoclonal NCAM (0B11, 1:250, Sigma, St. Louis, MO) for NCAM-IC in 5% milk/TBST. Membranes were then incubated with secondary antibody for 90 minutes at 25°C as follows: 1:3000 anti-rabbit for main isoforms and NCAM-EC, 1:1000 anti-mouse for PSA-NCAM, and 1:3000 anti-mouse for NCAM-IC in 5% milk/TBST. Membranes were developed by chemiluminescence (ECL, Amersham Pharmacia) and protein bands were detected on radiographic film (Hyperfilm ECL, Amersham Pharmacia). Images were scanned (Epson Expression 1680) into Adobe Photoshop 7.0 (Adobe systems, San Jose, CA) and densitometry was performed using the Bioquant Nova Prime system.

Membranes were stripped using IgG Elution Buffer (Fisher Scientific) and reprobed for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:20000, Imgenex, San Diego, CA). The density of each NCAM isoform was normalized to GAPDH. Separately, increasing amounts of total protein were applied to a set of gels to ascertain that the samples fell within the linear range of the densitometric curve for each protein.

Postmortem stability of NCAM isoforms

Thirteen 150–200g male Sprague Dawley rats (Zivic-Miller, Allison Park, PA) were anaesthetized with ether, decapitated, and separated into two groups. In the first group (n=6), frontal cortex was dissected and frozen immediately at −80°C, while in the second group (n=7) heads were kept at 25°C for 6 hours and then at 4°C for 18 hours before dissection and freezing. This experimental paradigm was used to approximate the postmortem condition for human brain tissue used in the current study since most subjects were brought to the medical examiner and placed in refrigerated storage prior to autopsy and brain tissue was dissected out and frozen within 24 hours after death. All rat cortical tissue was homogenized, sonicated, and Western blots analyzed as described above for human tissue. All primary antibodies recognized NCAM isoforms of rat and human origin.

pH Measurement

pH values for all brain tissue was ≥ 5.8, using previously established methods for pH determination (Romanczyk et al., 2002; Salimi et al., 2008). Briefly, about 250 mg cortical tissue was dissected from each brain sample and pulverized over an isopentane/dry ice slurry. Sterile water was added to the frozen powder (1:10 wt:vol) and homogenized for 10–15 s at 0°C. pH was measured with a Ross Sure Flow pH meter and Orion Thermo Ross Semimer perphect pH electrode.

Statistics

Statistical analysis was performed using GraphPad Prism Version 4.01 (GraphPad Software, San Diego, CA) and SAS version 9.1 (SAS, Cary, NC). Western blot data were analyzed by one-way analysis of variance (ANOVA) across age groups with significance set at $p<0.05$. Post-hoc Tukey's Multiple Comparison Test were performed with two-tailed p-values considered significant at <0.05. In a secondary analysis, NCAM values were regressed with age using linear and linear plus quadratic models with PMI and pH as covariates. The postmortem stability data was analyzed with an unpaired Student's t-test, with two-tailed p-values considered significant at $p<0.05$.

RESULTS

Sample Characteristics

Mean sample pH did not differ across the age groups by ANOVA (F=1.323, df=6, 41, p=0.273), see Table 1. PMI did vary significantly across the age groups by ANOVA (F=9.704, df=6, 41, p<0.0001), see Table 1. Post hoc Tukey's multiple comparison testing showed that the fetal group had lower PMI compared to each of the postnatal groups except for the 0–12 month group. The 0–12 month group also had lower PMI compared to the 6–10 and 11–15 year groups. In order to account for the potential confounding effects of pH and PMI, these variables were used as covariates in our statistical model.

Immunoreactive bands for each of the NCAM isoforms and cleavage fragments were identified by Western blotting in human prefrontal cortex, see Figure 1. Each NCAM protein demonstrated a distinct pattern of developmental regulation from mid-gestation into early adulthood. *A priori,* the brain samples were batched into seven age groups: fetal, 0–12 months, 1–5 years, 6–10 years, 11–15 years, 16–20 years, and 21–25 years. Subjects have been batched into similar age groups in previous studies of human cortical development (Romanczyk et al., 2002;Webster et al., 2002;Glantz et al., 2007). Batching subjects into age groups can help identify changes in the protein of interest during specific neurodevelopmental events such as axo-dendritic growth in the prenatal period, synaptogenesis during the early postnatal period, and synaptic elimination in early to mid adolescence.

In order to quantitate immunoreactive bands across a wide age range, it is important to normalize data to a gene product with stable levels across the same age interval. While both β-actin and α-tubulin bands exhibited substantial age-dependent changes (data not shown), GAPDH immunoreactive bands were remarkably stable from mid-gestation into early adulthood, by ANOVA (F=1.242, df=6, 41, p=0.309), see Figures 1,2. GAPDH was used to normalize data for each of the NCAM proteins.

Intact NCAM Isoforms (NCAM-180, NCAM-140, NCAM-120)

The antibody used for the main NCAM isoforms revealed immunoreactive bands at 180, 140 and 120 kDa molecular weights in nearly every cortical sample, see Figure 1. The highest NCAM-180 expression was found in fetal cortex although robust levels were also found in postnatal cortex through early adulthood, see Figures 1 and 3a. Similar to two other studies of NCAM isoforms in human frontal cortex, one or more weaker bands also appeared below the main 180 kDa band, likely representing heterogeneity of the NCAM-180 isoform (Barbeau et al., 1995;Vawter et al., 1998). Differences in levels of NCAM-180 showed a significant effect of age by ANOVA (F=3.674, df=6, 41, p=0.0062), see Figure 3A. A post hoc Tukey's multiple comparison test showed that the 1–5 year group (p<0.05) and the adult group (p<0.05) had lower NCAM-180 compared to the fetal group. To covary for the potential effects of PMI and tissue pH on these findings, a secondary regression analysis across all individual samples was also performed. A weak negative linear correlation emerged between age and NCAM-180 (F=4.62, p=0.0378, R-Square=0.1035). A similar relationship remained after covarying for PMI and pH (F=2.24, p=0.0989, R-square=0.1505).

