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Nitric oxide signaling in the development and evolution of language and cognitive circuits

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Abstract

The neocortex underlies not only remarkable motor and sensory capabilities, but also some of our most distinctly human cognitive functions. The emergence of these higher functions during evolution was accompanied by structural changes in the neocortex, including the acquisition of areal specializations such as Broca's speech and language area. The study of these evolutionary mechanisms, which likely involve species-dependent gene expression and function, represents a substantial challenge. These species differences, however, may represent valuable opportunities to understand the molecular underpinnings of neocortical evolution. Here, we discuss nitric oxide signaling as a candidate mechanism in the assembly of neocortical circuits underlying language and higher cognitive functions. This hypothesis was based on the highly specific mid-fetal pattern of nitric oxide synthase 1 (NOS1, previously nNOS) expression in the pyramidal (projection) neurons of two human neocortical areas respectively involved in speech and language, and higher cognition; the frontal operculum (FOP) and the anterior cingulate cortex (ACC). This expression is transiently present during mid-gestation, suggesting that NOS1 may be involved in the development of these areas and the assembly of their neural circuits. As no other gene product is known to exhibit such exquisite spatiotemporal expression, NOS1 represents a remarkable candidate for these functions.

Keywords

Broca's speech and language area; anterior cingulate cortex; neuronal nitric oxide synthase; neural circuit assembly; human fetal brain; neocortex evolution

1. Introduction

The higher cognitive functions that define humans as a unique species, including speech and language, decision making, and emotional and social processing, are centered in the neocortex, which is thought to be an important advance in mammalian evolution

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(Geschwind and Rakic, 2013). The emergence of higher cognitive functions in mammals was accompanied by structural changes that increased neocortical size, connectivity, and complexity, and led to the acquisition of areal specializations including the Broca's and Wernicke's areas for speech and language in the human brain (Keller et al., 2009). Although the molecular mechanisms that underlie these structural changes remain largely unknown, they are likely to involve changes in developmental processes (Rakic, 2009), which are required to coordinate the specification and differentiation of myriad cell types, their precise migratory localization, and their axonal connectivity (O'Leary and Koester, 1993, Rash and Grove, 2006, Molyneaux et al., 2007, Leone et al., 2008, Kwan et al., 2012b). The neurobiological study and modeling of species-dependent mechanisms present a particular challenge, as they are likely to be underlain by differences in gene expression and function. These species differences may preclude the straightforward use of animal models in some cases, but they also represent potential opportunities to reveal the mechanisms most pertinent to human brain evolution.

During 160 million years of mammalian evolution (Lister, 2004, Luo et al., 2011), the overall contribution of novel genes to phenotypic evolution has been hypothesized to be minor (Wray et al., 2003, Bird et al., 2007). Although examples of coding sequence evolution have been reported and their functional significance confirmed in some cases (Enard et al., 2002, Wang and Su, 2004) protein altering changes are constrained by the pleiotropic nature of gene function; specific coding changes may be beneficial to a particular phenotypic trait but severely deleterious to another (Williams, 1957). It has long been posited that evolutionary differences are likely to arise from changes in regulatory sequences that alter gene expression patterns as opposed to coding sequence variations (King and Wilson, 1975). Recent transcriptomic studies have revealed highly specific spatiotemporal patterns of gene expression in human brain regions, especially during development (Johnson et al., 2009, Colantuoni et al., 2011, Kang et al., 2011, Fertuzinhos et al., 2014, Miller et al., 2014, Pletikos et al., 2014). Potential species-dependent gene regulation, therefore, is likely to underlie at least some of the mechanisms that have rapidly evolved during the emergence of the primate neocortex. In a recent example of this possibility, a regulatory element of *GPR56* was found to be required for gene expression in the peri-Sylvian areas of the human neocortex (Bae et al., 2014). Interestingly, this element is functionally conserved in gyrencephalic mammals but absent in non-gyrencephalic mammals. This and other instances of species-difference represent tremendous opportunities for evolutionary studies. Furthermore, expression differences within specific neocortical regions may provide hypotheses on the potential functional consequences of these species-specific mechanisms. In this review, we discuss a newly-identified, species-dependent pattern of gene expression of neuronal nitric oxide synthase 1 (NOS1, previously nNOS) in the mid-fetal human neocortex, specifically within neural circuits involved in speech and language, and cognitive and emotional processing (Kwan et al., 2012a). The potential contribution of NOS1 to the assembly of these key circuits will be discussed in the context of other genes, neurons, and circuits that have been implicated in language and higher cognition.

