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Species-Dependent Posttranscriptional Regulation of *NOS1* by FMRP in the Developing Cerebral Cortex

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SUMMARY

Fragile X syndrome (FXS), the leading monogenic cause of intellectual disability and autism, results from loss of function of the RNA-binding protein FMRP. Here, we show that FMRP regulates translation of neuronal *nitric oxide synthase 1* (*NOS1*) in the developing human neocortex. Whereas *NOS1* mRNA is widely expressed, NOS1 protein is transiently coexpressed with FMRP during early synaptogenesis in layer- and region-specific pyramidal neurons. These include midfetal layer 5 subcortically projecting neurons arranged into alternating columns in the prospective Broca's area and orofacial motor cortex. Human *NOS1* translation is activated by FMRP via interactions with coding region binding motifs absent from mouse *Nos1* mRNA, which is expressed in mouse pyramidal neurons, but not efficiently translated. Correspondingly, neocortical NOS1 protein levels are severely reduced in developing human FXS cases, but not FMRP-deficient mice. Thus, alterations in FMRP posttranscriptional regulation of *NOS1* in developing neocortical circuits may contribute to cognitive dysfunction in FXS.

INTRODUCTION

The development of neural circuits is a precisely regulated process susceptible to genetic alterations that can lead to disorders affecting the most distinctively human aspects of cognition, including speech and language, theory of mind, and complex social behavior (Geschwind and Levitt, 2007; Lui et al., 2011; Ramocki and Zoghbi, 2008; State, 2010; Walsh et al., 2008). One such disorder, fragile X syndrome (FXS), is the leading inherited cause of intellectual disability and is often accompanied by autistic-like features, aggression, attention deficits, and delays in speech and language development (Abbeduto et al., 2007; Rogers et al., 2001; Willemsen et al., 2011). FXS is caused by loss of function of the *FMR1* gene, which encodes an RNA-binding protein (FMRP) involved in mRNA localization, stability, and translation (Ashley et al., 1993; Bagni and Greenough, 2005; Bassell and Warren, 2008; Zalfa et al., 2007). Many FMRP mRNA targets function in synaptic development and plasticity (Brown et al., 2001; Darnell et al., 2011). Concordantly, *Fmr1*-deficient mice show neural deficits also found in patients with FXS (The Dutch-Belgian Fragile X Consortium, 1994). However, FMRP target mRNAs and their role in human neurodevelopment are not as well understood.

The study of human FMRP function may also provide insights into the molecular mechanisms and neural circuits affected in autism spectrum disorders (ASDs), which are highly comorbid with FXS (Rogers et al., 2001). ASDs are a group of complex developmental syndromes characterized by impairments in social communication and language development, and repetitive behaviors. Multiple lines of evidence point to the dysfunction of neocortical circuits involved in social, emotional, and language processing in ASDs (Geschwind and Levitt, 2007; State, 2010; Walsh et al., 2008). Although no overt neuroanatomical alterations have been linked to the autistic brain, there is emerging evidence of abnormal organization of cortical minicolumns

(Casanova et al., 2002; Peters, 2010), which are composed of vertically arranged neurons connected into a local network and thought to originate from developmental radial units (Mountcastle, 1997; Rakic, 1988). Whether the molecular mechanisms altered in ASDs are associated with the development of specific human cortical circuits, including minicolumns, remains unknown.

Here, we report that FMRP binds human neuronal *nitric oxide synthase 1* (*NOS1*, also known as *nNOS*) mRNA and increases its translation in the developing neocortex in a species-dependent manner. NOS1 produces the gaseous signaling molecule nitric oxide (NO), which plays important roles in the development and function of the nervous system (Bredt and Snyder, 1994; Garthwaite, 2008). Our study of *NOS1* posttranscriptional regulation was instigated by our observation of a marked discrepancy between the midfetal human neocortex expression patterns of *NOS1* mRNA, which is widespread, and NOS1 protein, which is restricted to layer- and region-specific subpopulations of pyramidal neurons. These include layer 5 (L5) subcortically-projecting neurons with an alternating minicolumnar arrangement in the frontoparietal operculum (FOp). The FOp encompasses the prospective Broca's area and orofacial motor cortex, regions involved in speech production and language comprehension (Keller et al., 2009). After our screen for RNA-binding proteins revealed that FMRP is abundantly bound to human *NOS1* mRNA, we found that FMRP interacts with sequences in the *NOS1*-coding region that contain G-quartet (GQ) motifs and leads to increased NOS1 protein expression. These motifs are absent from mouse *Nos1* mRNA, and replacing the GQ-containing region of human *NOS1* with the mouse orthologous sequence abrogates FMRP-dependent activation of translation. Concordantly, neocortical NOS1 protein levels are dramatically reduced in human FXS, but not *Fmr1*-deficient mice. Thus, we identified a species-dependent posttranscriptional regulation of human *NOS1* by FMRP in specific neocortical circuits during column development and synaptogenesis, and showed it to be altered in FXS.

RESULTS

NOS1 Protein Is Transiently Expressed in Developing Human Pyramidal Neurons

The current research stems from our unexpected observation that strong NADPH-diaphorase (NADPH-d) activity, a reliable histoenzymatic marker of NOS (Dawson et al., 1991; Hope et al., 1991), is transiently present in subpopulations of pyramidal neurons in the developing human neocortex (Sestan and Kostović, 1994), in addition to its previously reported localization to interneurons and cortical plate (CP) neuropil (Fertuzinhos et al., 2009; Judas et al., 1999). Our comprehensive analysis of pre- and postnatal postmortem brains ranging from 8 postconceptional weeks (PCW) to adulthood identified transient expression of NOS1/NADPH-d in two layer- and region-specific populations of pyramidal neurons with a predominant localization to somata and apical dendrites (Figures 1A–1C; see Figure S1A available online). Specifically, morphologically immature pyramidal neurons expressing NOS1 were present in the middle of the CP corresponding to the future L5 exclusively in the ventrolateral frontal cortex of the FOp and the dorsal part of the anterior insula starting around 15 PCW. One week later, NOS1$^+$ pyramidal neurons were also found in the anterior cingulate cortex (ACC) and adjacent dorsolateral frontoparietal cortex in the upper CP corresponding to the future L2 and L3. NOS1 expression in both of these regions was also temporally regulated. The ACC L2/L3 expression of NOS1 was maintained at high levels throughout the late fetal ages, and decreased during early infancy (Figures 1B and S1A; data not shown). In contrast, L5 expression of NOS1 occurred in two waves. First, the L5 expression was restricted to the FOp, and started at 15 PCW, peaked at 18–20 PCW, and was rapidly downregulated at approximately 23 PCW, after which a small number of NOS1$^+$ pyramidal neurons were present in the ventral part of the anterior insula. Second, sparse pyramidal NOS1 expression was present throughout neocortical L5 in the weeks immediately prior to birth and was progressively downregulated after birth (Figures 1B and S1A; data not shown). Thus, in developing pyramidal neurons, NOS1 expression is precisely regulated, exhibiting temporal, laminar, and regional specificity.

Fetal L5 NOS1$^+$ Pyramidal Neurons Form Alternating Columns

Further analysis of the midfetal FOp L5 NOS1$^+$ pyramidal neurons revealed that they were arranged vertically into alternating arrays of intensely (NOS1$^+$) and lightly (NOS1$^-$) stained pyramidal neurons (Figures 1A and 1C) resembling previously described ontogenetic columns (Rakic, 1988). In contrast the ACC L2/L3 NOS1$^+$ pyramidal neurons were more densely distributed (Figure 1D; FOp L5, 67.86 $\pm$ 5.66 cells per 1,000 μm^2; ACC L3, 178.57 $\pm$ 27.95 cells per 1,000 μm^2; $p = 4.12 \times 10^{-5}$) and lacked this alternating columnar arrangement (Figure 1E; nearest neighbor distance between cell clusters: FOp L5, 27.87 $\pm$ 5.26 μm; ACC L3, 15.75 $\pm$ 4.68 μm; $p = 4.24 \times 10^{-9}$). In contrast to midgestation, perinatal NOS1$^+$ L5 neurons did not exhibit columnar organization (Figure S1A).

Because the FOp is structurally and functionally lateralized (Keller et al., 2009), we investigated whether L5 NOS1$^+$ columns exhibited left-right asymmetry in two whole midfetal brains (18 and 20 PCW). Serial reconstruction confirmed the two separate domains of NOS1$^+$ pyramidal neurons in the FOp and ACC of both hemispheres (Figures 1F and S1B) and provided approximate total numbers of FOp NOS1$^+$ columns (18 PCW: 41,380; and 20 PCW: 45,150). Although the number of NOS1$^+$ columns was not significantly different between the left and right hemispheres ($p = 0.569$), the distribution of NOS1$^+$ columns showed an asymmetric trend, peaking more rostrally in the right hemisphere, in both brains. Thus, the columnar organization of NOS1$^+$ neurons in the midfetal FOp L5 is bilaterally present.

