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NeuroD 2/6 регулируют баланс экспрессии транскрипционных факторов, контролирующих цитоархитектуру коры головного мозга

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АННОТАЦИЯ

Обоснование. Гены семейства *NeuroD*, включая *NeuroD1, NeuroD2, NeuroD6,* контролируют выживаемость, дифференцировку, созревание нейронов и формирование нейритов в нервной системе. В мозге мышей делеция *NeuroD1* приводит к полной потере зубчатой извилины гиппокампа вследствие апоптоза нейронов. *NeuroD2* необходим для выживания нейронов в мозжечке и интеграции таламокортикальных связей в новой коре, а также для формирования соматосенсорной коры. В недавних работах авторов показано, что у мышей с двойным дефицитом *NeuroD2/6* аксоны мозолистого тела дефектны из-за нарушений передачи сигналов, опосредованных белком EfnA4. Для того чтобы охарактеризовать молекулярные каскады, регулируемые белками NeuroD2/6, авторы исследовали экспрессию ключевых факторов транскрипции, которые контролируют различные аспекты развития коры головного мозга у мышей с дефицитом *NeuroD2* и *NeuroD6.*
Цель исследования — изучение возможных изменений программ дифференцировки транскрипционных факторов, связанных с NeuroD2/6.
Методы. В экспериментах использовали эмбрионы с двойным дефицитом *NeuroD2/6.* Генотип определяли методом полимеразной цепной реакции. Беременных мышей, несущих эмбрионы E13.5, оперировали для внутриутробной электропорации. Для изучения паттерна экспрессии генов-мишеней проводили *in situ* гибридизацию с синтетической РНК на разных стадиях эмбрионального развития. Для анализа активности промоторов соответствующих генов фрагменты геномной ДНК, содержащие мотивы связывания NeuroD2/6, клонировали в вектор pMCS-GL для измерения активности люциферазы. Относительные количественные данные анализировали с помощью парного одностороннего t-критерия Стьюдента. Графики сделаны с помощью программного обеспечения GraphPad Prism и представлены как среднее значение ± стандартная ошибка.
Результаты. Установлено, что экспрессия NeuroD1 эктопически активируется в постмитотических нейронах неокортекса и гиппокампа в двойном нокауте *NeuroD2/6.* Обнаружено также, что экспрессия транскрипционных факторов Cux1, Tbr1, Lhx2, Id2 нарушена в двойном нокауте *NeuroD2/6*. Показано, что Cux1 является прямой мишенью NeuroD2/6. Кроме того, выявлено, что количество предшественников $Olig2^+$ увеличивается в неокортексе мышей с двойным нокаутом *NeuroD2/6,* а экспрессия NeuroD2/6 и Olig2 является взаимоисключающей. Таким образом, установлено, что NeuroD2/6 регулируют экспрессию нескольких факторов транскрипции в развивающемся мозге.
Заключение. Кумулятивное действие обоих генов необходимо для инициации и поддержания экспрессии факторов транскрипции Cux1, Tbr1, Lhx2, Id2 и Olig2.
Ключевые слова: NeuroD; факторы транскрипции; эктопическая экспрессия; кора головного мозга.

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NeuroD 2/6 regulates expression balance of transcription factors controlling neurocortical cytoarchitecture