The developmental pattern of NCAM-140 was similar to NCAM-180, see Figure 1. The highest levels of NCAM-140 occurred in fetal and early postnatal subjects, although strong immunoreactive bands for NCAM-140 persisted into adulthood. In post hoc testing, NCAM-140 in the 0–12 month group was higher than in each the older age groups and NCAM-140 demonstrated a significant effect of age by ANOVA (F=6.497, df=6, 41, p<0.0001), see Figure 3B. A regression analysis across individual subjects showed a negative

linear correlation between age and NCAM-140 (F=10.42, p=0.0024, R-Square=0.2078) that remained significant after covarying for PMI and pH (F=4.80, p=0.0063, R-square=0.2747).

The pattern of expression of NCAM-120 was distinct from NCAM-180 and NCAM-140 in that NCAM-120 was almost absent in fetal cortex, see Figure 1. Furthermore, NCAM-120 levels were substantially higher in the early postnatal group and remained elevated into adulthood. NCAM-120 demonstrated a significant effect of age by ANOVA (F=5.311, df=6, 41, p=0.0006), see Figure 3C. In post hoc testing, NCAM-120 in the fetal group was lower than in the 0–12 month (p<0.01), 6–10 year (p<0.05), 11–15 year (p<0.001), and 21–25 year (p<0.05) groups. Across individual samples, NCAM-120 showed a positive nonlinear relationship with age (F=5.33, p=0.009, R-Square=0.2146), a relationship that remained significant after covarying for PMI and pH (F=5.31, p=0.0018, R-square=0.3645).

PSA-NCAM

High PSA-NCAM levels were strikingly associated with the earliest stages of cortical development, see Figure 1. Fetal samples were characterized by a broad immunoreactive band at 180 kDa and another strong band around 140 kDa. While the identity of the band at 140 kDa is uncertain, it may reflect differing length sialic acid chains or possibly a cleavage fragment. PSA-NCAM immunoreactive bands in all postnatal samples were much weaker than in the fetal samples. Fetal PSA-NCAM was higher than each of the older age groups (p<0.001), ranging from 5-fold higher compared to the 0–12 month group to greater than 30-fold higher compared to the adult group. PSA-NCAM showed a robust effect of age by ANOVA (F=20.57, df=6, 41, p<0.0001), see Figure 4. Across individual samples, PSA-NCAM showed a negative non-linear relationship with age (F=15.76, p<0.0001, R-Square=0.4470) that remained significant after covarying for PMI and pH (F=9.16, p<0.0001, R-square=0.4976).

Proteolytic Fragments of NCAM (NCAM-EC, NCAM-IC)

NCAM-EC has previously been found to migrate as a broad band in the 105–115 kDa range in human cortex (Vawter et al., 1998). A 105–115 kDa band separated from a 120 kDa band (NCAM-120) in most of the postnatal samples, see Figure 1. NCAM-EC bands were not visualized in most fetal samples, but they were detected with gradually increasing intensity in the infant and childhood samples. NCAM-EC reached a plateau in late childhood and early adolescence. NCAM-EC showed a significant effect of age by ANOVA (F=7.264, df=6, 41, p<0.0001), see Figure 5A. Across all individual samples, NCAM-EC showed a positive, linear correlation with age (F=31.52, p<0.0001, R-Square=0.4407) that remained significant after covarying for PMI and pH (F=11.63, p<0.0001, R-square=0.4787). To explore the relationship between NCAM-EC and the transmembrane NCAM isoforms, linear regression analyses were performed. While visual inspection of the levels of NCAM-180 versus NCAM-EC and NCAM-140 versus NCAM-EC suggested weak negative relationships, the correlations were not significant (NCAM-180 vs NCAM-EC, F=2.22, p=0.144, R-square=0.05254; NCAM-140 vs NCAM-EC, F=1.68, p=0.202, R-square=0.0404), see Figures 5C and 5D.

Immunoblotting for NCAM-IC demonstrated a band migrating to 30 kDa, consistent with the cytoplasmic domain of NCAM-140. Non-specific binding in the 70–80 kDa region prevented resolution of a potential intracellular fragment of NCAM-180. The highest NCAM-IC (30 kDa) levels were seen in the fetal and early postnatal cortex (0–12 month group), after which NCAM-IC was uniformly low. NCAM-IC in the 0–12 month group was significantly higher than each of the older groups in posthoc testing. NCAM-IC demonstrated a significant effect of age by ANOVA (F=8.529, df=6, 41, p<0.0001), see Figure 5B. Across individual samples, NCAM-IC showed a negative, non-linear relationship with age (F=9.85, p=0.0003, R-Square=0.3356) that remained significant after covarying for PMI and pH (F=7.17, p=0.0002, R-square=0.4368).

Postmortem Stability

Each of the NCAM proteins revealed good postmortem stability in a rat model. In this model, animals were sacrificed and frontal cortices were either dissected and frozen immediately or animals were kept at 25°C for 6 hours and then at 4°C for 18 hours before dissection and freezing. This paradigm modeled the typical postmortem course of many of the human subjects in this collection. Since none of the NCAM species showed a statistically significant change after a 24 hour PMI, it reduces the likelihood that the findings are significantly confounded by non-specific protein degradation of NCAM species. The numerical percentage changes and associated p values at 24 hours PMI compared to baseline were as follows: NCAM-180 (−25%, p=0.191), NCAM-140 (−1%, p=0.840), NCAM-120 (−17%, p=0.539), NCAM-EC (+18%, p=0.783), NCAM-IC (−15%, p=0.211), and PSA-NCAM (−12%, p=0.597).

DISCUSSION

This study is the first to examine major NCAM isoforms and proteolytic cleavage products in human cortex across development. The study demonstrated that NCAM-180, NCAM-140, NCAM-120, PSA-NCAM, and transmembrane-cleaved NCAM fragments are developmentally regulated from mid-gestation into early adulthood in human prefrontal cortex, see Figure 6 for summary comparison. These data represent the first analysis of NCAM in human prefrontal cortex across development, and suggest important roles for NCAM in both early and later stages of development.