2. Human mid-fetal neocortical development

The analysis of human developmental expression patterns that may be species-dependent necessitates the examination of the developing human brain. As part of a recent spatio-temporal analysis of the human brain transcriptome, Kang et al (Kang et al., 2011) generated a temporal classification system based on the timing and progression of benchmark developmental and maturation events in the human cerebral cortex, classifying the entire human lifespan from early embryonic development to adult aging into 15 periods. The processes that underlie the assembly of neocortical circuits largely take place during the mid-fetal periods (Fig. 1, periods 4–6, post-conceptional weeks 13–24) (Kang et al., 2011), in which the neocortex undergoes significant molecular, cellular, and anatomical changes critical to the wiring of its neural circuits. At the gross anatomical level, the mid-fetal human brain appears largely lissencephalic, as gyri and sulci are just beginning to form (Chi et al., 1977a). Arealization, the functional and cytoarchitectonic segregation of neocortical regions, is likely to be initiated during this period, as suggested by transcriptomic studies that have revealed early molecular gradients in both the anterior-posterior and medial-lateral axes (Johnson et al., 2009, Kang et al., 2011), which resemble the gradients important for arealization in the rodent neocortex (O’Leary and Koester, 1993, O’Leary and Nakagawa, 2002, Rash and Grove, 2006). Microscopically, the mid-fetal cerebral wall consists of distinct laminae containing neuronal progenitors (i.e. ventricular zone [VZ], inner and outer subventricular zones [iSVZ and oSVZ]), migrating neurons (intermediate zone [IZ]), and postmigratory neurons (subplate [SP] and cortical plate [CP]) (Angevine and Sidman, 1961, Marín-Padilla, 1992, Davis and Temple, 1994, Caviness and Takahashi, 1995). At these ages, the majority of pyramidal projection neurons, the principal neurons of the neocortex, have been generated (Fig. 1) (Rakic, 2002, de Graaf-Peters and Hadders-Algra, 2006). Pyramidal neurons are organized into six horizontal layers and extend axonal projections appropriate of their layer origin, including the long-range axons that connect distal regions within the neocortex (intracortical projections), as well as the neocortex with subcortical structures (corticofugal projections) (DeFelipe and Farinas, 1992, O’Leary and Koester, 1993, McConnell, 1995, Molyneaux et al., 2007). By period 4, or early mid-fetal development, the early-born pyramidal projection neurons have been generated, undergone migration, and settled into the deep cortical layers. The generation of the late-born neurons is partially complete, and many are migrating towards their upper-layer destinations. As newly post-migratory neurons differentiate, they undergo morphologic changes, including the extension and path finding of their axons, and the elaboration of dendrites and maturation of their spines. During these mid-fetal periods, both intracortical (e.g. corpus callosum) and corticofugal (e.g. corticothalamic and corticospinal) tracts are being established (Honig et al., 1996, Huang et al., 2009). Furthermore, the terminals of subcortical systems innervating the neocortex, including the dopaminergic and cholinergic systems and the thalamocortical afferents, also invade the neocortex during mid-gestation (Kostovic and Goldman-Rakic, 1983, Kostovi , 1986, Berger et al., 1991, Verney et al., 1993). During this time, neocortical interneurons originating from the ganglionic eminences are migrating into the neocortex and integrating into their destination cortical layers (Anderson et al., 1997, Markram et al., 2004, Wonders and Anderson, 2006, Fertuzinhos et al., 2009, Miyoshi and Fishell, 2011). These key events precede the peak period of

synaptogenesis and are critical for the hard-wiring of both local cortical circuits and long-range afferent and efferent connectivities. One evolutionarily relevant structure for coordinating these processes is the SP, which is a predominant layer in the mid-fetal cerebral wall containing the early-born pioneering neurons that are the first to achieve morphological maturity and form functional synapses (Molliver et al., 1973, Rakic, 1976, Kostovic and Rakic, 1980, 1990, Allendoerfer and Shatz, 1994, Herrmann et al., 1994, Hoerder-Suabedissen et al., 2013). The SP, a transient layer that persists throughout cortical development, plays critical roles in the migration of CP neurons and the coordination of both efferent and afferent projections, mediating early and transient synaptic activity thought to be important for the establishment of further synapses in the CP. The significant increases in the thickness and complexity of the primate SP compared to that of the rodent are consistent with the profound increases in the complexity of neocortical circuits between these species.

The molecular mechanisms that underlie distinct aspects of neocortical development are being unraveled, mostly through studies of the mouse (O'Leary and Koester, 1993, Rash and Grove, 2006, Molyneaux et al., 2007, Leone et al., 2008, Kwan et al., 2012b). Comparative studies of gene expression patterns suggest that many of the fundamental developmental mechanisms discovered in the mouse are well-conserved in the human brain (Monuki and Walsh, 2001, Abrahams et al., 2007, Clancy et al., 2007, Kwan et al., 2008, Ip et al., 2011, Saito et al., 2011). The relatively few cases of species-dependent gene expression patterns in the mid-fetal human neocortex, therefore, represent remarkable candidates for species differences in neocortical organization and circuitry, and present excellent opportunities to understand the developmental events most relevant to the assembly of human neocortex. Our examination of the mid-fetal neocortex revealed one such potential mechanism involving the protein NOS1.