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ABSTRACT

BACKGROUND: Genes of the *NeuroD* family, including *NeuroD1*, *NeuroD2*, and *NeuroD6*, control neuronal survival, differentiation, maturation, and neurite patterning in the nervous system. Deletion of NeuroD1 in the mouse brain results in complete loss of dentate gyrus because of neuronal apoptosis. NeuroD2 is required for neuron survival in the cerebellum and integration of thalamo-cortical connections into neocortex and formation of somatosensory whisker barrel cortex. In *NeuroD2/6* double deficient (DKO) mice, callosal axon projections are defective due to abnormal EfnA4 signaling. For further investigating the NeuroD2/6 controlled molecular cascade, we explored the expression of key transcription factors that control various aspects of cortical development in brains of *NeuroD2* and *NeuroD6* deficient mutants.
AIM: To investigate possible changes in differentiation programs downstream of NeuroD2/6 transcription factors.
METHODS: Embryos with *NeuroD2/6* double deficiency were used in the experiments, and pregnant mice carrying E13.5 embryos were operated for *in utero* electroporation. We performed *in situ* hybridization with preliminary synthesis of probes at various stages of embryonic development to study the expression pattern of target genes. Analyzing the activity of a gene promoter, genomic DNA fragments containing NeuroD2/6 motifs were cloned into pMCS-Gaussia Luc vector for luciferase assays. Charts were made with GraphPad Prism software and data were presented as mean ± standard error.
RESULTS: Our findings showed that NeuroD1 expression is ectopically upregulated in postmitotic neurons of *NeuroD2/6* DKO neocortex and hippocampus. We detected changes in expression of key transcription factors, Cux1, Tbr1, Lhx2, and Id2. Additionally, Cux1 was a direct target of NeuroD2/6. Moreover, $Olig2^+$ progenitors increased in *NeuroD2/6* DKO neocortex and expression of NeuroD2/6 and Olig2 was mutually exclusive. Thus, NeuroD2/6 regulates the expression of transcription factors in the developing brain.
CONCLUSION: Our findings indicate that cumulative action of *NeuroD2* and *NeuroD6* is required to initiate and maintain the expression of transcription factors Cux1, Tbr1, Lhx2, and Id2. Additionally, both genes are required to prevent premature differentiation of Olig2 positive glial precursors.
Keywords: NeuroD; transcription factors; ectopic expression, cerebral cortex.

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INTRODUCTION

The mammalian cerebral cortex is a highly organized structure, consisting of various cell types, including subclasses of progenitors, neurons, and glia cells [1, 2]. Neurons are the main functional cell

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populations and can be categorized into two major groups: ~20% GABAergic inhibitory interneurons and ~80% glutamatergic excitatory pyramidal neurons [3]. While interneurons establish local circuits with adjacent cells, cortical pyramidal neurons tend to form long-range projections. Glutamatergic projections contact and transmit signals to the contralateral hemisphere, thalamus, and spinal cord [4].

Helix-loop-helix (HLH) proteins are generally transcriptional activators involved in the regulation of cell survival, differentiation, migration, and fate specification. They can be categorized into two subclasses: I and II, the former termed E-box proteins and the latter as basic HLH (bHLH) transcription factors (TFs). E-box proteins and bHLH TFs form intersubclass heterodimers during DNA binding and recognize an E-box motif with CANNTG elementary sequence [5].

NeuroD1, NeuroD2, and NeuroD6 are three phylogenetically closely related bHLH TFs, collectively called NeuroD family TFs that control neuronal survival, differentiation, maturation, and neurite patterning in the nervous system. In the developing cerebral cortex, *NeuroD1*, *NeuroD2*, and *NeuroD6* genes are expressed in pyramidal neurons. Their expression initiates after a cell leaves the mitotic cycle and begins to differentiate while residing in the subventricular zone (SVZ). NeuroD1 expression decreases when a neuron begins migrating toward its final position, but the expression of NeuroD2 and NeuroD6 is maintained in postmigratory neurons [6].

NeuroD1 deletion in the mouse brain results in complete loss of the hippocampal dentate gyrus owing to neuronal apoptosis [7]. *NeuroD2* is required for neuron survival in the cerebellum, and it is essential for integrating thalamo-cortical connections into neocortex and formation of somatosensory whisker barrel cortex [8]. In *NeuroD2/6* double deficient (DKO) mice, callosal axon projections are defective due to abnormal EfnA4 signaling [9, 10].

To further investigate *NeuroD2/6* controlled molecular cascade, we explored the expression of key TFs that control various aspects of cortical development in brains of *NeuroD2* and *NeuroD6* deficient mutants. Our results indicated that cumulative action of both genes is required to initiate and maintain the expression of TFs Cux1, Tbr1, Lhx2, and Id2. Additionally, both genes are required to prevent premature differentiation of Olig2 positive glial precursors.

Aim — this study investigated the possible changes in differentiation programs downstream of NeuroD2/6 transcription factors.