Molecular and Projectional Identity of NOS1$^+$ Pyramidal Neurons

To molecularly characterize the identity of NOS1$^+$ neurons, we examined their expression of neuronal subtype markers. In the midfetal FOp L5, markers of subcortically-projecting pyramidal neurons, BCL11B (CTIP2) and *FEZF2* (*FEZL*, *ZFP312*) (Chen et al., 2005; Kwan et al., 2008; Leone et al., 2008; Molyneaux et al., 2007), were selectively coexpressed by L5 NOS1$^+$

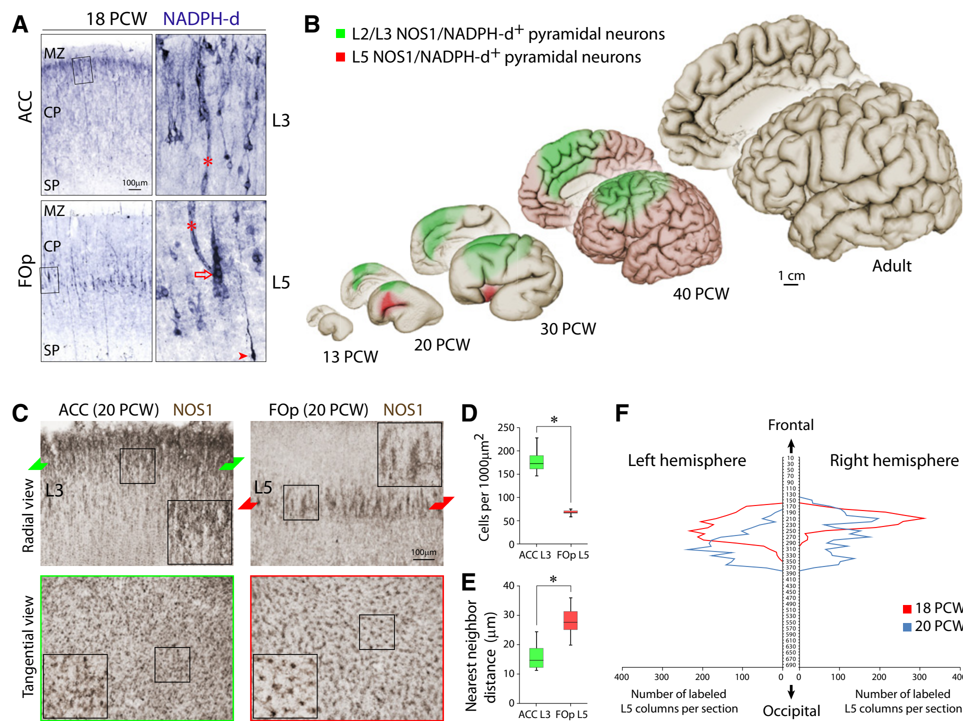

Figure 1. Spatiotemporal Dynamics of NOS1 Expression in Pyramidal Neurons of the Human Neocortex

(A) NADPH-d histochemistry at 18 PCW revealed intensely labeled pyramidal neurons in ACC L3 and FOp L5, where stained neurons were arranged into alternating vertical columns (open arrow). Interneurons (arrowhead) and blood vessels (asterisks) were also labeled. Boxes represent the 25th, 50th, and 75th percentiles.

(B) Schematic summary of the spatiotemporal dynamics of NOS1/NADPH-d staining in pyramidal neurons of L2/L3 (green) and L5 (red) in the developing and adult human neocortex.

(C) NOS1 immunohistochemistry of radial and tangential sections at 20 PCW. NOS1$^+$ pyramidal neurons exhibited a clear columnar organization in FOp L5, but not ACC L3.

(D and E) Analysis of cell density (D) and cluster spacing (E) revealed that NOS1$^+$ neurons in FOp L5 were significantly distinct in cytoarchitectonic organization from those in ACC L3. *$p < 0.05$. Boxes represent the 25th, 50th, and 75th percentiles. Error bars represent the 5th and 95th percentiles of 30 measurements.

(F) Serial section analysis of NADPH-d$^+$ L5 columns in two brains at 18 and 20 PCW. NADPH-d$^+$ columns were present bilaterally.

See also Figure S1.

neurons, forming an alternating columnar pattern identical to that of NOS1 (Figures 2A, 2B, and 2D). NOS1$^+$ neurons also coexpressed *FOXP2* (Figure 2E), a gene altered in a developmental disorder characterized by impaired speech and linguistic deficits (Lai et al., 2001). In contrast, SATB2, a marker of upper-layer corticocortical pyramidal neurons (Britanova et al., 2008), was highly expressed in NOS1$^-$, but not NOS1$^+$, neurons (Figures 2A and 2B), suggesting that NOS1$^-$ neurons were later born and likely migrating in between L5 NOS1$^+$ columns to the upper layers. Consistent with this, we observed vimentin (VIM)-positive radial glial fibers in between but not within NOS1$^+$ columns (Figure S2A). This suggests that glial-guided migration of upper-layer neurons occurs via corridors formed between L5 neuronal columns. In the ACC, all L2/L3 NOS1$^+$ neurons coexpressed SATB2 (Figure S2B), confirming their upper-layer identity and distinction from the FOp L5 NOS1$^+$ neurons.

To examine whether the midfetal pyramidal neurons of diverse subtypes have distinct cytoarchitectonic arrangements, we first measured the nearest neighbor ratio (NNR) and total path length ratio (TPLR) (Buxhoeveden et al., 1996) of NOS1$^+$ and NOS1$^-$ L5 neurons in the 20 PCW FOp (Figure 2C). This confirmed that NOS1$^+$ L5 neurons were significantly closer to being perfectly columnar (1.0) compared to NOS1$^-$ L5 neurons, as determined by both NNR (NOS1$^+$, 1.145 ± 0.146; NOS1$^-$, 1.528 ± 0.202; $p = 2.97 \times 10^{-5}$) and TPLR (NOS1$^+$, 1.122 ± 0.077; NOS1$^-$, 1.740 ± 0.369; $p = 2.41 \times 10^{-3}$). Next, we retrogradely traced projection neurons in a postmortem 20 PCW brain. We labeled subcortical projection neurons with Fast DiI inserted into the internal capsule and corticocortical projection neurons with Fast DiA inserted into the corpus callosum (Figure 2H). DiI-labeled subcortical projection neurons in the FOp formed columns similar in organization to the NOS1$^+$ columns (Figure 2I). In contrast, DiA-labeled callosal neurons in the ACC did not exhibit columnar organization (Figure 2J). Collectively, these results indicate that FOp NOS1$^+$ neurons exhibit the columnar organization and molecular identity of postmigratory L5 subcortically-projecting neurons.

Transient NOS1 Expression in Pyramidal Neurons Is Concomitant with Early Synaptogenesis

Previous studies have shown that significant neocortical synaptogenesis starts during midgestation (Molliver et al., 1973).

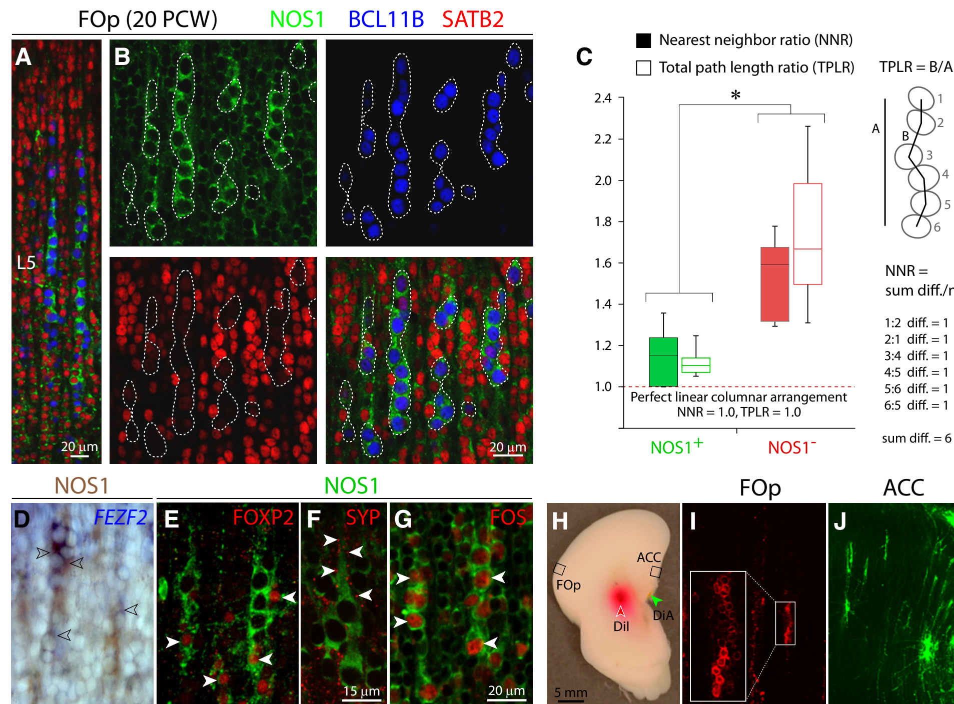

Figure 2. Molecular, Structural, and Axonal Connectivity Analyses of L5 NOS1$^+$ Columns in the Midfetal Human FOp

(A and B) Triple-immunofluorescent staining for NOS1 (green), BCL11B (blue), a marker of L5 subcortically-projecting neurons, and SATB2 (red), a marker of upper-layer corticocortical projection neurons, in 20 PCW FOp L5. NOS1$^+$ and BCL11B$^+$ pyramidal neurons formed alternating columns (outlined) separated by clusters of SATB2$^+$ neurons.

(C) Analysis of columnarity in FOp L5 neurons. NOS1$^+$ neurons were significantly more columnar in organization compared to NOS1$^-$ neurons. *$p < 0.05$. Boxes represent the 25th, 50th, and 75th percentiles. Error bars represent the 5th and 95th percentiles of 30 measurements. diff, difference.

(D) NOS1 immunostaining (brown) and *FEZF2* in situ hybridization (blue) of FOp L5 at 18 PCW. NOS1$^+$ neurons coexpressed *FEZF2* (open arrowheads).

(E–G) Immunofluorescent staining for NOS1 (green) and FOXP2, SYP, or FOS (red in E–G). NOS1$^+$ neurons coexpressed FOXP2 and FOS (arrowheads in E and G) and were encircled by SYP puncta (arrowheads in F).

(H–J) Retrograde axonal tracing at 20 PCW. Retrograde travel of Fast DiI inserted into dorsal internal capsule (red arrowhead) and Fast DiA inserted into the corpus callosum (green arrowhead) were examined after 7 months in incubation. In the FOp L5 (I), DiI-labeled subcortical projection neurons formed columns similar to those composed of NOS1$^+$ neurons. In the ACC (J), DiA-labeled corticocortical projection neurons did not exhibit obvious columnar organization. White box in (I) represents area enlarged in inset.

See also Figure S2.