3. Species-dependent expression of NOS1 in the mid-fetal ACC and FOP

Recently, we identified NOS1 to be expressed in a unique spatiotemporal and neuronal subtype-dependent pattern in the mid-fetal human neocortex. NOS1, an enzyme that mediates the tightly regulated biosynthesis of nitric oxide (NO) (Bredt and Snyder, 1994, Garthwaite, 2008), is well known as a specific marker of interneurons in the neocortex of multiple species (Vruwink et al., 2001). Unexpectedly, in the mid-fetal human neocortex, we found NOS1 to be expressed in a layer- and area-dependent manner in specific subpopulations of pyramidal projection neurons (Kwan et al., 2012a). The pyramidal expression of NOS1 is species-dependent, being present in the primate neocortex and absent from the rodent neocortex, in which NOS1 is restricted to the interneurons. We further found the cortical expression of NOS1 protein to be under post-transcriptional regulation mediated by the RNA-binding protein FMRP (Kwan et al., 2012a). Interestingly, this regulatory mechanism is species-dependent and correlates with the presence of G-quartet-forming sequences present in the *NOS1* mRNA of primate, but not rodent, species. The G-quartets are both necessary and sufficient for FMRP binding of *NOS1* mRNA and FMRP-dependent increase in NOS1 translation, and thus provide a potential mechanistic explanation for the species differences in cortical NOS1 protein expression.

This novel pattern of NOS1 expression in pyramidal cells is significant not only because of the potential evolutionary implications, but also because interneurons and pyramidal neurons, being derived from distinct progenitor lineages, express different molecular markers and use diverse neurotransmitters. The acquisition of NOS1 expression in the primate pyramidal neurons, therefore, is likely to have a functional significance. Perhaps the most intriguing and neurobiologically significant aspect of this NOS1 expression is its areal-specificity, being restricted to two key areas of the fetal neocortex, the frontal and frontoparietal operculum (FOp) and the anterior cingulate cortex (ACC). In the proceeding sections, we will discuss the key implications of these remarkable expression patterns.

3.1 NOS1 Expression in the Frontal Operculum (FOp)

One of the two neocortical areas in which NOS1 is present in pyramidal neurons is the FOp (Kwan et al., 2012a) (Fig. 2). In the mid-fetal FOp, NOS1 is specifically present in L5 neurons, which are organized into alternating minicolumns, and is absent from the upper cortical layers. Consistent with the possibility that L5 NOS1-expressing neurons project corticofugally, they coexpress the transcription factors FEZF2 (previously ZFP312) and BCL11B (previously CTIP2), which are each required for the development of corticofugal projection neurons (Arlotta et al., 2005, Chen et al., 2005a, Chen et al., 2005b, Molyneaux et al., 2005). Furthermore, retrograde tracing of corticofugal projection neurons in the mid-fetal brain by injection of a lipophilic tracer in the internal capsule labeled alternating neuronal columns highly similar to columns composed of NOS1 positive neurons (Kwan et al., 2012a). In addition to this exquisite laminar and cell-type specific pattern, L5 NOS1 expression in the FOp further exhibits remarkable temporal specificity, occurring only transiently within a small mid-fetal window, approximately from 16 to 24 weeks post-conception (Fig. 1), during which neocortical circuits are formed. Starting from late gestation, columnar NOS1 expression in L5 neurons becomes significantly down-regulated and remains absent throughout postnatal life. The functional significance of this highly precise spatiotemporal pattern remains to be explored. Several intriguing aspects of this pattern, however, may provide some insights.

Spatiotemporal expression—The FOp encompasses the fetal anlage of the adult Broca's speech and language area and the orofacial motor cortex. Since its first identification in the late 18th century via lesion studies (Broca, 1865), the Broca's area has been the focus of many investigations of speech and language circuitry (Judas and Cepanec, 2007, Keller et al., 2009). Prior to NOS1, however, no gene product has been found to exhibit selective expression in the developing Broca's area. Therefore, the highly selective expression of NOS1 in this region, specifically within the fetal window during which neural connectivity is established, suggests that it may play a role in the assembly of neuronal circuits underlying speech and language. Furthermore, the specific expression of NOS1 in the corticofugal projection neurons of the FOp suggests an involvement in the motor circuits required to generate speech. Amongst the afferent and efferent connections in Broca's area, many are intracortical, including the arcuate fasciculus that connects the Broca's and Wernicke's areas and is critical for speech patterns and processing (Fridriksson et al., 2013). The expression in L5 NOS1 neurons of markers of corticofugal projection neurons suggests that NOS1-expressing neurons may not be directly involved in these intracortical tracts.

Subcortical projections originating from L5 neurons of the FOp project to a number of brain structures, including the thalamus, caudate and putamen, and tectum (Kusama et al., 1985). Given the laminar location and marker expression of NOS1 neurons, they are likely to participate in these cortical efferent circuits.