MATERIALS AND METHODS

MOUSE MUTANTS

Generation of *NeuroD2/6* DKO embryos: a male and female in NRX line ($NeuroD6^{Cre/Cre/}NeuroD2^{+/-}$) were bred to produce embryos with mixed genotypes (25% incidence of *NeuroD2/6* double mutants: $NeuroD6^{Cre/Cre}/NeuroD2^{-/-}$). The embryos of the other genotypes ($NeuroD6^{Cre/Cre}/NeuroD2^{+/+}$ and $NeuroD6^{Cre/Cre}/NeuroD2^{+/-}$) were used as controls in the project. The mice of all genotypes were originally from C57BL/6 genetic background. The day on which the plug was detected was considered embryonic day 0.5 (E0.5). Male and female embryos were used in this study. The experiments were approved by the Bioethics Committee of National Research Lobachevsky State University of Nizhny Novgorod (protocol N 14 dated 01.19.2018).

IN UTERO ELECTROPORATION

Mostly, pregnant mice carrying E12.5 or E13.5 embryos were subjected to surgery. Plasmids (1 μg/μl) were mixed with fast green dye (1:20,000). During the entire process, the pregnant mice in surgery were laid down on a heating pad and anesthetized by constant inhalation of isoflurane mixed with oxygen. The abdominal region was cleaned with 70% ethanol and iodine followed by subcutaneous administration of Tamgesic before the operation started. An incision of ~15 mm was made on the fur and skin along the abdomen midline. The embryos wrapped in the uterine wall were gently pulled out with ring-headed forceps without damaging the blood vessels and visceral organs. DNA loaded in a fine glass capillary (pulled by HEKA-PIP6 capillary puller) was forced by a vacuum pico-pump into either of the cerebral lateral ventricles (fast green dye spreading over the injected hemisphere). Electrodes were placed on both sides of the head, with positive on the electroporated side. Electroporation was achieved by an electroporator (CUY21, Sonidel) using the following settings: 6 times pulses, 35 V voltage, 50 ms pulse duration, and 999 ms interval time. Consistent application of 1×PBS, containing antibiotics (1,000 units/ml of Penicillin-Streptomycin, Gibco, USA) to each operated embryo, was performed during and after electroporation. After all embryos were electroporated, they were returned carefully into the abdominal cavity filled with more 1×PBS

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containing antibiotics. The skin was sewn up with sterilized sutures. The fur was closed with surgical staples. After surgery, the mice were put back into individual cages marked with genes and electroporation dates. The operated mice were monitored every day for their health and sacrificed when embryos reached E18.5.

GENOTYPING AND POLYMERASE CHAIN REACTION

The double mutant genotype was determined by polymerase chain reaction (PCR) using the following primers: NDRFFw: 5′-TTC TCG CTC AAG CAG GAC-3′, NeoRFw: 5′-AGT GAC AAC GTC GAG CAC AG-3′, NDRFRev: 5′-CCC ACA GCT AAG AGA GCA CG-3′. A 20-µl PCR reaction was used containing 10× buffer 2 µl (Promega, USA), 0.4 µl 10 mM dNTPs (Invitrogen, USA), 10 nmol/ml each of 0.5 µl primers, 0.1 µl Go-taq polymerase (Promega, USA), 2 µl isolated tail DNA, and 14 µl distilled deionized water (ddH_2O). The amplification program was as follows: 94 °C for 15 s, 60 °C for 18 s, 72°C for 1 min; amplification was set for 35 cycles. The final elongation for 5 min at 72 °C and samples were cooled down to room temperature for DNA gel electrophoresis.