Expression of NCAM-180 and -140 isoforms were highest in fetal and infant cortex but each of the postnatal age groups showed substantial immunoreactivity into adulthood. This pattern is in general agreement with the developmental expression of NCAM-180 and -140 seen in rodent cortex (Chuong and Edelman, 1984). High level of NCAM-140 in early life is consistent with its localization to axon shafts and migratory growth cones of developing neurons and its role in regulating neurite outgrowth (Persohn et al., 1989; Stork et al., 2000). In contrast, NCAM-180 levels declined somewhat less than NCAM-140 across development, see Figure 3. The pattern of persistent NCAM-180 expression into adulthood is consistent with evidence that it is enriched in postsynaptic densities and contributes to synaptic plasticity, especially in mature neurons (Tomasiewicz et al., 1993; Wood et al., 1998; Dityatev et al., 2000). Notably, NCAM-180 expression appeared to decrease across adolescence into early adulthood. Given that NCAM-180 is enriched in postsynaptic densities, this developmental pattern across adolescence might in part reflect large-scale synaptic elimination as has been demonstrated in adolescent prefrontal cortex in both human (Huttenlocher and Dabholkar, 1997; Glantz et al., 2007) and non-human primate brains (Rakic et al., 1986; Anderson et al., 1995).

NCAM-120 was also developmentally regulated in the prefrontal cortex; however, its expression was virtually absent in fetal cortex followed by a robust increase in the infant cortex that remained elevated into adulthood. These data are corroborated by studies of NCAM-120 in mouse cortex and are unlikely to be the consequence of postmortem degradation. In mouse cortex, NCAM-120 is not expressed at high levels until postnatal development and its expression appears to be limited to cells of glial origin (Noble et al., 1985; Gennarini et al., 1986). Given that gliogenesis in human cortex is most active in the second half of gestation (Mrzljak et al., 1992; de Graaf-Peters and Hadders-Algra, 2006) and that the fetal samples in the current study were slightly under 20 gestational weeks, absence of NCAM-120 in the fetal group but presence in the earliest postnatal samples is consistent with data from mouse studies. The findings also suggest an important role for NCAM-120 in the adult cortex.

Each of the 3 main isoforms of NCAM can be post-translationally modified by PSA. Polysialylation of NCAM decreases NCAM-mediated cell adhesion, thereby promoting axon outgrowth and cell migration (Bruses and Rutishauser, 2001). This effect occurs via direct

steric inhibition of membrane-membrane apposition (Fujimoto et al., 2001). In the current study, PSA-immunoreactivity was observed in two broad bands that was highest in fetal cortex and then declined sharply after birth with stable low expression into adulthood. In fact, the subjects that were older than 3 weeks of age had PSA-NCAM expression that was comparable to adult levels, suggesting that postnatal downregulation of PSA-NCAM occurs rapidly during a narrow perinatal window preceding the onset of large-scale cortical synaptogenesis. The mechanism(s) that regulate PSA-NCAM levels across development are not currently understood.

NCAM-EC demonstrated a relatively slow and progressive increase starting in infancy and reaching a plateau in early adolescence, indicating that the shedding process is also developmentally regulated. While speculative, the apparent inverse relationship between NCAM-EC and both NCAM-140 and NCAM-180 suggests that these transmembrane isoforms could contribute to NCAM-EC in human cortex. In support of this hypothesis, synaptosomal preparations from human occipital cortex demonstrated an inverse relationship between NCAM-180 and NCAM-EC levels (Vawter et al., 2001). Likewise, studies of fibroblastoid cell lines transfected with individual NCAM isoforms have demonstrated that metalloproteinase-inducible shedding is associated with lower levels of full-length NCAM isoforms and higher levels of NCAM-EC (Diestel et al., 2005; Hubschmann et al., 2005; Hinkle et al., 2006). Future studies of ADAM protease expression and activity in human cortical development could further our understanding of NCAM-EC regulation.

Functionally, the importance of regulating NCAM-EC levels is demonstrated in transgenic mice that overexpress NCAM-EC, in which cortical neurons display reduced neurite outgrowth and branching and apical dendritic spines are reduced (Pillai-Nair et al., 2005; Brennaman and Maness, 2008), indicating that excessive shedding prevents normal neuronal architecture. NCAM-EC transgenic mice also show reduced GABAergic and excitatory synaptic contacts and associated sensory gating deficits (Pillai-Nair et al., 2005), suggesting an important role for NCAM-EC in synapse development as well. Since NCAM-EC appears to have a dominant negative effect on synapse development, the evidence for low NCAM-EC levels in infant cortex is consistent with large-scale cortical synpatogenesis during the first year of life. The subsequent rise in NCAM-EC levels may contribute to regulating synaptic formation in later development and the persistence of NCAM-EC into adulthood suggests it may exert an important function in synaptic maintenance and stability. One study has found changes in NCAM-EC levels in schizophrenia, with elevated levels in frontal cortex and hippocampus (Vawter et al., 1998). The current findings lend support to the hypothesis that changes in normal developmental regulation of NCAM-EC shedding represents a potential mechanism that could contribute to altered synaptic connectivity in schizophrenia.