Intriguingly, although speech and language functions are heavily biased to the left hemisphere in the adult neocortex, L5 NOS1 columns are present bilaterally during mid-gestation (Kwan et al., 2012a). The timing of the lateralization of speech circuitries and whether it has occurred by mid-gestation are not known. Although structural asymmetries may be present (Chi et al., 1977b), transcriptomic data from mid-to-late fetal development have shown no significant bias between hemispheres (Johnson et al., 2009). The bilateral expression of NOS1 during fetal development is compatible with the possibility of NOS1 involvement in the establishment of early bilateral circuits that are later functionally refined.

Minicolumnar pattern—NOS1-expressing L5 neurons in the FOp are organized into alternating minicolumns. Although the functional significance of cortical minicolumns has not been studied *in vivo*, they have been hypothesized to form a basic organizational unit of neocortical circuitry (Rakic, 1995, Mountcastle, 1997, Buxhoeveden and Casanova, 2002, Peters, 2010). The mid-fetal minicolumns have been proposed to be ontogenetically related to adult minicolumns, and may have formed as a result of glial-guided radial migration during corticogenesis, (Rakic, 1988, Rakic, 1995), although lateral dispersion, possibly regulated by Eph/ephrin signaling, may also contribute (Torii et al., 2009, Dimidschstein et al., 2013). Our data showed that neurons of the same minicolumn have a shared molecular identity (i.e. expression of subcortical projection neuron markers; Fig. 3) and corticofugal connectivity. Positioned in between the columns are potentially migratory corridors containing radial glial fibers and NOS1-negative neurons expressing the intracortical projection neuron marker SATB2 (Fig. 3) (Kwan et al., 2012a). That the majority of SATB2 neurons eventually settle in the upper cortical layers is consistent with the possibility that these NOS1-negative neurons are migratory, enroute to their upper layer destinations. Although the neurobiological function of minicolumns is not readily amenable to empirical investigation, studies have shown that clonally related pyramidal neurons preferentially connect with each other (Yu et al., 2009, Gao et al., 2013a) and have similar stimulus selectivity (Li et al., 2012). Other studies have suggested a role for minicolumns in neurodevelopmental disorders. Columnopathies, structural alterations in minicolumn organization, have been implicated in a range of neurological disorders, some of which involve speech and language impairments (Buxhoeveden and Casanova, 2002, Casanova et al., 2002, Casanova, 2013).

NOS1 neurons may further have some properties in common with an interesting neuronal subtype possibly involved in speech and language circuitry; the mirror neurons. Mirror neurons are active during both the observation and execution of specific goal-directed actions (Rizzolatti and Craighero, 2004). Because this dual function of mirror neurons is likely to form the basis for imitative behavior (Buccino et al., 2004), they have been proposed to contribute to the formation of learned speech patterns, and may have been an evolutionary substrate of communicative capabilities (Rizzolatti and Arbib, 1998). In the adult macaque monkey, mirror neurons, which project corticofugally, are present in area F5,

an area equivalent to the human Broca's speech and language area (Rizzolatti and Craighero, 2004, Kraskov et al., 2009). While direct recording from mirror neurons in humans has yet to be achieved, neuroimaging techniques have implicated a similar circuit in the human brain (Rizzolatti and Craighero, 2004). The shared location and axonal projections between NOS1 neurons and mirror neurons suggests a possible connection. However, as mirror neurons are functionally defined in the adult brain, long after NOS1 expression in FOp L5 neurons have been downregulated, this intriguing possibility is not amenable to being tested empirically.

FOXP2 Coexpression—Another aspect of NOS1 expression that is consistent with a potential role in language circuits is the coexpression of FOXP2 in some NOS1-expressing neurons within L5 minicolumns (Fig. 3). Since the discovery that the homozygous inheritance of a mutation in the *FOXP2* gene results in a severe speech impairment in a consanguineous family (Lai et al., 2001), *FOXP2* has been the focus of many studies of speech circuit development. Consistent with an involvement in human speech, disruption of a single copy of *Foxp2* in mice leads to impaired ultrasonic vocalization, as well as other alterations in cognitive abilities (Shu et al., 2005). Interestingly, studies in non-mammalian species that exhibit learned vocalization behaviors have corroborated the mammalian studies of FOXP2 function. In songbirds, the spatiotemporal expression of FOXP2 correlates with neural development and song learning, being present in brain areas that are critical to bird song (Haesler et al., 2007). Knockdown of *Foxp2* in these areas by RNA interference results in severe impairments in song learning ability (Haesler et al., 2007).