IN SITU HYBRIDIZATION

To perform *in vitro* transcription for synthesis of *in situ* hybridization (ISH) probes, the following reaction mixture was prepared before it was incubated for 3 h at 37 °C: 2 µg linearized plasmids as template, 2 µl 10× transcription buffer (Roche, Switzerland), 2 µl Digoxigenin-labeled RNA mix (Roche, Switzerland), 1 µl RNAse inhibitor (NEB), 2 µl T7 or Sp6 RNA polymerase (Roche, Switzerland), and up to 20 µl DEPC-treated H_2O. The synthesized anti-sense RNA probes were mixed with 3 µl lithium chloride (4 M) and incubated at −20 °C O/N. The probe precipitation was achieved by centrifugation (13.2 krpm at 4 °C for 20 min). RNA pellets were washed twice by centrifugation in 150 µl 70% ethanol before they were resuspended in 33 µl DEPC-treated H_2O. A volume of 3 µl of each probe was subjected to fast DNA gel electrophoresis to monitor RNA quality, and the remaining was mixed with 170 µl hybridization buffer (HB, 50% deionized formamide (AppliChem, Germany), 5× SSC, 1% blocking reagent (Roche, Switzerland), 5 mM EDTA, 0.1% Tween20 (Sigma-Aldrich, USA), 0.1% CHAPS (Sigma-Aldrich, USA), 0.1 mg/ml Heparin (Sigma-Aldrich, USA), and 100 µg/ml yeast RNA (Invitrogen, USA)). The probes were maintained at −20 °C. ISH: on day 1, tissue sections were dried in a vacuum for 30 min, and fixed in 4% paraformaldehyde (PFA) dissolved in DEPC-treated 1×PBS (DPBS) for 15 min followed by twice quick washes in DPBS (5 min each) before they were incubated in pK solution (20 mM Tris pH7.5, 1 mM EDTA pH8.0, 20 µg/ml proteinase K) for 2.5 min. Subsequently, sections were washed in 0.2% glycine (AppliChem, Germany) in DPBS, quickly washed twice in DPBS, and post-fixed in 4% PFA containing 0.2% glutaraldehyde (Sigma-Aldrich, USA) in DPBS for 15 min, quickly washed twice in DPBS, prehybridized in HB at 65 °C for 2 h, and eventually hybridized with the denatured probes (10 µl probe in 160 µl HB, heated at 90 °C for 5 min, kept on ice) at 68 °C O/N. On day 2, the sections were washed once in 2× SSC pH 4.5, incubated in RNase solution (0.5 M NaCl, 10 mM Tris pH8.0, 20 µg/ml RNase (Sigma-Aldrich, USA)) for 30 min at 37 °C, washed once in 2× SSC pH 4.5, stringently three times in 50% formamide/2×SSC pH 4.5 (30 min each) at 63 °C, and eventually three times in KTBT buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM KCl, 1% Triton X-100) for 10 min each. Subsequently, the sections were incubated in a blocking solution (KTBT containing 20% sheep serum (Sigma-Aldrich, USA)) for 2 h before they were incubated with anti-digoxigenin antibody (alkaline phosphatase (AP)-conjugated, Roche, 1:1,500) in blocking solution at 4 °C O/N. On day 3, the sections were washed four times in KTBT for 30 min each time, twice in NTMT buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween20) for 15 min each, and were eventually incubated in NTMT containing NBT/BCIP (AP substrates, Roche, Switzerland). The staining was monitored hourly until the signals showed up. The stained sections were subjected to an ascending alcohol series (50–100%), incubated in a clearing solution (benzyl alcohol:benzyl benzoate = 1:2) for 5 min, and finally mounted using Eukitt (O. Kindler).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry: on day 1, cryo-sections (16 µm thick) were dried in a vacuum for 30 min, and fixed in 4% PFA (dissolved in 1×PBS) for 15 min followed by twice quick washes in 1×PBS. Subsequently, the sections were incubated in a blocking solution (blocking solution, 1×PBS containing 0.5% Triton X-100, 2% BSA, and 10% horse serum (Sigma-Aldrich, USA)), before they were incubated with primary antibodies in blocking solution at 4 °C O/N. The following day, sections were quickly washed three times in 1× PBS, and incubated with secondary antibodies in blocking solution

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for 1.5 h and three times for 10 min each. The slides were then mounted with DAKO anti-artifact medium (DAKO).

