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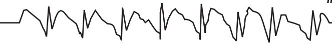

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Neuro2D Lies at the Nexus of Autism, Epilepsy, and Intellectual Disabilities

Disruption of NEUROD2 causes a neurodevelopmental syndrome with autistic features via cell-autonomous defects in forebrain glutamatergic neurons

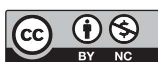
Runge K, Mathieu R, Bugeon S, et al. *A. Mol Psychiatry* 2021;26:7852. doi: 10.1038/s41380-021-01179-x.

Although the transcription factor *NEUROD2* has recently been associated with epilepsy, its precise role during nervous system development remains unclear. Using a multi-scale approach, we set out to understand how Neurod2 deletion affects the development of the cerebral cortex in mice. In Neurod2 KO embryos, cortical projection neurons over-migrated, thereby altering the final size and position of layers. In juvenile and adults, spine density and turnover were dysregulated in apical but not basal compartments in layer 5 neurons. Patch-clamp recordings in layer 5 neurons of juvenile mice revealed increased intrinsic excitability. Bulk RNA sequencing showed dysregulated expression of many genes associated with neuronal excitability and synaptic function, whose human orthologs were strongly associated with autism spectrum disorders (ASD). At the behavior level, Neurod2 KO mice displayed social interaction deficits, stereotypies, hyperactivity, and occasionally spontaneous seizures. Mice heterozygous for Neurod2 had similar defects, indicating that Neurod2 is haploinsufficient. Finally, specific deletion of Neurod2 in forebrain excitatory neurons recapitulated cellular and behavioral phenotypes found in constitutive KO mice, revealing the region-specific contribution of dysfunctional Neurod2 in symptoms. Informed by these neurobehavioral features in mouse mutants, we identified eleven patients from 8 families with a neurodevelopmental disorder including intellectual disability and ASD associated with NEUROD2 pathogenic mutations. Our findings demonstrate crucial roles for Neurod2 in neocortical development, whose alterations can cause neurodevelopmental disorders including intellectual disability and ASD.

Commentary

NEUROD2 belongs to the family of Neuron Differentiation (NEUROD) basic helix-loop-helix (bHLH) proteins that are transcription factors involved in central and peripheral nervous system development.[1] More specifically, these proteins regulate early neuronal differentiation as well as maturational processes such as dendritic patterning for *NeuroD2*. The NeuroD family contains 4 closely related proteins: Neurod1 (or NeuroD), Neurod2 (also called NeuroD-related factor NDRF), Neurod4, and Neurod6 (also called Nex and Math2) displaying overlapping expression patterns that are not identical in the developing cortex.[1] All 4 genes appear around embryonic day 12/13 in mouse. NeuroD4 has the most restricted expression confined to the ventricular zone of the dorsal telencephalon. NeuroD1 is expressed in both mitotic and post-mitotic neuronal cells during development and ultimately is restricted to upper pyramidal neurons. NeuroD1 and 6 are abundantly expressed in post-mitotic pyramidal neurons. Although their expression decreases after birth, NeuroD6 persists in deep layer neurons while NeuroD2 persists in pyramidal neurons across all cortical layers. NeuroD2 has recently attracted attention because 2 recent studies identified de novo mutations in the DNA binding domain of NeuroD2 associated with early infantile epileptic encephalopathy in 2 children[2] and with neurodevelopmental delay in 1 child.[3] The first study confirmed the causative role of *NeuroD2* mutation for the induction of epilepsy by using *X. laevis* tadpoles. CRISPR/Cas9 knockdown to mimic loss-of-function mutations led to seizures in tadpoles. In addition, overexpression of the NeuroD2 mutant gene failed to induce ectopic neuronal differentiation that occurs with overexpression of a wildtype *NeuroD2* gene. The second study did not examine whether the identified *NeuroD2* mutation led to neurodevelopmental delay. Further examining a connection of de novo *NeuroD2* mutations to neurodevelopmental delay and their causative roles is the goal of the present study.

To achieve this goal, the authors identified 6 patients with missense mutations in *NEUROD2*, 5 with de novo and 1 with germline mutations, that displayed the disabilities listed above. Non-penetrant phenotypes in these patients included ADHD symptoms (5/7 patients) and epilepsy (3/7 patients). It is not fully understood how specific gene variants lead to different behavioral phenotypes, but it is thought that the different pathogenicity of gene variants contributes to the observed variability. The authors developed a versatile assay to determine the pathogenicity of each *NeuroD2* variant. They took advantage of the fact that



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overexpression of wildtype human *NEUROD2* in P19 mouse embryonic carcinoma cells induces neuronal differentiation (90% of the cells), which relies on DNA binding. Some *NEUROD2* variants did not induce neuronal differentiation while others gave an intermediate phenotype (45% of the cells). This assay is thus sensitive enough to detect differences among variants and suggest that the identified variants are pathogenic due to a loss of NEUROD2 transcription factor activity.