Studies seeking to understand the biological role of FOXP2 have suggested that it may be important in the development of dendritic morphology, synaptic plasticity, and cortico-striatal connectivity (Enard et al., 2009, Vernes et al., 2011). A recent study of the genomic sequences that interact with FOXP2 has further identified genes involved in dendritic and axonal outgrowth, providing additional support that FOXP2 mediates these processes in circuit development (Vernes et al., 2011). At the level of the amino acid sequence, FOXP2 is remarkably well-conserved across species (Campbell et al., 2009, Fisher and Scharff, 2009). Evolutionary studies have suggested that the two amino acids differentiating the human protein from that of the chimpanzee have undergone recent selection during human evolution and may have a functional significance, possibly by mediating changes in the transcription of downstream genes (Enard et al., 2002, Zhang et al., 2002, Konopka et al., 2009). FOXP2 targets further include *SRPX2* and *CNTNAP2*, two genes that have been implicated in disorders that impair speech. *SRPX2* has been shown to play a role in cortical synaptogenesis, and its disruption alters ultrasonic vocalizations in mice (Roll et al., 2006, Sia et al., 2013). *CNTNAP2* has also been shown to play a role in mouse vocalization and behavior (Peñagarikano et al., 2011, Fujita et al., 2012). In humans, mutations in *CNTNAP2* have been identified in language disorders in autism spectrum disorder patients (Alarcón et al., 2008, Bakkaloglu et al., 2008).

The precise location at which the loss of FOXP2 function is most significant for speech impairment remains unknown. FOXP2 is widely expressed in multiple brain regions (Lai et al., 2001, Takahashi et al., 2008). In both the human and the mouse neocortex, FOXP2 is mostly restricted to L6 projections neurons, and is sparsely expressed in L5 (Lai et al., 2001,

Ferland et al., 2003). Unlike NOS1, FOXP2 expression in the neocortex does not exhibit areal preference for the speech and language areas (Lai et al., 2001, Takahashi et al., 2008). Interestingly, FOXP2 has been shown to directly bind to the *NOS1* locus and transcriptionally regulates its expression (Spiteri et al., 2007). Although the significance of this potential relationship between FOXP2 and NOS1 has not been elucidated, the existing data are consistent with the possibility that the L5 columns, within which FOXP2 and NOS1 are coexpressed, may contribute to speech and language circuitry.

3.2 NOS1 Expression in the Anterior Cingulate Cortex (ACC)

In the mid-fetal ACC, a significant number of upper layer (L2/L3) neurons express NOS1 at high levels (Kwan et al., 2012a). Unlike their counterparts in the FOp, however, L5 neurons of the ACC do not express NOS1. Consistent with their upper layer neuron location, NOS1-expressing neurons coexpress SATB2, a transcription factor that is present in intracortical projection neurons and required for their development (Alcamo et al., 2008, Britanova et al., 2008). ACC pyramidal NOS1 expression is also temporally regulated, being present at high levels from mid to late gestation and downregulated postnatally (Kwan et al., 2012a). A small number of upper layer pyramidal neurons in the ACC retain NOS1 expression into the adult ages (Gao et al., 2013b). The significance of pyramidal NOS1 expression in the adult neocortex, and whether its function there parallels its fetal role, remains unexplored. The reduction in pyramidal expression of NOS1 in the ACC in psychiatric disorders, as discussed below, may provide some insights.

The ACC is crucial for the integration of cognitive and emotional processes, encompassing executive, social, and affective functions (Devinsky et al., 1995). The cognitive component of the ACC, anatomically located in the dorsal portion, is connected with both the motor and sensory systems of the neocortex and therefore centrally located in the integration of bottom-up stimuli with top-down commands (Paus et al., 1993, Picard and Strick, 1996, Williams et al., 2004, Crottaz-Herbette and Menon, 2006). The emotional component, which is located in the ventral part of the ACC, is connected with the nucleus accumbens, amygdala, anterior insula, and hypothalamus, and involved in emotion and motivation processing (Bush et al., 2002, Etkin et al., 2011). Much of the knowledge on ACC function has come from functional imaging and electrical studies (van Veen and Carter, 2002, Sheth et al., 2012, Hyman et al., 2013). Based on these functional data, it has been proposed that the ACC is important for performance monitoring, error detection, and action selection, perhaps using the dopaminergic system to reinforce adaptive behavior (Brown and Braver, 2005, Schweimer and Hauber, 2006, Ko et al., 2009).

The areal distribution of NOS1 pyramidal neurons, being highly enriched in the ACC, is somewhat reminiscent of the distribution of von Economo neurons (VENs, also known as spindle neurons) in the adult neocortex (Nimchinsky et al., 1999, Allman et al., 2010). VENs are morphologically identified by their elongated cell soma and highly bipolar morphology, which, in contrast to the elaborate dendritic system of the pyramidal neurons, may enable higher selectivity in information integration by narrowing the dendrite field (Nimchinsky et al., 1999, Watson et al., 2006). The VENs exhibit remarkable areal preference, being present in the highest numbers in the ACC and less numerous in the

frontal insula and dorsolateral prefrontal cortex, and significant species-dependence, being restricted to the great apes, as well as species of cetaceans and elephants (Nimchinsky et al., 1999, Butti et al., 2009, Hakeem et al., 2009, Allman et al., 2010, Stimpson et al., 2011). This species selectivity has led to the hypothesis that VENs may have been an adaptation that enables fast information processing, perhaps important in complex social behaviors (Craig, 2009, Allman et al., 2010). VENs, which are specifically present in neocortical L5, are not likely to overlap with NOS1-expressing neurons in the upper layers of the ACC. However, because of their shared areal localization and overlap in developmental timing, both being present in the late gestational and early post-natal ages, these two groups of neurons may be functionally interconnected (Allman et al., 2010). Furthermore, it is possible for the apical dendrites of the VENs, which extend into the upper layers, to receive NO signaling from the L2/L3 pyramidal neurons. As functional studies in the species that have VENs are prohibitively challenging, the possibility that NOS1 neurons and VENs may have a functional relationship has not yet been experimentally addressed.