LUCIFERASE PROMOTER ASSAY

Seven genomic DNA fragments containing 3–5 potential NeuroD2/*6* motifs (E-boxes) within 5 kbp upstream region of Cux1 transcriptional origin were cloned into pMCS-Gaussia Luc vector for luciferase assays (pMCS-GL vector) (Thermo Scientific, USA) for luminescence measurement. The following primers were used for PCR amplification:
E1Fw: 5′-AGGCTGGCCCTGAACTTAGAGATCG-3′
E1Rv: 5′- CAGAGGACCTGAGTTCAGCTCCCAG-3′
E2Fw: 5′- AAAATTTCACTTCTTTATCATTAGT-3′
E2Rv: 5′-AATTCTTTTCCTTATTATTCTAACA-3′
E3Fw: 5′-TGAGTAGATAACAGGAATGCACCAC-3′
E3Rv: 5′-TCTTGTATTATTACCCAAAACAGTG-3′
E4Fw: 5′-AAGCTTTTTTTCCTGCGTCAGAGCC-3′
E4Rv: 5′-GATGTTTAGAACAAGTATAGTCATA-3′
E5Fw: 5′-AAAGCTTAAGCTCTGGGCCTCCCAA-3′
E5Rv: 5′-TAAAGGTGGCTAGACAAAATGGGCA-3′
E6Fw: 5′-CCGAGGTCCGCAGTGTCCCGCCTGG-3′
E6Rv: 5′-GTTTGAGGGCTTAGCGTTATTCGGT-3′
E7Fw: 5′-AGTCGCGGCGGCAGCAGCCGCAGGC-3′
E7Rv: 5′-TGCGCTGCGGCAGCGGGGCGCGGGC-3′
A mass of 100 ng pCMV-AP (for normalization), 250 ng pCAG-GFP or pCAG-NeuroD2, and 250 ng pMCS-GL containing each of the genomic fragment (empty pMCS-GL vector used as baseline) were co-transfected into HEK293 cells in triplicate wells of a 24-well plate by lipofectamine 2000. After a 48 h incubation, the supernatants were collected and used for assays. The assay was performed with Secrete-Pair Dual Luminescence assay kit (GeneCopoeia, USA), as previously described [10, 11]. Luminescent signals were measured by Glomax luminescence reader (Promega, USA). We used ApE plasmid editor program, generated at Erik Jorgensen's lab, MIT, for bioinformatic analysis [12].

MICROSCOPY AND IMAGE ACQUISITION

Bright field images (for ISH results) were obtained with the microscope Olympus BX60 and software AxioVision 4.8 (Carl Zeiss, Germany). Fluorescent images (for IHC results) were obtained with confocal microscope Leica TCS SL and configured software (Leica Microsystems, Germany). The same manipulations (such as background subtraction, contrast adjustment, and photomerge) were performed on the paired images of the control and DKO brain sections by Adobe photoshop CS3 or ImageJ.

STATISTICS

The relative quantitative data of luciferase assays were analyzed with a paired one-tailed t-test. The charts were made with GraphPad Prism software and presented as mean ± standard error. P values <0.05 were considered significant: $0.01< p < 0.05$ (*), $0.001< p < 0.01$(**), and $p <0.001$(***).

RESULTS

NEUROD1 EXPRESSION IS ECTOPICALLY UPREGULATED IN POSTMITOTIC NEURONS OF *NEUROD2/6* DOUBLE DEFICIENT NEOCORTEX AND HIPPOCAMPUS

NeuroD family TFs promote cell survival and differentiation. The phenotype of *NeuroD2/6* DKO mice was unexpectedly mild [9, 10]. The size and layering of neocortex were not significantly affected. These results indicate that a compensation mechanism could have been activated in DKO. The closest member of NeuroD family, NeuroD1, is normally expressed in SVZ and intermediate zone (IZ); it is immediately downregulated in neurons, once they begin moving toward CP [9, 10]. We expected NeuroD1 expression to be maintained in DKO brains. Indeed, we found that NeuroD1 expression was maintained in migrating neurons of *NeuroD2/6* DKO CP (Fig. 1, *a'–c'*). Additionally, co-immunofluorescent (IF) staining of NeuroD1 with either Ctip2 or Satb2 at E15.5 showed that NeuroD1 upregulation occurred in $Ctip2^+$ and emerging $Satb2^+$ neurons (not shown) suggesting that NeuroD1 was activated in deep layer (DL) and upper-layer (UL) neurons of *NeuroD2/6* DKO brains.

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NEUROD2/6 REGULATE EXPRESSION OF TRANSCRIPTION FACTORS IN THE DEVELOPING BRAIN

To identify downstream molecular mechanisms responsible for the previously described developmental defects, including corpus callosum agenesis, caused by genetic deletion of *NeuroD2/6* [9, 10], we performed an extensive ISH-based gene expression screen using E16.5 control and DKO brain tissues. We tested the expression of 300 genes (including cell skeleton proteins, transcriptional factors, adhesion molecules, and intracellular kinases) in developing DKO brains.