This study demonstrated that NCAM-IC (30 kDa) is also developmentally regulated in human frontal cortex, with high levels in fetal and early postnatal period and then decreasing to a stable plateau after about one year of age. Early development may represent a window in which ectodomain shedding begins to yield NCAM-IC, implicating cleavage in the development of normal cortical cytoarchitecture. Support for this notion comes from cell culture studies which have demonstrated that NCAM-IC (30 kDa) competitively interacts with the non-receptor tyrosine kinase p59^{fyn} to inhibit neurite outgrowth and cell migration (Prag et al., 2002; Buttner et al., 2004), while the 80 kDa fragment competes with NCAM-180 for spectrin to promote neurite outgrowth (Buttner et al., 2004). On the other hand, shedding downregulates PSA-NCAM from the cell surface, enabling interactions of noncleaved NCAM with heterophilic or homophilic receptors, and other molecules that promote synaptogenesis. Somewhat paradoxically, our study found a weak inverse relationship between NCAM-IC and NCAM-EC levels. One possibility is that the main source of NCAM-EC could shift from NCAM-140 to another NCAM isoform across development. This could yield progressively less NCAM-

IC (30 kDa), potentially generating an ~80 kDa NCAM-IC fragment derived from NCAM180 that we were unable to resolve, even as NCAM-EC remains elevated. Alternatively, a secreted isoform of NCAM (NCAM-SEC, 108–115 kDa) may not be readily distinguishable from NCAM-EC (Poltorak et al., 1996; Vawter et al., 1999), and could contribute to higher NCAM-EC levels across development. Differential clearance rates of NCAM-EC and NCAM-IC could also contribute to their apparent inverse relationship.

A number of potentially confounding variables were considered in this study. First, mean PMI differed across the groups by ANOVA, due primarily to the short PMI in the fetal group. However, mean tissue pH did not vary across the age groups and evidence suggests that pH is a better predictor than PMI of protein and mRNA stability in postmortem brain tissue (Harrison et al., 1995). Several methods were used to assess the impact of PMI and pH on the results. Applying linear and quadratic regression analysis to each NCAM protein and then covarying for PMI and pH demonstrated that neither PMI nor pH had a significant effect on the NCAM findings. To further examine the potential impact of PMI on NCAM, a postmortem stability study was performed using a rat model to approximate the human postmortem condition. None of the NCAM isoforms or cleavage products showed significant changes over a 24 hour PMI, indicating that the results were not likely to have been influenced by postmortem degradation. Second, the distribution of subject ages was uneven within each of the age groups. For example, the fetal group was represented solely by mid-second trimester subjects, while the 0–12 month group had primarily neonates. In a future study, more subjects and a more even distribution of subjects for targeted age intervals may help to more precisely identify the inflection points of NCAM regulation. Other potential confounds include undiagnosed physical and psychiatric disorders (including substance abuse), dietary issues and effects of medications that subjects could have been taking.

In conclusion, each of the NCAM proteins and cleavage products studied – NCAM-180, NCAM-140, NCAM-120, PSA-NCAM, NCAM-EC and NCAM-IC – were developmentally regulated in human frontal cortex. For the main isoforms (NCAM-180, -140, and -120) and PSA-NCAM, the developmental patterns in human cortex were similar to patterns found in rodent studies. Most up- or down-regulation occurred across the fetal and early postnatal ages, but NCAM-EC also demonstrated modest regulation across adolescence. These results support the hypothesis that NCAM exerts important roles in axonal/dendritic growth and synaptic plasticity in early human cortical development. Beyond informing on normal developmental processes, these data suggest developmental age ranges during which NCAM signaling might be particularly susceptible to perturbation and, in turn, contribute to altered neurodevelopment. The specific mechanisms that govern the developmental trajectories of each NCAM isoform remain unknown but are likely to be highly complex and dependent upon cell- and location-specific factors. Immunohistochemical approaches will be used in future studies to localize NCAM isoform expression to specific cell types to further enhance our understanding of the role of NCAM signaling in normal and altered neurodevelopment.