The importance of ACC function is notable given the disorders that have been associated with ACC dysfunction. In schizophrenia, for example, pathological studies have identified altered cellular architecture in the ACC (Salgado-Pineda et al., 2014). Furthermore, neural imaging studies identified ACC abnormalities that were reversed with treatment response. Similarly, in major depressive disorders, reduced neuronal density has been found in the ACC (Rajkowska et al., 1999, Rajkowska et al., 2001) and successful psychotherapy was found to correlate with increased activity in the ACC (Mayberg et al., 1997, Quidé et al., 2012).

Interestingly, disrupted NO signaling and NOS1 dysfunction have been implicated in the pathogenesis of several neuropsychiatric disorders. In genetic studies, a hypomorphic polymorphism in *NOS1* was found to confer significantly increased susceptibility to depression, schizophrenia, and attention deficit hyperactivity disorder (Reif et al., 2006, Reif et al., 2009). This *NOS1* hypomorphic allele has also been linked to hypoactivity in the ACC (Reif et al., 2006, Reif et al., 2009), which is consistent with the possibility that reduced nitric oxide signaling can lead to impaired ACC function. A recent genome-wide association study revealed an additional *NOS1* polymorphism to be associated with general psychological distress (Luciano et al., 2012). Examination of post-mortem patient materials have further implicated disrupted NO signaling. In fragile X syndrome, the leading inherited form of intellectual disability, we have found NOS1 expression to be severely reduced in the developing neocortex (Kwan et al., 2012a). Furthermore, a recent study of depressive patient materials revealed significant decreases in NO metabolites in the cerebrospinal fluid and in NOS1 expression in pyramidal neurons of the ACC (Gao et al., 2013b), an area with the highest pyramidal NOS1 expression in the fetal human cortex (Kwan et al., 2012a). Together, these data corroborate the possibility that NOS1 plays a key function in the ACC and that its disruption can contribute to multiple neuropsychiatric disorders that affect higher cognitive abilities.

The spatiotemporal expression pattern of NOS1 is consistent with its possible involvement in the development of neural circuitry related to specific neocortical areas. In addition to direct connections that can be revealed by anatomical studies, recent advances in neural

imaging have facilitated the examination of functional connectivity. Functional connectivity can be inferred by the imaging of resting state coordinated activity (Power et al., 2011), which measures synchronized activation in a task-independent manner. Interestingly, the two neocortical areas with mid-fetal pyramidal NOS1 expression, the FOp and ACC, exhibit coordinated activity and form a cingulo-opercular network (Power et al., 2011). The cingulo-opercular network is one of the predominant default mode networks. In addition to resting-state activity, this network is also activated during speech recognition tasks and perceptual and memory search tasks (Vaden et al., 2013, Sestieri et al., 2014). Interestingly, disruption in the activity of the cingulo-opercular network has been found in schizophrenia (Tu et al., 2012). Although the cingulo-opercular network has not been directly examined in patients with the hypomorphic *NOS1* allele associated with schizophrenia, these data suggest that reduced NOS1 function may contribute to the dysfunction of a default mode network. This possibility would be further consistent, given the spatiotemporal expression of NOS1, with a potential role of NOS1 in the wiring of key neocortical neural circuitry.

4. NOS1 Contribution to Neocortical Development

Because the expression of NOS1 in pyramidal neurons has only recently been discovered, the functional role of NOS1 in these neurons has not been explored. Given the highly specific spatiotemporal pattern of NOS1 expression, it is likely to play a specific role in a subset of pyramidal neurons. The transient nature of NOS1 expression during development and prior to significant neuronal activity is consistent with the possibility that NOS1 contributes to early wiring and synaptogenesis in the neocortex, perhaps mediating functions specifically required to establish opercular and cingulate circuitry.

Due to the ephemeral nature of NO, which has a half-life in the millisecond range (Liu et al., 1998, Tsikas, 2005), its ability to affect biological processes is limited to a small radius from the site of its production. Furthermore, the diffusion of NO is not amenable to regulation and can occur randomly. The expression and sub-cellular localization of NOS1 is, therefore, particularly indicative of the function of NO signaling. This suggests that the species-dependent expression of NOS1 in pyramidal neurons is significant, and likely to have a localized functional effect on neocortical circuits, as NO would be present at sites near pyramidal neurons in the primate neocortex that would have been too far removed from interneurons to have received NO signaling in the rodent neocortex, which lack pyramidal NOS1.