Most tested genes showed normal expression patterns in *NeuroD2/6* DKO brains, but some were downregulated in DKO cerebral cortex. Herein, we will present results of changes in expression patterns of TFs only. One of the TFs, whose expression was changed in DKO brains, was *Cux1*, a widely used gene marker of UL neurons. Its expression was reduced in DKO, as shown by ISH (Fig. 1, *a*, *a*'). Additionally, we tested the expression level by IHC staining (Fig. 1, *d*, *d*'). Most Brn2$^+$ UL neurons co-expressed Cux1 in controls; however, Brn2+ cells in DKO brains rarely showed Cux1 staining (not shown). Interestingly, Brn2 expression, a gene marker of upper neocortical layer neurons, was not changed.

Expression of another TF, Id2, was dramatically reduced in CP of E16.5 DKO embryos (Fig. 2, *b*, *b*'). Id2 encodes an HLH protein that lacks a basic DNA binding domain, but is still able to interact with E-box proteins. Id2 is, thus, considered a dominant negative repressor of class II bHLH TFs [13]. Id2 might be downstream of *NeuroD2/6* to serve as a regulator of *NeuroD2/6* function through a negative feedback loop. It would be interesting to investigate the mechanisms by which NeuroD factors regulate Id2 expression and other potential antagonists. Lhx2 (Fig. 2, *c*, *c*',) was also downregulated in CP of DKO brains at E16.5.

Tbr1 is essential for specifying layer VI neurons and patterning of corticothalamic axon projection [14]. To establish which subpopulations of DL neurons were reduced in *NeuroD2/6* DKO brains, we performed IF staining of this marker on E18.5 sections control and DKO brains. Tbr1$^+$ neurons were markedly reduced in DKO brains (Fig. 2, *e*, *e*').

To test if *NeuroD2/6* controls Cux1 cell expression level autonomously, we reintroduced NeuroD2 into developing brains of *NeuroD2/6* DKO through *in utero* electroporation. Cux1 expression was restored in cortical neurons electroporated with a respective genetic construct (Fig. 3).

To test, if *NeuroD2/6* directly controls Cux1 expression, we performed a bioinformatical analysis of the genomic region 5 kbp upstream of the mouse Cux1 gene (Fig. 4). We identified seven regions containing consensus sequences for E-boxes (CANNTG). These DNA fragments (E1-7 of 290–530 bps length) were cloned into luciferase expression plasmids (pMCS-GL). Each fragment contained 3–5 E-boxes with the consensus sequence. After co-transfection of each genomic fragment and NeuroD6 relative to cells that had been co-transfected with pMCS-GL and GFP, we tested for luciferase activity. Two fragments, E6 and E7, the closest regions to 5′TATA box of Cux1, had a threefold increase in luciferase activity (See Fig. 4). These results suggest that Cux1 is a direct target of *NeuroD2/6* genes in the developing neocortex.

OLIG2$^+$ PROGENITORS ARE INCREASED IN *NEUROD2/6* DOUBLE DEFICIENT NEOCORTEX

To investigate possible effects of NeuroD2/6 on gliogenesis, we performed IHC staining of the glial progenitor marker, TF Olig2. During embryonic development, Olig2 is mainly expressed in progenitors that will differentiate into astrocytes and oligodendrocytes [15, 16]. We found that the numbers of Olig2$^+$ cells were substantially higher at E18.5, but not at E16.5 and DKO brains relative to controls (Fig. 5, *b*, *b*'). We quantified the total number of Olig2$^+$ cells for three pairs of E18.5 littermate control and DKO brains (at similar coronal planes in each pair). The number of Olig2$^+$ cells in DKO was approximately twice as high as control samples [control: 234±53; DKO: 456±35; p=0.0053] (Fig. 5, *d*).

NEUROD2/6 AND OLIG2 EXPRESSION IS MUTUALLY EXCLUSIVE

To test if an increase in the number of Olig2$^+$ cells has a direct effect on *NeuroD*2/6 inactivation, we tested if *NeuroD* genes would be expressed in glial progenitors. To address this, we performed co-staining of Cre (co-expressed with NeuroD6) and Olig2 to examine their colocalization. Cre and Olig2 expression did not overlap in neocortex of E18.5 control or DKO brains, despite the increase of Olig2$^+$ cells in DKO (Fig. 5, *a*, *c* and *a*', *c*'). The results indicated that production of Olig2$^+$ cells in electroporated has a secondary effect caused by the premature differentiation of SVZ/VZ (ventricular zone) progenitors forced by NeuroD2 overexpression.