Consistent with the possibility that NOS1 expression is associated with synaptogenesis, we found presynaptic synaptophysin (SYP) puncta encircling the cell membrane of FOp NOS1$^+$ L5 neurons at the soma and apical dendrite (Figure 2F). Our pre-embedding NOS1 immuno-EM in the 18 and 20 PCW FOp, however, revealed only sparse mature synapses in the CP (Figures S2C and S2D), suggesting that the majority of SYP$^+$ puncta on L5 NOS1$^+$ columns were immature terminals. Concordantly, immature synapses that have not yet become electron dense or accumulated vesicles and nonsynaptic contacts were observed in L5 and on NOS1$^+$ dendrites (Figures S2E–S2H). Interestingly, FOS (C-FOS), a marker of recent neuronal activity, was expressed by virtually all NOS1$^+$ columnar neurons and was mostly absent from NOS1$^-$ intercolumnar neurons in the 20 PCW FOp (Figure 2G), suggesting that NOS1$^+$ L5 neurons may be active. Together, these findings suggest that the expression of NOS1 in L5 FOp neurons is concomitant with early synaptogenesis and neuronal activity.

Cross-Species Comparison of Neocortical NOS1 Expression

To determine whether the spatiotemporal expression pattern of NOS1 exhibits species differences, we examined NADPH-d/NOS1 expression in the gyrated macaque monkey neocortex and lissencephalic mouse neocortex (Figure 3; data not shown). In the macaque, NADPH-d$^+$ pyramidal neurons were present in L2/L3 of the ACC and adjacent frontoparietal regions, starting as early as embryonic day (E) 62, an age equivalent to human midgestation (Kostovic and Rakic, 1990), and persisting until the late fetal period (Figures 3B and S3; data not shown). NADPH-d$^+$ pyramidal neurons were present in L5 columns of the FOp and adjacent regions (Figures 3B and 3C), starting as early as E73, peaking near E82, and persisting until at least E113 (Figures 3B and S3; data not shown). Consistent with previous studies, our analyses of the mouse neocortex from E18.5 to P14, a period equivalent to human midfetal to early postnatal development, revealed that intense NADPH-d activity was present exclusively in interneurons and neuropil (Figure 3A;

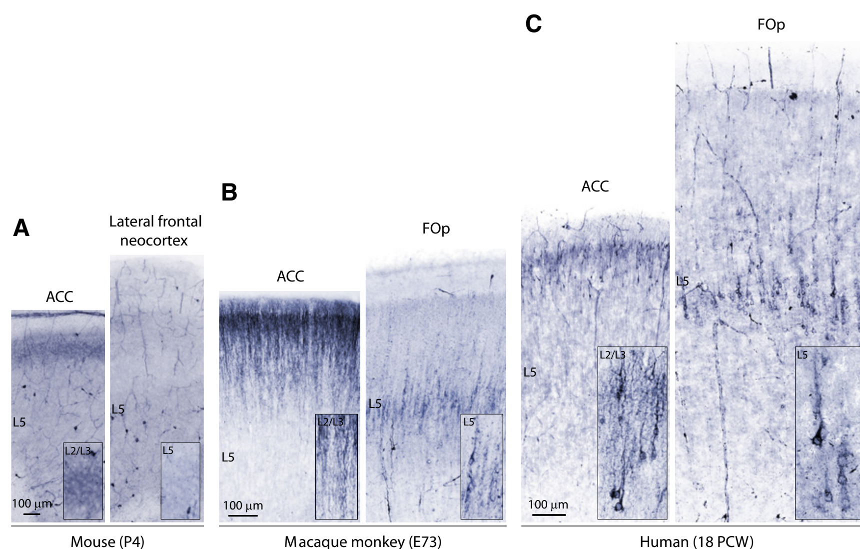

Figure 3. Comparative Analysis of NADPH-d Activity in Mouse, Macaque, and Human Neocortex at Equivalent Developmental Ages

In the P4 mouse ACC and lateral frontal cortex (A), intense NADPH-d staining was restricted to interneurons, with neuropil staining in ACC L2/L3. In the E73 macaque neocortex (B), intense NADPH-d activity was present in ACC L2/L3 pyramidal neurons similar to those labeled in the human 18 PCW ACC (C). In the macaque FOp, NADPH-d$^+$ L5 pyramidal neurons were arranged into vertical columns similar in organization to the human FOp columns (C). Strong interneuronal and weak neuropil NADPH-d staining was present in all cortical areas in mouse, macaque, and human neocortex. See also Figure S3.

data not shown), indicating that pyramidal expression of NOS1 is species dependent.

Discordant *NOS1* mRNA and Protein Expression Patterns in the Fetal Neocortex

To examine the expression pattern of *NOS1* mRNA, adjacent tissue sections of the midfetal neocortex were analyzed with *NOS1* in situ hybridization, NADPH-d, and NOS1 immunostaining (Figure 4A). Surprisingly, *NOS1* mRNA was abundantly and widely present in the CP in all cortical layers and regions examined, including the great majority of pyramidal neurons that did not express NOS1 protein. This striking difference between the highly restricted NOS1 protein and widespread *NOS1* mRNA expression suggests that *NOS1* is posttranscriptionally regulated.

Remarkably, *Nos1* mRNA was also abundantly and widely expressed in the early postnatal mouse neocortex (Figure 4B). Pyramidal expression of mouse *Nos1* mRNA was confirmed by quantitative RT-PCR of fluorescently sorted pyramidal neurons fate mapped in mice doubly transgenic for *Emx1-Cre* and a CRE-responsive *Gfp* (*CAG-Cat-Gfp*) (Figure 4C). Therefore, whereas *NOS1* mRNA is expressed in pyramidal neurons of both human and mouse neocortex, its efficient translation into NOS1 protein occurs in subpopulations of human, but not mouse, pyramidal neurons. This indicates that pyramidal NOS1 expression is driven by species-dependent posttranscriptional regulation.

NOS1 mRNA Associates with FMRP in Human Fetal Neocortex

To identify potential *NOS1* mRNA-binding proteins, we used immobilized full-length human *NOS1* mRNA to pull down candidate proteins from the human frontal CP at 20 and 21 PCW. To facilitate the isolation of sequence-dependent RNA-binding proteins, we used three negative control RNAs (*GAPDH*, *EGFP*, and *NeoR*). *NOS1* mRNA-interacting proteins showed a distinct enrichment at a molecular weight of approximately 75 kDa (Figure 5A). To identify the protein present in this band, we analyzed our human brain transcriptome data set (http://www.humanbraintranscriptome.org; Johnson et al., 2009; Kang et al., 2011) for RNA-binding proteins near 75 kDa that are expressed in the midfetal frontal neocortex. Analysis of four candidates (FMRP, FXR1, CPEB3, and EIF2C2) by immunoblotting of pulled-down proteins revealed that FMRP, but not the others, was strongly and specifically associated with *NOS1* mRNA (Figures 5A and S4A). The presence of FMRP in this *NOS1*-enriched band was confirmed by mass spectrometry (data not shown), which also revealed the putative presence of PABPC4, a poly-adenylate-binding protein, and HSPA8, a chaperone protein. Double-immunofluorescent staining showed that FMRP was highly coexpressed in NOS1$^+$ pyramidal neurons in the midfetal FOp and ACC (Figures 5B and S4B). Subcellularly, FMRP and NOS1 colocalized to the soma and apical dendrite. Interestingly, most NOS1$^+$ interneurons in the SP and CP did not express FMRP at high levels during midgestation. Together, these results suggest a potential role of FMRP in the posttranscriptional regulation of *NOS1* in fetal human pyramidal neurons.

Species Differences in FMRP-*NOS1* mRNA Association in the Developing Neocortex

To confirm the putative FMRP-*NOS1* mRNA interaction, we performed RNA-binding protein immunoprecipitation (RIP) using 21 PCW frontal CP lysate. RNAs coimmunoprecipitated with FMRP were analyzed using quantitative RT-PCR (Figure 5C). Compared to rabbit immunoglobulin (IgG) control, anti-FMRP antibodies immunoprecipitated 6.8- ± 0.8-fold more *NOS1* mRNA, a level of enrichment similar to *MAP1B* mRNA (7.1- ± 0.6-fold), a well known target of FMRP (Darnell et al., 2011), and significantly higher than *GAPDH* mRNA (1.5- ± 0.4-fold), a negative control. In contrast in the early postnatal mouse neocortex (Figure 5D), *Nos1* mRNA was enriched only 2.7- ± 0.3-fold by anti-FMRP immunoprecipitation, markedly lower than the 8.6- ± 0.5-fold enrichment for *Map1b* mRNA and comparable to *Gapdh* mRNA (2.0- ± 0.1-fold). Consistent with