In neurons, NO signaling leads to two major downstream events, the activation of NO-sensitive soluble guanylyl cyclase, which synthesizes the secondary messenger cGMP, and the post-translational modification of proteins via S-nitrosylation (Jaffrey et al., 2001, Garthwaite, 2008). Given that pyramidal NOS1 expression has remained virtually unstudied, the signaling events downstream have also yet to be explored.

Significant synaptogenesis in the human neocortex starts during mid-gestation (Fig. 1) (Molliver et al., 1973). Consistent with the possibility of NOS1 involvement in synaptogenesis, a small number of presynaptic synaptophysin puncta encircle the cell membrane of FOp NOS1 neurons (Kwan et al., 2012a). Despite these apparent early

contacts, electron microscopy revealed that the synapses present in the CP at these ages are largely immature (Kwan et al., 2012a). Interestingly, NOS1-expressing neurons, despite their likely lack of functional synaptic transmission, selectively co-expressed high levels of FOS (previously c-Fos; Fig. 3), which is an immediate early gene that is transcriptionally activated downstream of neuronal activity. These data suggest the possibility that NOS1 may mediate a form of neuronal activity prior to the establishment of functional synapses. The ability of NO to mediate neurotransmission without synaptic contact or vesicular release (Garthwaite, 2008) is consistent with this possibility. Furthermore, a recent report suggests that cGMP production downstream of NOS1 activates a signaling cascade that directly leads to the activation of multiple immediate-early activity-dependent genes, likely independently of synaptic transmission (Gallo and Iadecola, 2011). Such a form of early, pre-synaptogenic activity may contribute to the establishment of neural circuitry in cortical development, perhaps by mediating neuronal synchrony. A few studies have shown that nitric oxide mediates neuronal synchronization within a local circuit (Rörig and Sutor, 1996, O'Donnell and Grace, 1997, Sammut et al., 2007). Therefore, nitric oxide may play a similar role in the establishment of connectivity between pyramidal neurons, perhaps mediating synchrony that may be important for the wiring of minicolumns.

A number of studies suggest the possibility that NOS1 may play a role in establishing synapses and neural circuits. The blockade of NOS1 function has been shown to disrupt synapse formation and result in spine loss (Sánchez-Islas and León-Olea, 2004, Morales-Medina et al., 2007, Nikonenko et al., 2008). Furthermore, a recent study found that NOS1 is important in the local activity-dependent development of excitatory synapses (Nikonenko et al., 2013).

In future experiments, it would be valuable to elucidate the location and biological function of NOS1 in pyramidal neurons. These studies can include the assessment of cGMP activation and protein S-nitrosylation in the fetal human neocortex. To understand the neurobiological function of NOS1, it would be interesting to assess the consequence of the forced expression of NOS1 in pyramidal neurons *in vivo*, which may be achieved by *in utero* electroporation. Pyramidal neurons derived from human induced pluripotent stem cells may also be used to assess the morphological or synaptogenic consequence of NO signaling.

5. Discussion

The origins of human uniqueness have long been a subject of fascination for scientists and philosophers alike. With recent advances in genetics and molecular biology, aspects of this question may slowly become experimentally tractable. Given the functional regionalization of the brain, in particular of neocortical areas, analysis of genes that are expressed in spatially-restricted patterns is expected to yield important insights into specific functional circuits. NOS1, being spatiotemporally and species-dependently expressed in key neocortical areas, may provide a valuable opportunity to understand some of the molecular biology underpinning the assembly and evolution of circuits involved in speech and language and higher cognition. With the increasing availability of large datasets surveying the spatiotemporal specificity of mRNA and protein expression in the primate and rodent brains, additional interesting gene expression patterns will surely be revealed in the near

future. Thus far, however, as no other gene product has been shown to be expressed with such specificity, NOS1 represents a somewhat unique opportunity to understand the wiring of frontal-opercular and anterior cingulate neural circuits.

Recent advances in sequencing technology will, without a doubt, continue to reveal additional genes that are disrupted in speech and language disorders, as well as those affecting higher cognition. Identifying the neurobiological roles of these candidate genes, however, will remain challenging. Key mechanistic insights can be gained from the study of intact brain circuits using animal models. Although model animals have been tremendously valuable in giving us functional insights, their use in the study of cognitive functions is impeded by the difficulty of identifying the appropriate readout for these functions in animal models. Learned vocalization has been assessed in several model species. Song birds have become a well-studied model for vocal learning (Fee et al., 2004, Bolhuis et al., 2010, Fee and Scharff, 2010) and the emerging use of ultrasonic vocalization analysis in mice shows great promise, especially given the wealth of genetic lines available from mouse consortia. To understand the neurobiological relevance of animal vocalization to human speech, or draw similar conclusions regarding cognitive functions, it is important to synergize human and animal studies to frame animal findings in the proper evolutionary context. Such studies necessitate the examination of the human brain. Although functional analyses in the human brain are not possible, analysis of mRNA and protein expression, biochemical studies of interactions and pathways, and histochemical localization are feasible. Furthermore, the emerging use of induced pluripotent cells further provides a system of live human neurons that can help bridge the gap between the use of post-mortem tissue and the study of live animal models.