DISCUSSION

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NeuroD family TFs redundantly regulate cell differentiation in genetically linked pathways. Stages of neuronal differentiation in the mammalian telencephalon are defined by sequential, but overlapping expression of neuronal bHLH proteins. Ngn1 and Ngn2 are expressed in proliferating neuronal progenitors that reside in VZ or SVZ. Ngn1/2 synergizes as the earliest neuron-specific determinants and facilitates the initial phase of radial migration away from the neurogenic territory [17–19]. Young neurons begin to express NeuroD1 as they enter SVZ, shortly after leaving VZ. There is still some controversy as to whether NeuroD1 is active in mitotically dividing SVZ cells or expressed in postmitotic cells. The dynamics of NeuroD1 expression suggest a function in neuronal differentiation and delineate a border between precursor and postmitotic compartments. Sequential expression of Ngn1/2 and NeuroD1 is conserved and found in VZ of frogs' and rats' developing spinal cords [20]. The onset of NeuroD2/6 expression followed by NeuroD1 and robust NeuroD2/6 expression was mostly confined to postmitotic neurons that migrate in IZ and CP. Thus, switch from NeuroD1 to NeuroD2/6 expression marked the maturation of pyramidal neurons. In contrast, in Ngn1/2 deficient mice, neuronal specification was disrupted [18]. *NeuroD2*/6 DKO mice exhibited mainly defects in later neuronal functions, including impaired commissural tract formation, disorganized dendritic morphology, and a reduction in glutamatergic synapses [9]. Ngn1/2, NeuroD1, and NeuroD2/6, thus, coordinate the sequential phases of pyramidal neuron development in the neocortex: determination, differentiation, and maturation (Fig. 6).

Neocortical neurogenesis was much more severely affected in Ngn1/2 DKO mice than either of the single mutant mice, suggesting that they share a largely redundant function [17]. Likewise, the genetic inactivation of NeuroD2 and NeuroD6 results in callosal agenesis and some abnormalities in the developing cerebral cortex [9]. Our results confirmed that the functional redundancy of NeuroD2 and NeuroD6 in single restoration of either NeuroD2 or NeuroD6 expression *in vivo* was sufficient to rescue pyramidal neuron differentiation and callosal axon growth in *NeuroD2*/6 DKO mice [10]. NeuroD family TFs are assumed to promote neuronal survival and differentiation. Genetic deletion of *NeuroD1* led to a nearly complete loss of the hippocampal dentate gyrus because of activated Bax dependent apoptosis of immature granule neurons [21, 22]. *NeuroD2* single-deficient mice exhibit a high prevalence of programmed cell death in cerebellum and dentate gyrus [22]. Our findings indicated that NeuroD1 was activated ectopically upon *NeuroD2* and *NeuroD6* deletions. Ectopic NeuroD1 expression in CP of *NeuroD2/6* DKO embryos might contribute to the partial rescue of *NeuroD2/6* deficiency and protect *NeuroD2/6* null pyramidal neurons from apoptosis and mis-differentiation. To investigate the contribution of all three *NeuroD* genes in various aspects of neocortical development, one would need to generate *NeuroD1*/2/6 triple deficient mice. Nevertheless, downregulation of expression of many TFs involved in cortical development, such as Lhx2, Id2, and Cux1, indicates merely partial substitution of NeuroD1 for *NeuroD2/6*. The survival of most UL and DL pyramidal neurons, despite defective inter-hemispheric connectivity in *NeuroD2/6* DKO mice, suggests that excitatory pyramidal neurons do not depend on extrinsic trophic factors secreted by innervated contralateral axons, unlike in cerebellar granule cells [23]. Instead, the survival of cortical pyramidal neurons might largely depend on intrinsic regulation or locally diffusible signals.