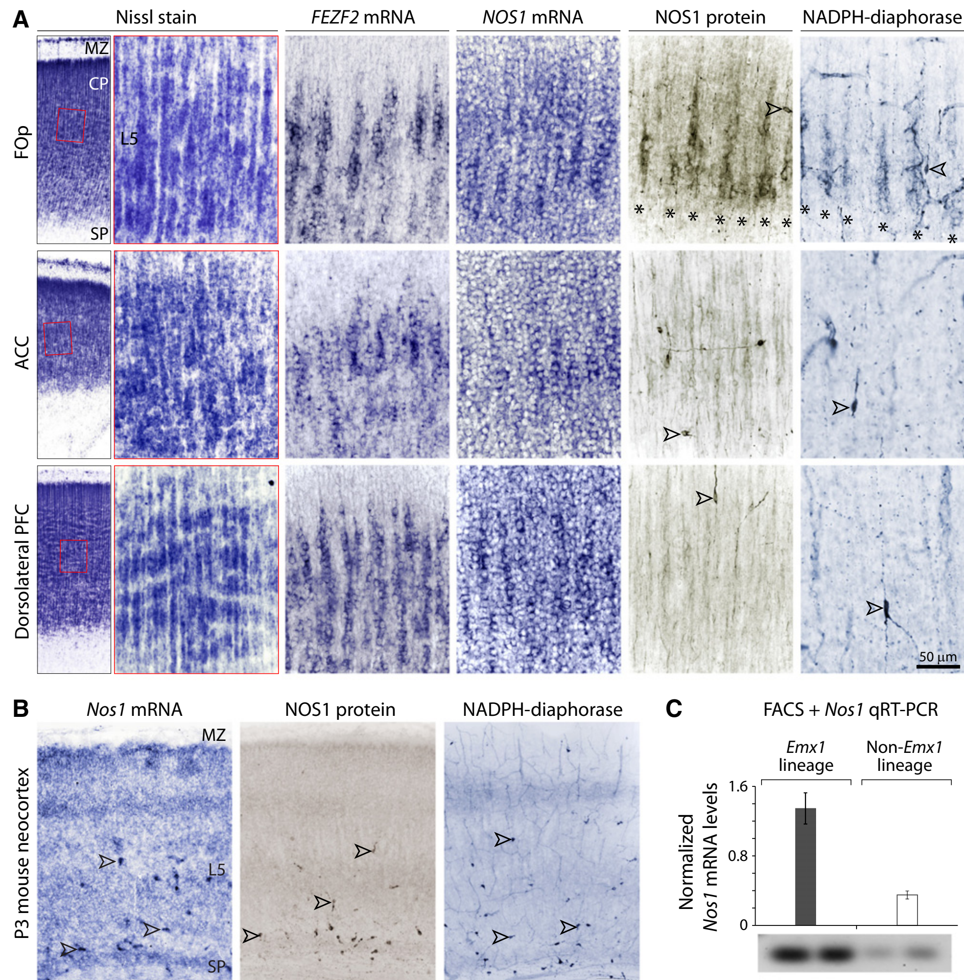

Figure 4. Discordant *NOS1* mRNA and NOS1 Protein Expression Patterns in the Developing Human and Mouse Neocortex

(A) Nissl staining, *FEZF2* and *NOS1* in situ hybridization, NOS1 immunohistochemistry, and NADPH-d histochemistry in adjacent sections from 18 PCW FOp, ACC, and dorsal lateral prefrontal cortex (PFC). *NOS1* mRNA was abundantly present in all cortical regions and layers examined. Intense NOS1 and NADPH-d labeling in L5 pyramidal columns (asterisks) were present in the FOp. In all examined cortical regions, NOS1 and NADPH-d were present in interneurons (open arrowheads) and neuropil. Red boxes represent areas enlarged.

(B) *Nos1* in situ hybridization, NOS1, and NADPH-d staining in adjacent sections from P3 mouse frontal neocortex. *Nos1* mRNA was widely present; intense NOS1 and NADPH-d stainings were exclusively present in interneurons (open arrowheads). Neuropil was weakly stained. MZ, marginal zone; SP, subplate.

(C) Pyramidal neurons of the *Emx1* lineage were isolated from the P3 mouse neocortex by fluorescent cell sorting (FACS) and analyzed by quantitative (q) RT-PCR. *Nos1* mRNA was abundantly present in pyramidal neurons. Error bars represent the 5th and 95th percentiles of four measurements.

this, *Nos1* was absent from the FMRP targets identified in a recent, comprehensive HITS-CLIP analysis of the mouse brain (Darnell et al., 2011). Thus, FMRP strongly associates with human but not mouse *NOS1* mRNA in the developing neocortex, suggesting that FMRP may underlie species differences in *NOS1* translation.

FMRP Binds GQ-Containing Sequences in the Human *NOS1*-Coding Region

FMRP can interact with specific mRNA sequences including GQ structures (Darnell et al., 2001; Schaeffer et al., 2001) and poly-uridine stretches (Chen et al., 2003). Analysis of human *NOS1* mRNA revealed three putative GQ motifs and six poly-uridine stretches (Figure 6A). RNA pull-down assays from 21 PCW frontal CP lysate revealed that FMRP had strong affinity for each of the two *NOS1*-coding region GQs (GQ1 and GQ2), but not GQ3 or the U-rich regions (UR1–UR6) (Figure 6B). To confirm this, we synthesized a fragment of RNA representing both GQ1 and GQ2 and performed an electrophoretic mobility shift assay (EMSA; Figure 6C). In the presence of FMRP, this RNA exhibited a significant shift that was abolished by the addition of excess nonbiotinylated "cold" RNA or a neutralizing FMRP antibody. To determine whether human GQ1 and GQ2 form RNA G-quadruplex structures, we used a reverse-transcription termination assay (Figure 6D). Reverse-transcriptase activity pauses at sites of GQ structures in a cation-dependent manner (Schaeffer et al., 2001). Reverse transcription from both GQ1 and GQ2 RNA exhibited a significant pause at the expected GQ site in the

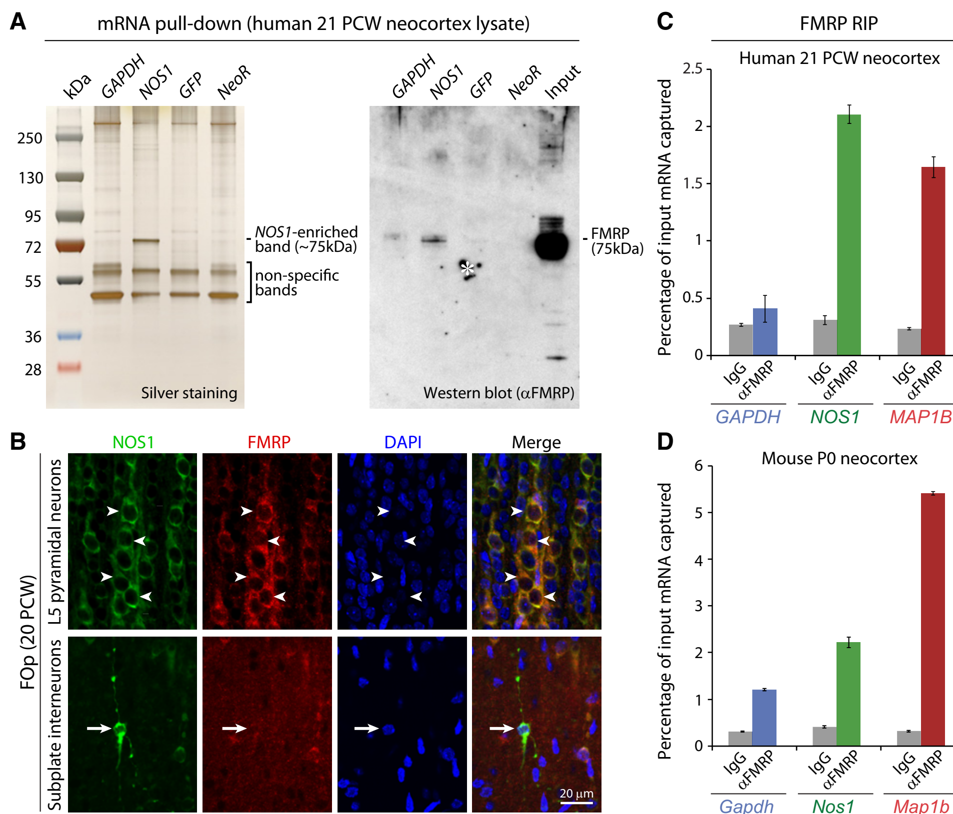

Figure 5. FMRP Binds *NOS1* mRNA in the Human Fetal Neocortex

(A) Proteins eluted from an mRNA pull-down assay using lysates of a 21 PCW human neocortex were analyzed by silver staining and immunoblotting. *NOS1*, but not control (*GAPDH*, *GFP*, and *NeoR*), mRNA specifically associated with an ~75 kDa protein that was immunopositive for FMRP. Asterisk indicates an artifact of gel transfer.

(B) NOS1 (green), FMRP (red), and DAPI (blue) staining of 20 PCW FOp. FMRP was coexpressed by L5 columnar NOS1$^+$ pyramidal neurons (solid arrowheads), but not most interneurons (arrows).

(C and D) FMRP immunoprecipitated mRNAs from a 21 PCW human and P0 mouse CP were analyzed by quantitative RT-PCR. Relative to control *GAPDH* and *MAP1B* mRNAs, FMRP strongly associated with *NOS1* mRNA (green bar) in human, but not mouse. Error bars represent the 5th and 95th percentiles of four measurements.

See also Figure S4.

presence of potassium, which facilitates GQ formation, but not lithium, which abrogates it. Therefore, FMRP interacts with GQ-forming sequences found within the coding region of human *NOS1* mRNA.

Evolution of *NOS1* mRNA GQ-Containing Sequences

To investigate whether GQ motifs are present in other mammals, we analyzed the 21 species for which *NOS1* mRNA sequence was available. Highly stable tetrads at both GQ1 and GQ2 positions were predicted only in the great apes and macaque monkey (Figure S5A). Among great apes, which otherwise have perfectly conserved GQs, only orangutan has a point mutation that leads to a less stable two-stack GQ1 quartet, but a fully conserved GQ2 quartet. In marmoset, a New World monkey, and nonprimate mammals, with the exception of the guinea pig that exhibited one quartet, they are absent from both positions. Further analysis of the entire *NOS1* coding region in nine placental mammals revealed a very high degree of conservation (Figure S5B), with the vast majority of substitutions being synonymous. The few nonsynonymous substitutions, however, were selectively clustered in the GQ region. This marked reduction in amino acid identity in an otherwise highly conserved protein is consistent with the hypothesis that the sequences containing the GQ motifs evolved and made possible posttranscriptional regulation by FMRP. Furthermore, these sequences have remained quite stable since their emergence in catarrhine primates, which is consistent with the expression of NOS1 in human and macaque pyramidal neurons.