Acknowledgments

Abbreviations

NCAM

neural cell adhesion molecule

PSA

polysialic acid

ADAM

a disintegrin and metalloprotease

NCAM-EC

extracellular cleaved NCAM

NCAM-IC

intracellular cleaved NCAM

TBST

Tris-buffered saline Tween-20

PVDF

polyvinylidene difluoride

GAPDH

glyceraldehyde-3-phosphate dehydrogenase

ANOVA

one-way analysis of variance

PMI

postmortem interval

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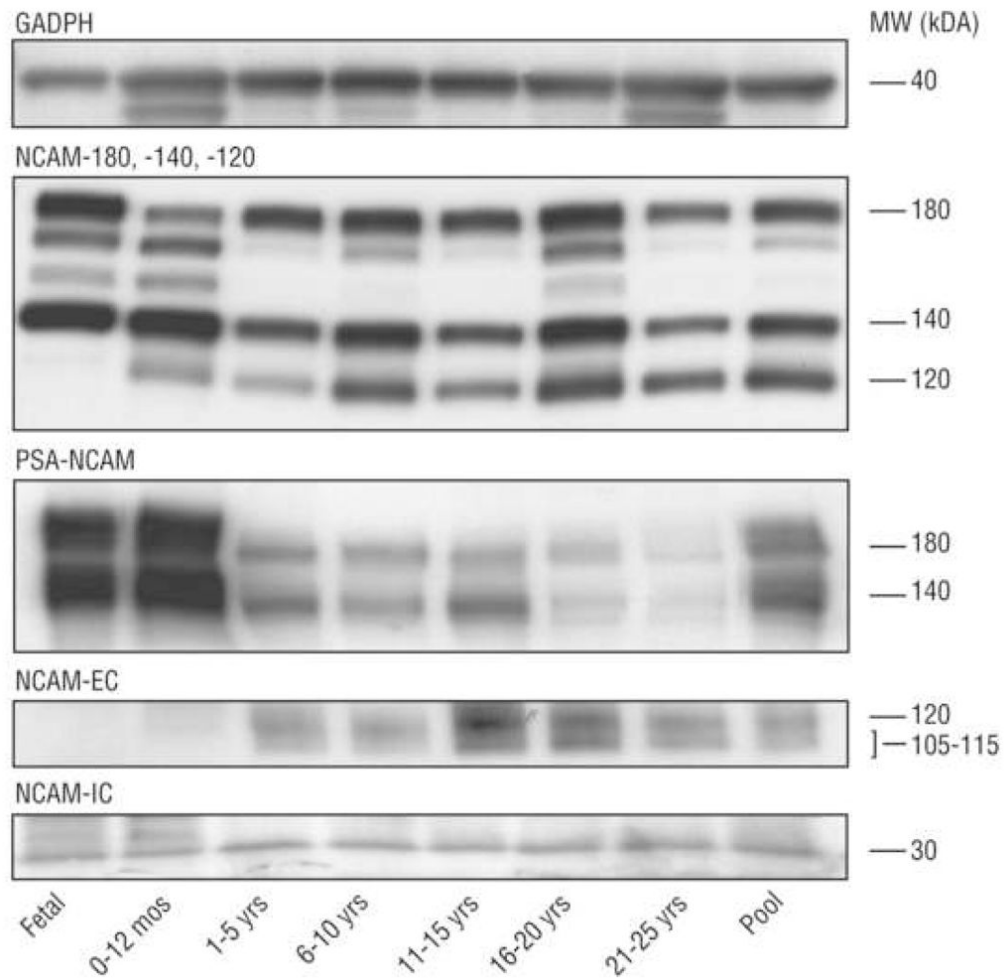
Figure 1.
Representative Western blots of GAPDH, NCAM-180, -140, -120, PSA-NCAM (polysialic acid-NCAM), NCAM-EC (extracellular cleaved NCAM), and NCAM-IC (intracellular cleaved NCAM) in human prefrontal cortex (areas 9/46) across development. An equal amount of total protein homogenate was loaded to each lane for each respective protein. Lanes 1–7 contained one subject from each of the following age groups: fetal, 0–12 mos (months), 1–5 yrs (years), 6–10 yrs, 11–15 yrs, 16–20 yrs, and 21–25 yrs; lane 8 contained a pooled sample applied to all gels. Protein bands are marked with molecular weights (MW) in kilodaltons (kDa). The NCAM-180, -140, -120 gel shows immunoreactive bands at 180, 140 and 120 kDa at all ages except in the fetal group for NCAM-120. Consistent with other studies of NCAM in human brain, several weaker bands are also seen between 180 and 140 kDa, likely representing isoforms of NCAM-180. The NCAM-EC gel shows the 105–115 kDa band representing NCAM-EC and a band at 120 kDa representing NCAM-120 in most postnatal samples.
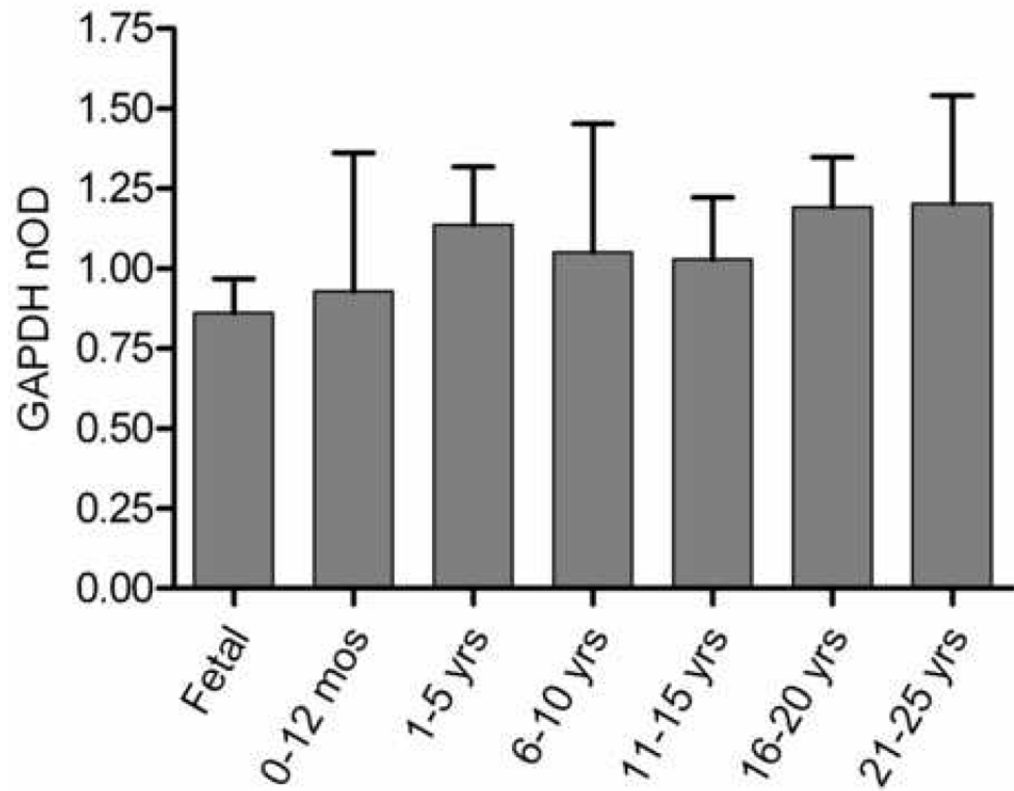
Figure 2.
GAPDH in prefrontal cortex did not demonstrate an effect of age by ANOVA (F=1.242, df=6, 41, p=0.309) from mid-gestation through early adulthood by Western blot. Error bars reflect standard deviations.
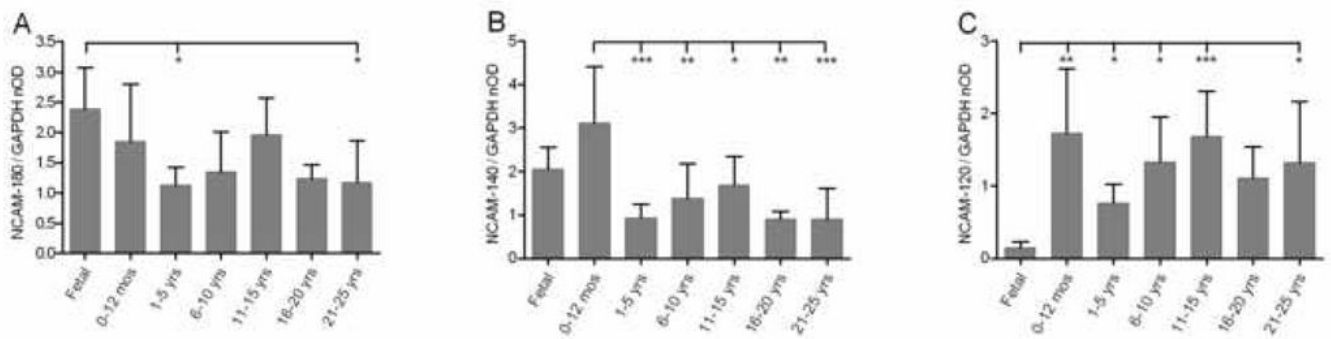
Figure 3.
NCAM-180, -140, and -120 protein(GAPDH normalized) measured by Western blot in human prefrontal cortex spanning from fetal to early adult ages. Post hoc Tukey's multiple comparison testing demonstrated changes at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) significance levels, as indicated. (A) NCAM-180 showed a significant effect of age by ANOVA (F=3.674, df=6, 41, p=0.0062). NCAM-180 was higher in the fetal group compared to the 1–5 and 21–25 year groups (*). (B) NCAM-140 demonstrated an effect of age by ANOVA (F=6.497, df=6, 41, p<0.0001). Compared to the 0–12 months (mos) age group, NCAM-140 was lower in the 11–15 year (*), 6–10 and 16–20 year (**), and in the 1–5 and 21–25 year (***) groups. (C) NCAM-120 demonstrated an effect of age by ANOVA (F=5.311, df=6, 41, p=0.0006). NCAM-120 in the fetal group was lower than in the 6–10 and 21–25 year (*), 0–12 mos (**) and 11–15 year (***) groups.