To validate a causative link between NEUROD2 loss-of-function and the behavioral symptoms, the authors used *Neurod2* knockout (KO) mice. These mice displayed autism-relevant social abnormalities, hyperactivity, and seizures. In addition, a co-expression network analysis in humans using psychENCODE[4] revealed that *NEUROD2* is positioned as a hub in a cortical transcriptional regulatory network associated with neurodevelopmental disorders including autism and intellectual disabilities. Considering that some gene variants had an intermediate phenotype in PC19 cells, it would be intriguing to generate CRISPR/Cas9 mice with specific variants and examine whether the different mouse lines display similar or more severe behavioral defects. This would support the idea that different gene variants with different pathogenicity led to different behavioral phenotypes. One limitation is that these mice display seizure activity, which could affect social and repetitive behavior. It can be difficult to untangle seizures and abnormal behavior.

Mechanistically, at postnatal day 30, they found no gross abnormalities of the cortex and no gross axon targeting defect in the *Neurod2* KO mice. However, there was a small reduction in corpus callosum size, and a superficial shift of pyramidal neuron positioning in both *Neurod2* KO and heterozygote mice. *NeuroD2/NeuroD6* double KO displayed the total absence of corpus callosum.[5] The absence of major corpus callosum defects in *Neurod2* KO mice is likely due to the functional redundancy between NeuroD family members. In utero electroporation of Cre recombinase in conditional *NeuroD2* KO mice (*Neurod2*$^{fl/fl}$) recapitulated the over-migration phenotype confirming that it was a cell-autonomous defect. The authors provide data supporting an altered cell shape in the intermediate zone leading to an acceleration of the transition from multipolar cell to more elongated cells that can rapidly migrate. Their data are in contrast with another study using *Neurod2* shRNA that suggested altered terminal translocation.[6] The reason for the discrepancy remains unclear, and it may be worth checking other shRNA sequences to rule out off-target effects. Other defects include alterations in spines and excitability. Intriguingly, *Neurod2* removal differentially affected synaptic activity in layer (L) 2/3 and L5 cortical pyramidal neurons, while intrinsic excitability was increased in both. Finally, they also found alterations in spine density and in vivo turnover in L5 neurons but not in L2/3 neurons. These data suggest that we cannot generalize findings in pyramidal neurons of different cortical layers.

Considering the identified alterations in pyramidal neurons and the fact that NeuroD2 is exclusively expressed in these neurons in the cortex (confirmed in this study), they generated conditional *Neurod2* KO mice in forebrain excitatory neurons using *Emx1*-Cre mice crossed with *Neurod2*$^{fl/fl}$ mice. These mice displayed the behavioral abnormalities observed in *Neurod2* KOs suggesting that *Neurod2* loss-of-function in pyramidal neurons drives the neurodevelopmental abnormalities. However, the *Emx1*-Cre mice also induce recombination in the hippocampus and some parts of the amygdala. Whether Neurod2 loss in these regions contributes to the behavioral abnormalities remains to be examined. It would also be intriguing to examine layer specific loss of *Neurod2* in the neocortex on behavior as well as the impact of specific gene variant on neuronal development using in utero electroporation combined with CRISPR/Cas9 system.

The authors also attempted to identify potential molecular mechanisms accounting for the cellular defects by performing gene ontology analyses of a Chip-seq dataset from E14.5 cortex.[7] They found specific genes involved in multipolar-to-bipolar cell transition. They also found that the *Neurod2* KO mice displayed 39 altered synaptome genes consistent with the identified defects in spines and synaptic activity. One such gene of particular interest is the gluococorticoid receptor Nr3c1 that requires Neurod2 as a cofactor and regulates spine plasticity upon stress. Ultimately, it will be critical to perform rescue experiments of selective genes and assess the impact on the cellular and behavioral phenotypes.

Collectively, the present study provides strong evidence that *NeuroD2* loss-of-function mutations resulting in loss of DNA binding and transcriptional activity leads to neurodevelopmental disorders characterized with autistic features, epilepsy, hyperactivity, and speech disturbances depending on the gene variants. It will remain important to further investigate the molecular mechanism responsible for specific cellular defects in each cortical layer and ultimately behavioral abnormalities. It is also important to sort out the contribution of brain regions other than the cortex (e.g., amygdala) to the behavioral defects. Intriguingly, examination of the DECIPHER human genome database identified a duplication in the NEUROD2 gene associated with intellectual disability and delayed language development. This remarkably highlights that too much or too little Neurod2 as reported for other genes (e.g., FLNA[8]) leads to behavioral abnormalities reinforcing the importance of *NEUROD2* as a critical hub gene in the etiology of neurodevelopmental disorders.

By Angelique Bordey, PhD

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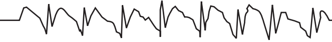

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