FMRP Increases NOS1 Expression via Interaction with a GQ-Containing Sequence

To test the functional consequences of FMRP on *NOS1* translation, we cotransfected human expression constructs of FMRP (CAG-h*FMR1*) and NOS1 (CAG-h*NOS1*) into Neuro-2a cells and quantified NOS activity (Figure 6E). With CAG-h*FMR1* cotransfection, NOS1 activity was increased in a dose-dependent manner, by up to 3.6- ± 0.9-fold (p = 0.043), indicating that

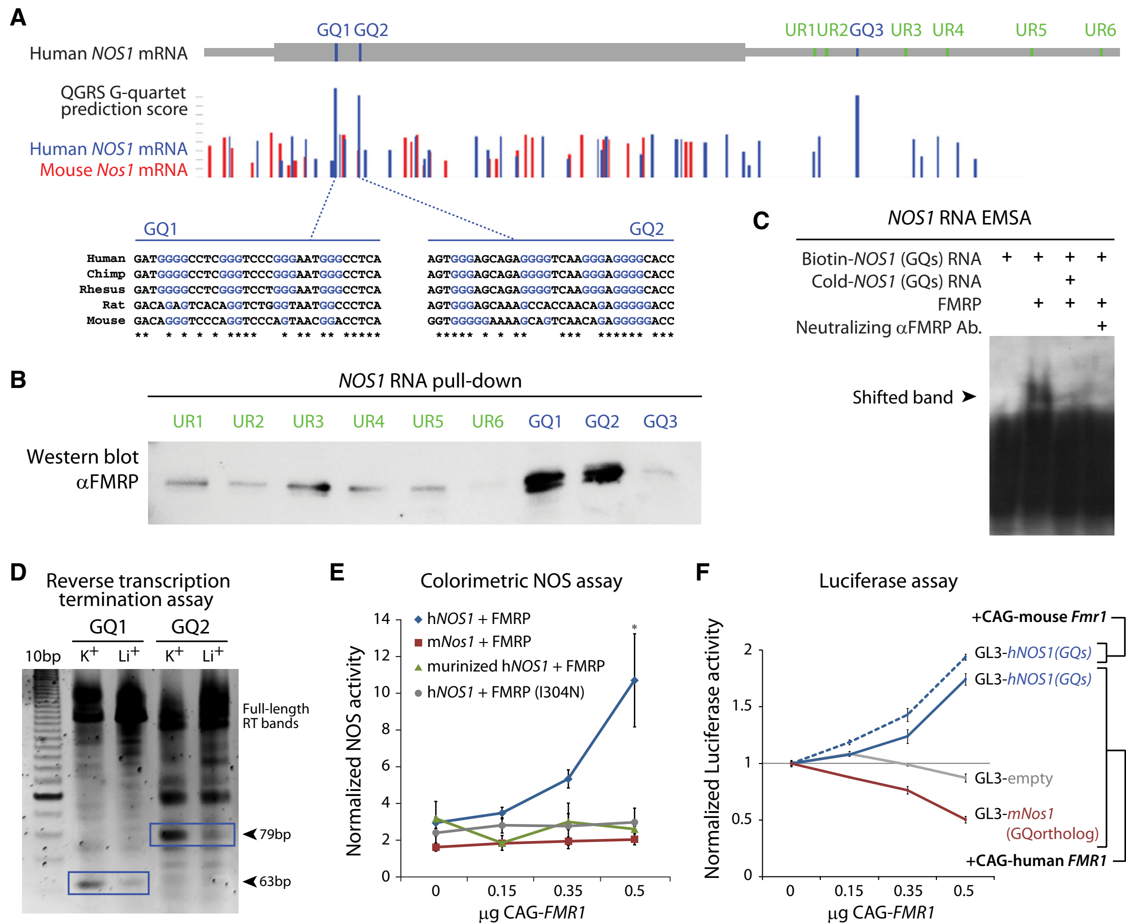

Figure 6. FMRP Binds Human *NOS1* GQ-Containing Sequences and Enhances Human, but Not Mouse, *NOS1* mRNA Translation

(A) Prediction of putative FMRP-binding GQ and U-rich (UR) motifs in the human *NOS1* mRNA sequence and alignment of GQ1 and GQ2. GQ1 and GQ2 were highly conserved in primates, but not rodents.

(B) FMRP association with each putative binding motif was analyzed by an mRNA pull-down assay using 21 PCW human neocortex lysates. FMRP selectively associated with GQ1 and GQ2.

(C) EMSA of GQ1 and GQ2. RNA containing both GQs exhibited a significant shift in mobility in the presence of FMRP. This shift was abolished by addition of excess unbiotinylated ("cold") RNA or a neutralizing anti-FMRP antibody.

(D) Reverse-transcription termination assay. Reverse transcription paused at the expected GQ sites for both GQ1 and GQ2 in the presence of K^+, which facilitates GQ formation, but not Li^+, which disrupts it.

(E) Colorimetric NOS assays in Neuro-2a cells cotransfected with CAG-h*FMR1* or CAG-h*FMR1*(I304N) and one of CAG-h*NOS1*, CAG-m*Nos1*, or CAG-murinized-h*NOS1*. NOS activity from h*NOS1*, but not m*Nos1* or murinized h*NOS1*, increased dose dependently with increasing wild-type FMRP. The I304N mutation in FMRP abolished its activation of h*NOS1* translation. *$p < 0.05$. Error bars represent the 5th and 95th percentiles of four measurements.

(F) Luciferase assays in Neuro-2a cells transfected with an empty reporter construct (GL3-empty), or constructs containing the human *NOS1* GQs (GL3-h*NOS1*[GQs]) or the orthologous sequence in mouse *Nos1* (GL3-m*NOS1*[GQortholog]). Luciferase activity in cells transfected with GL3-h*NOS1*(GQs) increased dose dependently with cotransfection of human CAG-h*FMR1* (solid blue line) or mouse CAG-m*Fmr1* (broken blue line). Luciferase activity from the GL3-m*Nos1* (GQortholog) decreased with increasing amounts of CAG-h*FMR1* (solid red line). Error bars represent the 5th and 95th percentiles of six measurements.

See also Figure S5.

FMRP acts as a positive regulator of NOS1 expression. No increase in NOS1 activity occurred when a mouse *Nos1* construct (CAG-m*Nos1*) or a human *FMR1* construct harboring the I304N mutation (CAG-h*FMR1* [I304N]) (Siomi et al., 1994) was used, or when the GQ-containing sequence of the human *NOS1* was replaced with the orthologous sequence from mouse *Nos1* (CAG-murinized-h*NOS1*). Therefore, the FMRP-mediated increase in NOS1 expression is dependent on the species of the *NOS1* sequence, the intact KH2 domain of FMRP, and the presence of GQ-containing sequences in the *NOS1* mRNA. To specifically examine the GQ region, we cloned the *NOS1* sequences containing GQ1 and GQ2 into the 3′ UTR of SV40-GL3 and performed luciferase assays in Neuro-2a cells (Figure 6F). The inclusion of the human *NOS1* GQs (SV40-GL3-h*NOS1*-GQs) led to significant dose-dependent increases in luciferase activity in response to CAG-h*FMR1*, indicating that FMRP increases *NOS1* translation via binding to these sequences. Importantly, a mouse FMRP expression construct

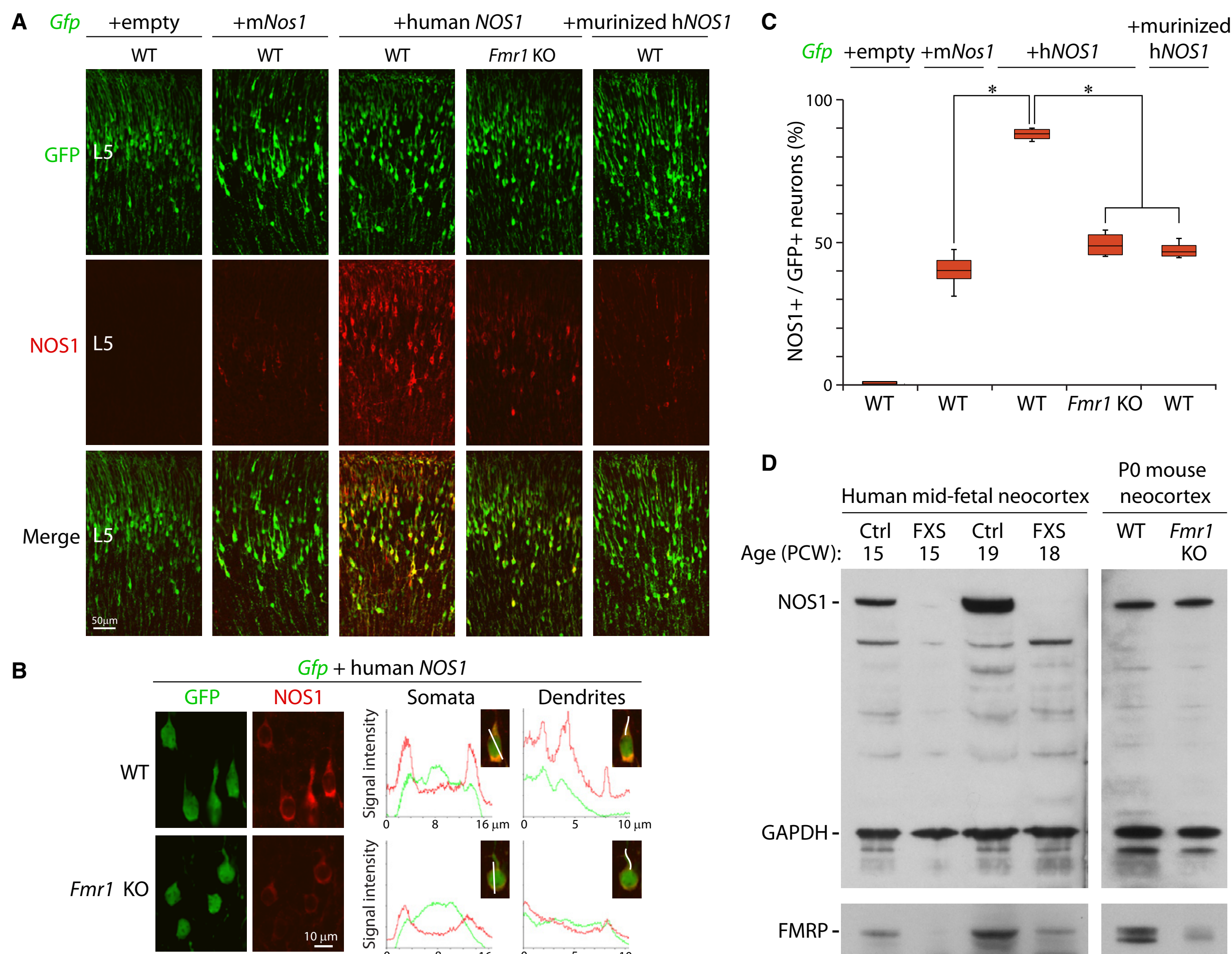

Figure 7. Efficient Translation of Human *NOS1* in Pyramidal Neurons Requires FMRP and Is Severely Reduced in Fetal FXS Neocortex