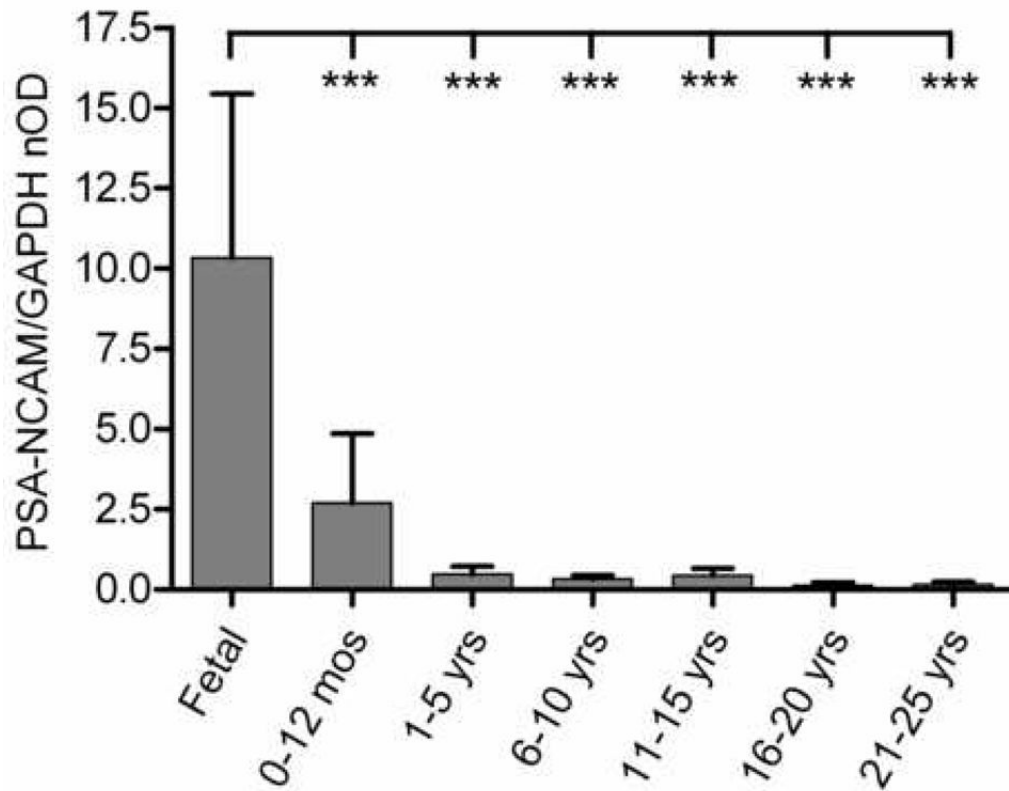**Figure 4.**
PSA-NCAM (polysialic acid-NCAM) in prefrontal cortex showed a strong effect of age by ANOVA (F=20.57, df=6, 41, p<0.0001) as measured by Western blot (GAPDH normalized). Fetal PSA-NCAM was from 5- to 30-fold higher compared to each of the postnatal groups (***p<0.001).
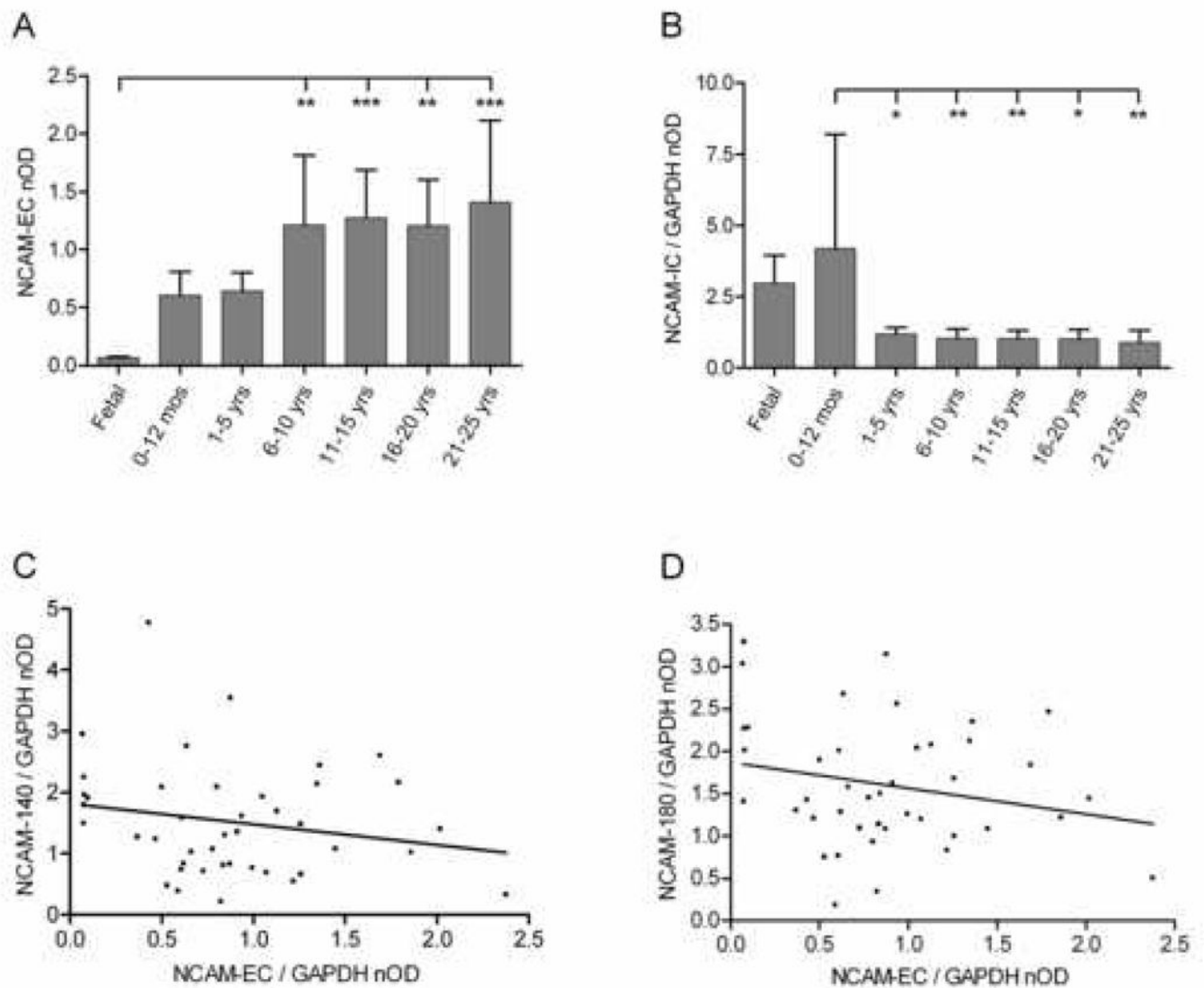
Figure 5.
(A) NCAM-EC (extracellular cleaved NCAM) in prefrontal cortex showed an effect of age by ANOVA (F=7.264, df=6, 41, p<0.0001) as measured by Western blot (GAPDH normalized). NCAM-EC in the fetal samples was lower compared to the 16–20 year (*p<0.05) and 6–10, 11–15, and 21–25 year (***p<0.001) groups. (B) NCAM-IC (intracellular cleaved NCAM) in prefrontal cortex showed an effect of age by ANOVA (F=8.529, df=6, 41, p<0.0001) by Western