Recent advances in recombineering, transgenesis, and genome editing are transforming functional genetics, as changes in the genome can be customized and engineered with high precision and relative ease. Humanized transgenics and knock-ins may be generated to recapitulate the expression and function of human genes, providing a potential means to study the neurobiological role of human genes in an intact circuit *in vivo*. Since the advent of transgenic primates (Chan et al., 2001), the last decade has seen tremendous advances in genome editing technologies, which have made it possible to engineer the genomes of non-traditional model species, for which a working protocol for traditional homologous recombination using cultured embryonic stem cells does not exist. The CRISPR/Cas9 system, for example, has recently been adapted for use in the generation of macaque transgenics (Niu et al., 2014), and zinc-finger nuclease and vector-based methods have been refined and used to generate marmoset transgenics (Sasaki et al., 2009, Kishi et al., 2014). The use of genetically engineered primates and other gyrencephalic species for functional studies may lead to new avenues of exploration that can illuminate the evolutionary and developmental mechanisms most pertinent to the human brain.

These and other recent techniques represent exciting opportunities to explore and model the neurobiological significance of NOS1 expression in the human neocortex, as well as the molecular cascades involving its synthesis and signaling, and may lead to new hypotheses about the role of NOS1 in the evolution of speech and cognitive circuits.

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Highlights

- NOS1 is expressed in specific subsets of pyramidal neurons in the human fetal brain
- Pyramidal NOS1 expression is transient, areal-dependent, and species-dependent
- Spatiotemporal expression of NOS1 implicates a role in language and cognitive circuit assembly

Figure 1.

Developmental events and spatiotemporal NOS1 expression in the mid-fetal human neocortex. The timing and duration of selected neocortical developmental processes, as defined by anatomical (Bourgeois, 1997, de Graaf-Peters and Hadders-Algra, 2006, Clancy et al., 2007, Workman et al., 2013) and transcriptome data (Johnson et al., 2009, Kang et al., 2011), are illustrated. The human lifespan is categorized into 15 periods based on the timing of developmental, maturation, and aging events in the cerebral cortex (Kang et al., 2011). During the mid-fetal ages, periods 4–6, NOS1 is expressed in the pyramidal neurons of FOp L5 and ACC L2/L3 (Kwan et al., 2012a). Unlike the transient pyramidal expression, NOS1 expression in interneurons persists through adulthood. Parts of this figure were modified from Kang et al., 2011.

Figure 2.

Species -dependent NOS1 expression in the developing neocortex. **A.** In the mid-fetal human neocortex, NOS1 is expressed in the pyramidal neurons of two key regions underlying language and cognitive functions. NOS1 expression is revealed by NADPH-diaphorase staining (blue), a histochemical technique based on the enzymatic activity of NOS to catalyze the NADPH-dependent conversion of a soluble tetrazolium salt to aformazan precipitate (Hope et al., 1991). In the frontal operculum (FOp), NOS1 is selectively present in the pyramidal neurons of neocortical layer 5 (L5). NOS1-expressing neurons form alternating minicolumns separated by NOS1 -negative neurons. In the anterior cingulate cortex (ACC), many L2 and L3 pyramidal neurons express high levels of NOS1, which is absent from the deep layers of the ACC. NOS1-expressing interneurons (open arrowhead) are present throughout neocortical areas and layers with no significant selectivity. **B.** In the early postnatal mouse neocortex, which is developmentally equivalent to the human mid -fetal neocortex, NOS1 is exclusively expressed in interneurons (open arrowheads) and absent from pyramidal neurons. Parts of this figure were modified from Kwan et al., 2012a.

Figure 3.

A schematic summary of molecular expression in NOS1-expressing L5 pyramidal neurons of the mid-fetal FOp. **A.** In the mid-fetal FOp, NOS1-expressing pyramidal neurons (green) within L5 minicolumns express BCL11B (blue), a marker of L5 corticofugal projection neurons. In between the alternating columns of NOS1-expressing neurons are numerous NOS1-negative pyramidal neurons that express SATB2 (red) a marker of intracortical projection neurons and vimentin (VIM)-positive radial glia (orange-red lines). These data are consistent with the possibility that migratory corridors supporting the migration of upper-layer neurons are present in between NOS1-expressing minicolumns. **B.** A proportion of NOS1-expressing neurons, mostly positioned in the deeper portions of the minicolumns, express FOXP2 (red), loss-of-function of which results in a severe speech and language disorder. A majority of NOS1-expressing neurons coexpress FOS (previously c-Fos), an immediate early gene that is upregulated in response to neuronal activity.