(A–C) Neocortex of wild-type or *Fmr1* KO mouse electroporated in utero at E13.5 and immunostained for NOS1 (red) at P0. In wild-type neocortex the majority of pyramidal neurons transfected with h*NOS1* expressed high levels of NOS1 properly localized to the soma and apical dendrite. NOS1 protein expression from m*Nos1* or murinized-h*NOS1* in wild-type and from h*NOS1* in *Fmr1* KO neocortex was dramatically reduced in comparison. The fluorescent intensity of NOS1 staining normalized to GFP (B) and the proportion of GFP^+ cells expressing NOS1 (C) were quantified. *$p < 0.05$. Boxes represent the 25th, 50th, and 75th percentiles. Error bars represent the 5th and 95th percentiles of at least four animals.

(D) Immunoblots of human fetal FXS and P0 mouse *Fmr1* KO neocortex. Normalized to *GAPDH* levels, the neocortical expression of NOS1 protein was severely reduced in both human fetal FXS cases. In neonatal mouse neocortex, loss of *Fmr1* did not alter neocortical NOS1 expression.

See also Figure S6.

(CAG-m*Fmr1*) dose dependently increased luciferase expression in a manner highly similar to CAG-h*FMR1*. However, when the human *NOS1* GQ sequences were replaced with the orthologous region of the mouse *Nos1* (SV40-GL3-m*Nos1*-GQortholog), FMRP failed to enhance luciferase activity. These results indicate that FMRP activates NOS1 protein expression via binding to a sequence containing GQ motifs and that this interaction exhibits species differences. Together, these data strongly support a scenario wherein FMRP activation of *NOS1* translation evolved through *NOS1* nucleotide substitutions that gave rise to a GQ-containing sequence targeted by FMRP.

Mouse Pyramidal Neurons Efficiently Translate Human NOS1 in an FMRP-Dependent Manner

Because mouse FMRP is able to enhance human NOS1 expression, we hypothesized that exogenous human *NOS1* mRNA can be efficiently translated in mouse pyramidal neurons, likely in an *Fmr1*-dependent manner. To test this in vivo, we introduced a *NOS1* expression construct with the CAG-*Gfp* reporter into mouse neocortical ventricular zone (VZ) using in utero electroporation (IUE) at E13.5 to target L5 pyramidal neurons. At P0, the majority of CAG-h*NOS1*-electroporated L5 pyramidal neurons expressed NOS1 protein at high levels (Figures 7A–7C). In contrast those electroporated with CAG-m*Nos1* or

CAG-murinized-h*NOS1* expressed only low levels of NOS1 in a minority of neurons, indicating reduced protein expression efficiency consistent with our in vitro assays. Human and mouse NOS1 exhibited similar somatodendritic localization (Figures 7A and 7B). Therefore, our results show that mouse pyramidal neurons possess all of the cellular machinery necessary for the translation of human NOS1 protein and suggest that their diminished expression of endogenous NOS1 is a result of differences in the *NOS1* mRNA sequence between human and mouse.

To assess FMRP dependence, we further electroporated *Fmr1* knockout (KO) mice with CAG-h*NOS1* and CAG-*Gfp* (Figure 7A). Both the number of GFP$^+$ neurons expressing NOS1 and the levels of NOS1 protein decreased significantly compared to wild-type (Figures 7B and 7C). In addition, neurons cultured from E14.5 *Fmr1* KO and transfected with CAG-h*NOS1* also exhibited a significant reduction in NOS1 levels compared to control (Figure S6A; 43.8% ± 7.8% reduction; p = 0.0065). These data show that FMRP is required for the efficient expression of human NOS1 protein in pyramidal neurons.

Severe Reduction in NOS1 Protein Levels in Developing Human FXS but Not Mouse *Fmr1* KO Neocortex

To determine whether NOS1 protein levels are altered in human FXS cases, we performed immunoblotting of neocortex from confirmed midfetal and postnatal FXS cases (15 and 18 PCW; and 9, 22, and 85 years) and age-matched controls. Neocortical lysates normalized to *GAPDH* levels were immunoblotted for NOS1, FMRP, and GAPDH (Figure 7D). Remarkably, in both fetal cases of FXS, neocortical NOS1 protein levels were severely reduced compared to matched controls. Furthermore, this deficit was age dependent, being very dramatic in the fetal cases, less so in the cases aged 9 and 22 years, and absent in the 85 years' specimen (Figure S6C). These results indicate that NOS1 protein expression is greatly reduced in the developing human FXS neocortex. Notably, neocortical NOS1 levels were not affected in early postnatal *Fmr1* KO mice (Figures 7D and S6B), indicating that the requirement of FMRP for NOS1 expression is species dependent.

DISCUSSION

In this study we demonstrate that human neocortical NOS1 expression is posttranscriptionally regulated by FMRP in a species-dependent manner. Molecular analyses revealed that FMRP binds GQ motif-containing sequences present in the coding region of human, but not mouse, *NOS1* mRNA and facilitates NOS1 protein expression. Concordantly, NOS1 expression is severely reduced in the developing FXS human, but not FMRP-deficient mouse, neocortex. In the human neocortex, NOS1 and FMRP are transiently coexpressed during synaptogenesis in subpopulations of pyramidal neurons in regions involved in speech, language, and complex social behaviors. Together, these findings provide a novel candidate mechanism and insights into the potential connectional pathology of FXS and possibly ASD.

Our analyses indicate that the FMRP-*NOS1* interaction emerged as result of closely clustered nucleotide substitutions within the otherwise highly conserved coding sequence of *NOS1* that gave rise to the GQ-containing motifs, occurring at the potential expense of protein integrity. FMRP binding to GQ motifs has been associated with translational repression (Bechara et al., 2009; Schaeffer et al., 2001). There is, however, a precedent for positive, activity-dependent posttranscriptional regulation in *PSD-95* (*DLG4*), which has an FMRP-binding GQ motif (Todd et al., 2003; Zalfa et al., 2007). Interestingly, NOS1 and PSD-95 are functionally related. NOS1 is anchored to the synaptic membrane via a physical interaction with PSD-95 (Brenman et al., 1996) and its enzymatic product, NO, *S*-nitrosylates PSD-95 (Ho et al., 2011). Although the abundant presence of *NOS1* mRNA in pyramidal neurons suggests that translational regulation is involved, FMRP may also control the stability of the *NOS1* transcript in a manner similar to its control of *PSD-95* mRNA stability (Zalfa et al., 2007). Furthermore, the GQ motif has been shown to mediate the dendritic localization of *PSD-95* (Dictenberg et al., 2008) and may also play a role in *NOS1* mRNA targeting. The possibility that *NOS1* and *PSD-95* are similarly regulated by FMRP is consistent with their shared postsynaptic localization, physical interaction, and related functions. The binding of FMRP to GQs has been demonstrated both in vitro (Bagni and Greenough, 2005; Bassell and Warren, 2008) and in vivo (Rackham and Brown, 2004; Iioka et al., 2011). Recently, however, it was shown that the presence GQ motifs is not predictive of FMRP binding (Darnell et al., 2011). Therefore, the context dependence of FMRP interactions with GQs remains to be fully elucidated, and individual potential interactions should be validated empirically.

Animal models of FXS exhibit multiple phenotypes present in human FXS, indicating that many aspects of FMRP function are well conserved. Therefore, any contribution of NOS1 to the FXS phenotype would likely involve the higher cognitive functions that are absent from mouse. This possibility is supported by the coexpression of NOS1 and FMRP in projection neurons of the FOp and the ACC and adjacent dorsal frontoparietal neocortex. The FOp encompasses the future Broca's area and its contralateral hemisphere equivalent, as well as the orofacial motor cortex, regions involved in speech production, language comprehension, and action recognition (Keller et al., 2009). The ACC is involved in decision making, attention, emotional processing, and social awareness (Devinsky et al., 1995). NOS1 expression in these regions is also temporally regulated from midgestation to early infancy, a developmental period critical for early synaptogenesis, dendritic spine formation, and ingrowth of cortical afferents (Kang et al., 2011). Therefore, the neuroanatomical localization and timing of the FMRP-*NOS1* interaction are consistent with a putative role in the development of neocortical circuits, including those involved in linguistic and social functions likely affected in FXS and ASD.