blot (GAPDH normalized). NCAM-IC in the 0–12 mos (months) group was higher than in each of the older age groups (***p<0.001). (C) Scatterplot of NCAM-140 versus NCAM-EC in all cortical samples. A linear regression analysis between the two variables was not statistically significant (F=1.68, p=0.202, R-square=0.0404). (D) Scatterplot of NCAM-180 versus NCAM-EC in all cortical samples. A linear regression analysis between the two variables was not statistically significant (F=2.22, p=0.144, R-square=0.05254).
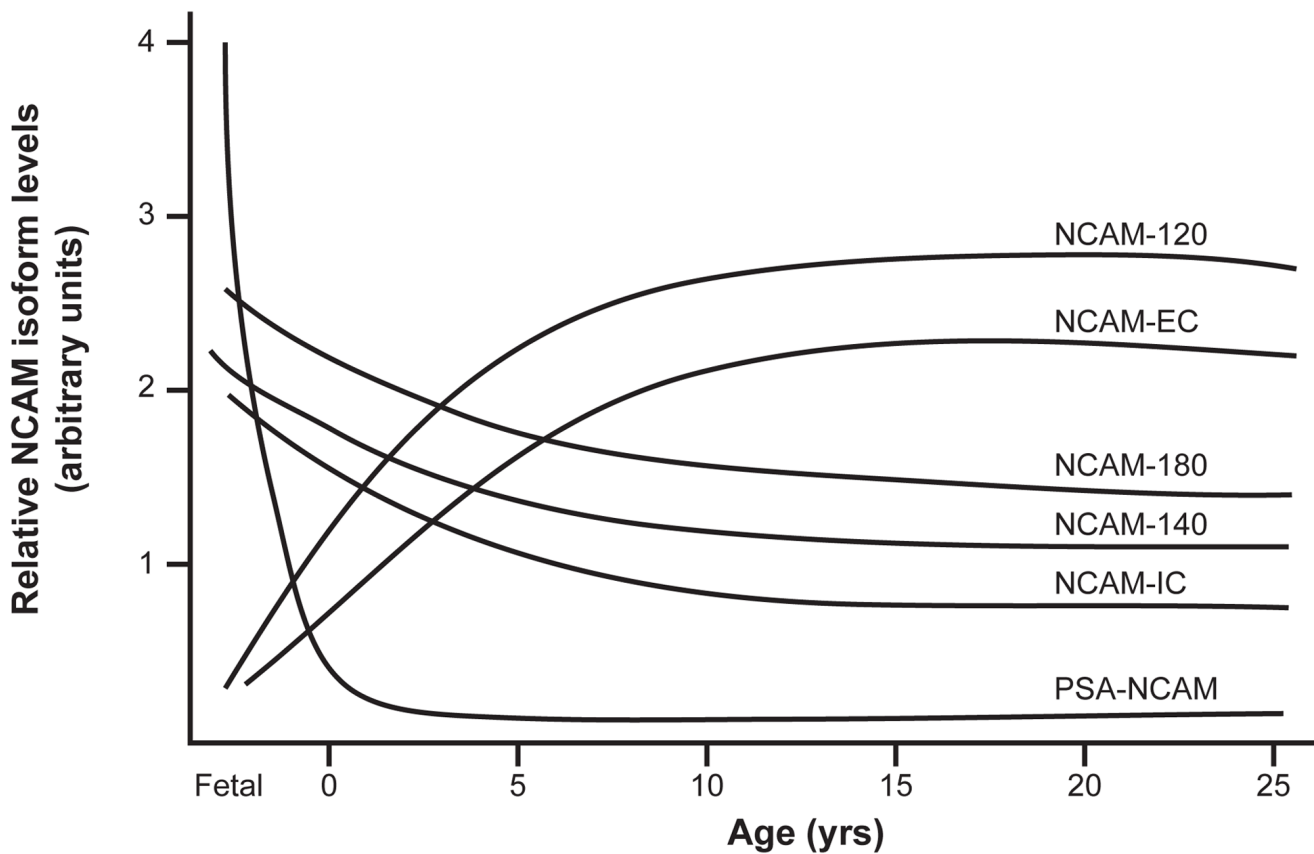
Figure 6.
A summary figure of normalized NCAM species in human cortical development. These smoothed lines allow for a qualitative comparison of changes in each of the normalized NCAM species relative to each other from mid-gestation into early adulthood.

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Table 1

Demographic information

Age Group	Subject #	Gender	Ethnicity	Age	PMI (hrs)^a	pH^b	Cause of Death
Fetal	1	M	AA	18(GA,wks)	1.0	6.5	Elective abortion
	2	F	AA	18.0	1.0	6.1	Elective abortion
	3	F	AA	19.0	1.0	6.0	Elective abortion
	4	M	C	19.0	1.0	6.0	Elective abortion
	5	M	AA	19.0	1.0	6.2	Elective abortion
	6	F	AA	19.0	1.0	6.0	Elective abortion
Mean				18.7 ± 0.5	1.0 ± 0.0	6.1 ± 0.2	
Infant	7	M	AA	0.01(yrs)	5.0	6.3	Heart defect
(0–12 months)	8	M	AA	0.05	9.0	6.8	Asphyxia, probable SIDS
	9	M	C	0.1	7.0	6.2	Idiopathic pulmonary hemorrhage
	10	F	C	0.3	11.0	5.8	SIDS
Mean				0.12 ± 0.13	8.0 ± 2.6	6.3 ± 0.4	
1–5 years	11	M	AA	1.7	10.0	5.9	Asthma
	12	F	AA	2.2	21.0	6.0	Acute myocarditis
	13	F	AA	2.8	12.0	6.1	Drowning
	14	F	AA	3.3	11.0	6.5	Drowning
	15	M	C	4.7	17.0	5.8	Drowning
	16	F	C	5.9	20.0	6.3	Drowning
Mean				3.4 ± 1.6	15.2 ± 4.8	6.1 ± 0.3	
6–10 years	17	M	C	6.9	18.0	6.3	MVA, multiple injuries
	18	F	C	8.0	5.0	6.4	Cardiac arrhythmia
	19	F	AA	8.1	20.0	6.4	MVA, multiple injuries
	20	F	AA	8.6	20.0	6.5	Allograft transplantation rejection
	21	M	C	8.9	36.0	5.8	Hypothermia and drowning
	22	F	AA	9.1	20.0	6.5	Asthma
Mean				8.3 ± 0.8	19.8 ± 9.8	6.3 ± 0.3	
11–15 years	23	M	C	11.6	25.0	6.7	MVA, multiple injuries
	24	M	AA	11.7	19.0	6.0	Drowning
	25	M	AA	12.5	22.0	6.2	Cardiac arrhythmia
	26	F	C	13.0	18.0	6.7	Accident, multiple injuries

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Age Group	Subject #	Gender	Ethnicity	Age	PMI (hrs)[a]	pH[b]	Cause of Death
	27	M	AA	13.3	28.0	6.1	Fire, thermal injuries
	28	F	AA	13.4	17.0	6.8	Asthma
	29	M	C	13.7	18.0	6.3	MVA, multiple injuries
	30	M	C	14.2	16.0	6.5	Cardiac arrhythmia
	31	M	C	15.2	20.0	6.5	Drowning
Mean				13.2 ± 1.1	20.3 ± 4.0	6.4 ± 0.3	
16–20 years	32	M	AA	16.5	21.0	6.0	Cardiac arrhythmia
	33	M	AA	17.2	14.0	6.3	Congenital heart defect
	34	F	AA	18.4	16.0	6.1	Asthma
	35	M	C	20.4	8.0	6.1	Gunshot wound to chest
Mean				18.1 ± 1.7	14.8 ± 5.4	6.1 ± 0.1	
21–25 years	36	M	AA	21.0	13.0	6.2	Drowning
	37	F	AA	21.5	22.0	6.1	Asthma
	38	M	AA	22.8	12.0	5.8	Cardiac arrhythmia
	39	M	AA	23.0	8.0	6.5	Cardiomyopathy
	40	M	C	23.3	21.0	6.3	Drowning
	41	M	C	25.0	8.0	6.2	MVA, compressional asphyxia
	42	M	AA	25.4	19.0	6.4	Asthma
Mean				23.1 ± 1.6	14.7 ± 5.9	6.2 ± 0.2	

Group means are presented with standard deviations. M = male, F = female, AA = African American, C = Caucasian, GA = gestational age, SIDS = sudden infant death syndrome, MVA = motor vehicle accident.

[a] Mean PMI differed across age groups by ANOVA (F=9.704, df=6, 41, p<0.0001).

[b] Mean pH did not differ across age groups by ANOVA (F=1.323, df=6, 41, p=0.273).