This potential role of *NOS1* in the development and function of human neural circuits is further supported by studies of a human *NOS1* hypomorphic allele, which has been associated with attention deficit hyperactivity disorder (ADHD), impulsivity, and aggression (Reif et al., 2009), behavioral features often comorbid with FXS (Rogers et al., 2001). This *NOS1* hypomorphism has also been associated with hypoactivity in the ACC (Reif et al., 2009), a cortical region with prominent midfetal pyramidal

expression of NOS1 protein. Functional imaging studies revealed a similar reduction in ACC activation in patients with FXS and ADHD during attentional-processing tasks (Bush et al., 1999; Menon et al., 2004) and in autistic children in response to a familiar face (Pierce and Redcay, 2008). The overlapping deficits between NOS1 hypomorphism, FXS, and FXS comorbidities are consistent with a functional role of *NOS1* in human brain circuitry related to FXS and ASD. Furthermore, the two midfetal neocortical regions with prominent pyramidal NOS1 expression, the ACC and FOp, exhibit highly coordinated resting state activity, suggesting functional connectivity between the two areas and the presence of a cingulo-opercular cognitive network (Power et al., 2011). Interestingly, the connectivity of this cingulo-opercular network has been reported to be impaired in schizophrenia (Tu et al., 2012). Remarkably, multiple sequence variations in *NOS1* have been associated with schizophrenia (Cui et al., 2010; Reif et al., 2006; Shinkai et al., 2002), supporting a potential role of NOS1 in the formation of cognitive circuits and in disorders that affect cognition.

Structural alteration in the organization of minicolumns has been reported in autism and other psychiatric disorders (Casanova et al., 2002). In this study we found that within the midfetal FOp, alternating L5 columns coexpress FMRP and NOS1, as well as FOXP2, which is implicated in the development of speech, language, and cognition (Lai et al., 2001), functions affected in FXS and ASD. We also showed that neurons within the same column have a shared subcortical molecular identity and connectivity. Positioned in between the columns are migratory corridors containing radial glial fibers and corticocortical projection neurons en route to the superficial layers. Thus, this fetal organization may have implications for the developmental basis of normal minicolumns (Rakic, 1988), as well as columnopathies (Casanova et al., 2002). Interestingly, the NOS1$^+$ columnar neurons of the fetal FOp share some areal and projection properties with adult mirror neurons, which are present in macaque area F5 (Rizzolatti and Craighero, 2004), an area equivalent to the human Broca's area, and project subcortical axons (Kraskov et al., 2009). Mirror neurons, which are activated during both the observation and execution of a particular goal-directed action, are thought to contribute to theory of mind and language abilities (Rizzolatti and Craighero, 2004), and in autistic children the mirror neuron activity that is normally observed in the FOp is absent (Dapretto et al., 2006). Therefore, the molecular profile of NOS1$^+$ columns, as well as their shared location and connectivity with mirror neurons, is consistent with a potential role in motor and cognitive development.

The synthesis of NO, a short-lived gas that cannot be stored or transported, must be precisely regulated and amenable to rapid, localized activation. Because FMRP controls both the dendritic localization and translation of target mRNAs, it is well suited to contribute to the dynamic regulation of NOS1 activity. It should be noted, however, that *NOS1* mRNA may also be under additional, perhaps negative, posttranscription control, as suggested by the lack of NOS1 protein expression in the majority of *NOS1* mRNA-expressing pyramidal neurons. The modulation of neuronal function by NO in the brain has been widely studied, and postsynaptic NO is thought to represent a retrograde signal that promotes presynaptic differentiation (Bredt and Snyder, 1994; Garthwaite, 2008). Blockade of NOS1 function has been shown to disrupt synapse formation and result in spine loss (Nikonenko et al., 2008). Given the potential role of NO in synapse development, the loss of NOS1 expression in the fetal FXS brain during early synaptogenesis may contribute to the dendritic spine phenotype of human FXS (Irwin et al., 2000). Studies have also shown that NO mediates neuronal synchronization (O'Donnell and Grace, 1997) and can modulate protein function via *S*-nitrosylation (Jaffrey et al., 2001), including that of histones (Nott et al., 2008), which can mediate transcriptome changes. In future studies it will be important to characterize the mechanisms of NO function in the developing human neocortex and their potential contribution to FXS.

EXPERIMENTAL PROCEDURES

Human Brain Tissue Processing

The sources and methods for the collection, dissection, and fixation of control and fragile X postmortem human tissues are described in the Extended Experimental Procedures. All specimens were collected under guidelines approved by institutional review boards and anonymized prior to our receipt. Fixed tissues were sectioned by vibratome or cryostat. For NADPH-d staining, sections were incubated in β-NADPH, nitro blue tetrazolium, and Triton X-100. Sections were preincubated in hydrogen peroxide for immunohistochemistry or directly preblocked in blocking solution for immunofluorescent staining prior to incubation with primary antibodies followed by biotinylated or fluorophore-conjugated secondary antibodies. For immunohistochemistry, sections were further incubated in avidin-biotin-peroxidase complex and visualized using DAB.

RNA Pull-Down Assay and RIP

For pull-down assays, RNAs were transcribed from cDNA or PCR products, biotinylated, and captured using streptavidin beads. Human midfetal CP lysates were added, and bound proteins were analyzed by SDS-PAGE, silver staining, and immunoblotting. For RIP, FMRP-bound mRNAs were immunoprecipitated from midfetal human and neonatal mouse CP lysates and analyzed using quantitative RT-PCR.

Expression Assays and IUE

The generation of DNA constructs is described in the Extended Experimental Procedures. Neuro-2a cells were transfected by lipofection. Luciferase or NOS activity was assayed 48 hr after transfection and normalized to transfection efficiency controls. For electroporation, DNA was injected into the lateral ventricles of embryonic mice and transferred into VZ cells by 40V pulses. Electroporated brains were analyzed at P0 by immunostaining. All experiments using animals were carried out in accordance with a protocol approved by Yale University's Committee on Animal Research and National Institutes of Health guidelines.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cell.2012.02.060.

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REFERENCES

Abbeduto, L., Brady, N., and Kover, S.T. (2007). Language development and fragile X syndrome: profiles, syndrome-specificity, and within-syndrome differences. Ment. Retard. Dev. Disabil. Res. Rev. *13*, 36–46.

Ashley, C.T., Jr., Wilkinson, K.D., Reines, D., and Warren, S.T. (1993). FMR1 protein: conserved RNP family domains and selective RNA binding. Science *262*, 563–566.

Bagni, C., and Greenough, W.T. (2005). From mRNP trafficking to spine dysmorphogenesis: the roots of fragile X syndrome. Nat. Rev. Neurosci. *6*, 376–387.

Bassell, G.J., and Warren, S.T. (2008). Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. Neuron *60*, 201–214.

Bechara, E.G., Didiot, M.C., Melko, M., Davidovic, L., Bensaid, M., Martin, P., Castets, M., Pognonec, P., Khandjian, E.W., Moine, H., and Bardoni, B. (2009). A novel function for fragile X mental retardation protein in translational activation. PLoS Biol. *7*, e16.

Bredt, D.S., and Snyder, S.H. (1994). Nitric oxide: a physiologic messenger molecule. Annu. Rev. Biochem. *63*, 175–195.

Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., et al. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell *84*, 757–767.

Britanova, O., de Juan Romero, C., Cheung, A., Kwan, K.Y., Schwark, M., Gyorgy, A., Vogel, T., Akopov, S., Mitkovski, M., Agoston, D., et al. (2008). Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. Neuron *57*, 378–392.

Brown, V., Jin, P., Ceman, S., Darnell, J.C., O'Donnell, W.T., Tenenbaum, S.A., Jin, X., Feng, Y., Wilkinson, K.D., Keene, J.D., et al. (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell *107*, 477–487.

Bush, G., Frazier, J.A., Rauch, S.L., Seidman, L.J., Whalen, P.J., Jenike, M.A., Rosen, B.R., and Biederman, J. (1999). Anterior cingulate cortex dysfunction in attention-deficit/hyperactivity disorder revealed by fMRI and the Counting Stroop. Biol. Psychiatry *45*, 1542–1552.

Buxhoeveden, D., Lefkowitz, W., Loats, P., and Armstrong, E. (1996). The linear organization of cell columns in human and nonhuman anthropoid Tpt cortex. Anat. Embryol. (Berl.) *194*, 23–36.

Casanova, M.F., Buxhoeveden, D.P., Switala, A.E., and Roy, E. (2002). Minicolumnar pathology in autism. Neurology *58*, 428–432.

Chen, J.G., Rasin, M.R., Kwan, K.Y., and Sestan, N. (2005). *Zfp312* is required for subcortical axonal projections and dendritic morphology of deep-layer pyramidal neurons of the cerebral cortex. Proc. Natl. Acad. Sci. USA *102*, 17792–17797.

Chen, L., Yun, S.W., Seto, J., Liu, W., and Toth, M. (2003). The fragile X mental retardation protein binds and regulates a novel class of mRNAs containing U rich target sequences. Neuroscience *120*, 1005–1017.

Cui, H., Nishiguchi, N., Yanagi, M., Fukutake, M., Mouri, K., Kitamura, N., Hashimoto, T., Shirakawa, O., and Hishimoto, A. (2010). A putative cis-acting polymorphism in the *NOS1* gene is associated with schizophrenia and NOS1 immunoreactivity in the postmortem brain. Schizophr. Res. *121*, 172–178.

Dapretto, M., Davies, M.S., Pfeifer, J.H., Scott, A.A., Sigman, M., Bookheimer, S.Y., and Iacoboni, M. (2006). Understanding emotions in others: mirror neuron dysfunction in children with autism spectrum disorders. Nat. Neurosci. *9*, 28–30.

Darnell, J.C., Jensen, K.B., Jin, P., Brown, V., Warren, S.T., and Darnell, R.B. (2001). Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. Cell *107*, 489–499.

Darnell, J.C., Van Driesche, S.J., Zhang, C., Hung, K.Y., Mele, A., Fraser, C.E., Stone, E.F., Chen, C., Fak, J.J., Chi, S.W., et al. (2011). FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell *146*, 247–261.

Dawson, T.M., Bredt, D.S., Fotuhi, M., Hwang, P.M., and Snyder, S.H. (1991). Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc. Natl. Acad. Sci. USA *88*, 7797–7801.

Devinsky, O., Morrell, M.J., and Vogt, B.A. (1995). Contributions of anterior cingulate cortex to behaviour. Brain *118*, 279–306.

Dictenberg, J.B., Swanger, S.A., Antar, L.N., Singer, R.H., and Bassell, G.J. (2008). A direct role for FMRP in activity-dependent dendritic mRNA transport links filopodial-spine morphogenesis to fragile X syndrome. Dev. Cell *14*, 926–939.

The Dutch-Belgian Fragile X Consortium. (1994). *Fmr1* knockout mice: a model to study fragile X mental retardation. Cell *78*, 23–33.

Fertuzinhos, S., Krsnik, Z., Kawasawa, Y.I., Rasin, M.R., Kwan, K.Y., Chen, J.G., Judas, M., Hayashi, M., and Sestan, N. (2009). Selective depletion of molecularly defined cortical interneurons in human holoprosencephaly with severe striatal hypoplasia. Cereb. Cortex *19*, 2196–2207.

Garthwaite, J. (2008). Concepts of neural nitric oxide-mediated transmission. Eur. J. Neurosci. *27*, 2783–2802.

Geschwind, D.H., and Levitt, P. (2007). Autism spectrum disorders: developmental disconnection syndromes. Curr. Opin. Neurobiol. *17*, 103–111.

Ho, G.P., Selvakumar, B., Mukai, J., Hester, L.D., Wang, Y., Gogos, J.A., and Snyder, S.H. (2011). S-nitrosylation and S-palmitoylation reciprocally regulate synaptic targeting of PSD-95. Neuron *71*, 131–141.

Hope, B.T., Michael, G.J., Knigge, K.M., and Vincent, S.R. (1991). Neuronal NADPH diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. USA *88*, 2811–2814.

Iioka, H., Loiselle, D., Haystead, T.A., and Macara, I.G. (2011). Efficient detection of RNA-protein interactions using tethered RNAs. Nucleic Acids Res. *39*, e53.

Irwin, S.A., Galvez, R., and Greenough, W.T. (2000). Dendritic spine structural anomalies in fragile-X mental retardation syndrome. Cereb. Cortex *10*, 1038–1044.

Jaffrey, S.R., Erdjument-Bromage, H., Ferris, C.D., Tempst, P., and Snyder, S.H. (2001). Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat. Cell Biol. *3*, 193–197.

Johnson, M.B., Kawasawa, Y.I., Mason, C.E., Krsnik, Z., Coppola, G., Bogdanović, D., Geschwind, D.H., Mane, S.M., State, M.W., and Sestan, N. (2009). Functional and evolutionary insights into human brain development through global transcriptome analysis. Neuron *62*, 494–509.

Judas, M., Sestan, N., and Kostović, I. (1999). Nitrinergic neurons in the developing and adult human telencephalon: transient and permanent patterns of expression in comparison to other mammals. Microsc. Res. Tech. *45*, 401–419.

Kang, H.J., Kawasawa, Y.I., Cheng, F., Zhu, Y., Xu, X., Li, M., Sousa, A.M.M., Pletikos, M., Meyer, K.A., Sedmak, G., et al. (2011). Spatio-temporal transcriptome of the human brain. Nature *478*, 483–489.

Keller, S.S., Crow, T., Foundas, A., Amunts, K., and Roberts, N. (2009). Broca's area: nomenclature, anatomy, typology and asymmetry. Brain Lang. *109*, 29–48.

Kostovic, I., and Rakic, P. (1990). Developmental history of the transient subplate zone in the visual and somatosensory cortex of the macaque monkey and human brain. J. Comp. Neurol. *297*, 441–470.

Kraskov, A., Dancause, N., Quallo, M.M., Shepherd, S., and Lemon, R.N. (2009). Corticospinal neurons in macaque ventral premotor cortex with mirror properties: a potential mechanism for action suppression? Neuron *64*, 922–930.

Kwan, K.Y., Lam, M.M., Krsnik, Z., Kawasawa, Y.I., Lefebvre, V., and Sestan, N. (2008). SOX5 postmitotically regulates migration, postmigratory differentiation, and projections of subplate and deep-layer neocortical neurons. Proc. Natl. Acad. Sci. USA *105*, 16021–16026.

Lai, C.S., Fisher, S.E., Hurst, J.A., Vargha-Khadem, F., and Monaco, A.P. (2001). A forkhead-domain gene is mutated in a severe speech and language disorder. Nature *413*, 519–523.

Leone, D.P., Srinivasan, K., Chen, B., Alcamo, E., and McConnell, S.K. (2008). The determination of projection neuron identity in the developing cerebral cortex. Curr. Opin. Neurobiol. *18*, 28–35.

Lui, J.H., Hansen, D.V., and Kriegstein, A.R. (2011). Development and evolution of the human neocortex. Cell *146*, 18–36.

Menon, V., Leroux, J., White, C.D., and Reiss, A.L. (2004). Frontostriatal deficits in fragile X syndrome: relation to FMR1 gene expression. Proc. Natl. Acad. Sci. USA *101*, 3615–3620.

Molliver, M.E., Kostović, I., and van der Loos, H. (1973). The development of synapses in cerebral cortex of the human fetus. Brain Res. *50*, 403–407.

Molyneaux, B.J., Arlotta, P., Menezes, J.R., and Macklis, J.D. (2007). Neuronal subtype specification in the cerebral cortex. Nat. Rev. Neurosci. *8*, 427–437.

Mountcastle, V.B. (1997). The columnar organization of the neocortex. Brain *120*, 701–722.

Nikonenko, I., Boda, B., Steen, S., Knott, G., Welker, E., and Muller, D. (2008). PSD-95 promotes synaptogenesis and multiinnervated spine formation through nitric oxide signaling. J. Cell Biol. *183*, 1115–1127.

Nott, A., Watson, P.M., Robinson, J.D., Crepaldi, L., and Riccio, A. (2008). S-Nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. Nature *455*, 411–415.

O'Donnell, P., and Grace, A.A. (1997). Cortical afferents modulate striatal gap junction permeability via nitric oxide. Neuroscience *76*, 1–5.

Peters, A. (2010). The morphology of minicolumns. In The Neurochemical Basis of Autism: from Molecules to Minicolumns, G.J. Blatt, ed. (New York: Springer), pp. 45–68.

Pierce, K., and Redcay, E. (2008). Fusiform function in children with an autism spectrum disorder is a matter of "who". Biol. Psychiatry *64*, 552–560.

Power, J.D., Cohen, A.L., Nelson, S.M., Wig, G.S., Barnes, K.A., Church, J.A., Vogel, A.C., Laumann, T.O., Miezin, F.M., Schlaggar, B.L., et al. (2011). Functional network organization of the human brain. Neuron *72*, 665–678.

Rackham, O., and Brown, C.M. (2004). Visualization of RNA-protein interactions in living cells: FMRP and IMP1 interact on mRNAs. EMBO J. *23*, 3346–3355.

Rakic, P. (1988). Specification of cerebral cortical areas. Science *241*, 170–176.

Ramocki, M.B., and Zoghbi, H.Y. (2008). Failure of neuronal homeostasis results in common neuropsychiatric phenotypes. Nature *455*, 912–918.

Reif, A., Herterich, S., Strobel, A., Ehlis, A.C., Saur, D., Jacob, C.P., Wienker, T., Töpner, T., Fritzen, S., Walter, U., et al. (2006). A neuronal nitric oxide synthase (NOS-I) haplotype associated with schizophrenia modifies prefrontal cortex function. Mol. Psychiatry *11*, 286–300.

Reif, A., Jacob, C.P., Rujescu, D., Herterich, S., Lang, S., Gutknecht, L., Baehne, C.G., Strobel, A., Freitag, C.M., Giegling, I., et al. (2009). Influence of functional variant of neuronal nitric oxide synthase on impulsive behaviors in humans. Arch. Gen. Psychiatry *66*, 41–50.

Rizzolatti, G., and Craighero, L. (2004). The mirror-neuron system. Annu. Rev. Neurosci. *27*, 169–192.

Rogers, S.J., Wehner, D.E., and Hagerman, R. (2001). The behavioral phenotype in fragile X: symptoms of autism in very young children with fragile X syndrome, idiopathic autism, and other developmental disorders. J. Dev. Behav. Pediatr. *22*, 409–417.

Schaeffer, C., Bardoni, B., Mandel, J.L., Ehresmann, B., Ehresmann, C., and Moine, H. (2001). The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif. EMBO J. *20*, 4803–4813.

Sestan, N., and Kostović, I. (1994). Histochemical localization of nitric oxide synthase in the CNS. Trends Neurosci. *17*, 105–106.

Shinkai, T., Ohmori, O., Hori, H., and Nakamura, J. (2002). Allelic association of the neuronal nitric oxide synthase (*NOS1*) gene with schizophrenia. Mol. Psychiatry *7*, 560–563.

Siomi, H., Choi, M., Siomi, M.C., Nussbaum, R.L., and Dreyfuss, G. (1994). Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell *77*, 33–39.

State, M.W. (2010). The genetics of child psychiatric disorders: focus on autism and Tourette syndrome. Neuron *68*, 254–269.

Todd, P.K., Mack, K.J., and Malter, J.S. (2003). The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent translation of PSD-95. Proc. Natl. Acad. Sci. USA *100*, 14374–14378.

Tu, P.C., Hsieh, J.C., Li, C.T., Bai, Y.M., and Su, T.P. (2012). Cortico-striatal disconnection within the cingulo-opercular network in schizophrenia revealed by intrinsic functional connectivity analysis: a resting fMRI study. Neuroimage *59*, 238–247.

Walsh, C.A., Morrow, E.M., and Rubenstein, J.L. (2008). Autism and brain development. Cell *135*, 396–400.

Willemsen, R., Levenga, J., and Oostra, B.A. (2011). CGG repeat in the FMR1 gene: size matters. Clin. Genet. *80*, 214–225.

Zalfa, F., Eleuteri, B., Dickson, K.S., Mercaldo, V., De Rubeis, S., di Penta, A., Tabolacci, E., Chiurazzi, P., Neri, G., Grant, S.G., and Bagni, C. (2007). A new function for the fragile X mental retardation protein in regulation of PSD-95 mRNA stability. Nat. Neurosci. *10*, 578–587.