Complementarily expressed bHLH TFs can regulate cellular behaviors in a genetically related fashion. For instance, Ngn1/2 and Mash1 are mainly expressed in dorsal and ventral subpopulations of telencephalic progenitors [17]. Their reciprocally graded expression contributes to neocortical patterning, as indicated by the observation that Mash1 is aberrantly expressed in the dorsal lineage and activates normally ventrally specific markers in Ngn1/2 double mutant embryos [15]. Additionally, overexpression of Ngn2 *in vivo* can induce the expression of later bHLH TFs, such as NeuroD1 and NeuroD2, suggesting that Ngn2 acts upstream of NeuroD1/2 to induce neuron differentiation [16]. This hypothesis was further substantiated by another finding that NeuroD1 expression was disrupted in Ngn2/Mash1 double mutants [17]. However, to date, little attention has been paid to the genetic relationship between NeuroD1 and NeuroD2/6. In wild-type brains, migrating projection neurons immediately lose NeuroD1 expression as soon as they reach CP. In this study, the results show that NeuroD1 is ectopically upregulated in NeuroD2/6 null UL and DL neurons, indicating that NeuroD2/6 may regulate NeuroD1 expression *in vivo*. Considering that NeuroD2/6 frequently serves as transcription activators, we speculate that it induces other immediate transcription regulator(s) that in turn repress NeuroD1 expression (See Fig. 5). The hypothesized repressor(s) is/are yet to be identified.

CONCLUSION

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Our findings indicated that the cumulative action of *NeuroD2* and *NeuroD6* genes is required to initiate and maintain the expression of TFs Cux1, Tbr1, Lhx2, and Id2. Additionally, both genes are required prevention of premature differentiation of $Olig2^{+}$ glial precursors.

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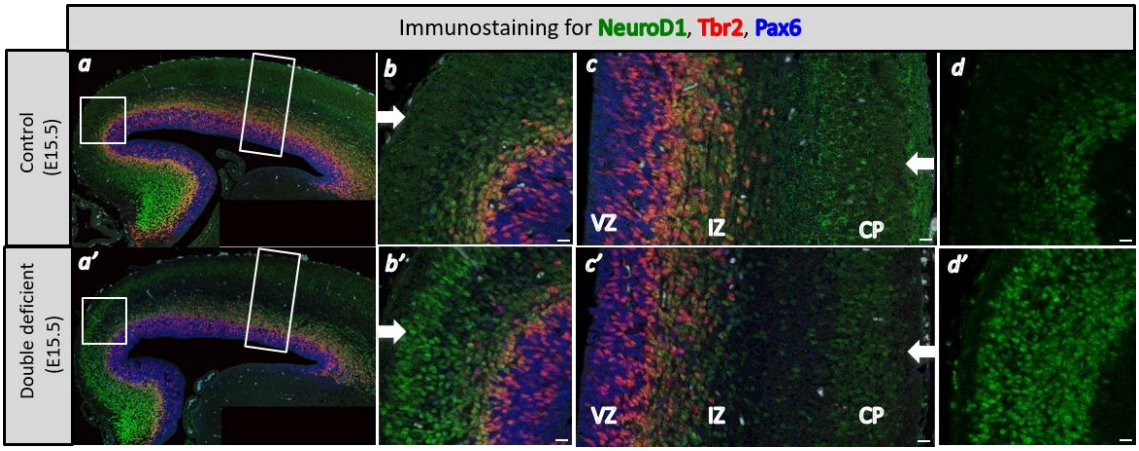

Fig.1. Ectopic expression of NeuroD1 in the developing neocortex and hippocampus caused by inactivation of NeuroD2/6: *a*, *a*' — immunofluorescent staining of NeuroD1 (green), Tbr2 (red), and Pax6 (blue); *b*, *b*' — enlarged views of the areas in square frames; *c*, *c*' — enlarged views of areas in rectangular frames; *d*, *d*' — immunofluorescent staining for NeuroD1 (only green); note the lack of NeuroD1 expression in the wild type (*d*) and ectopic expression in the double deficient (*d*'). VZ — ventricular zone, IZ — intermediate zone, CP — cortical plate. Arrowheads indicate: *b*, *c* — area of the cortical plate in which NeuroD1 expression is absent; *b*', *c*' represents a similar region in cortical plate of double deficient that exhibits ectopic expression of NeuroD1. IF staining was performed on sections of E15.5 littermate control and double deficient brains. Scale bars: 10 μm.

Рис. 1. Инактивация NeuroD2/6 вызывает эктопическую экспрессию NeuroD1 в развивающемся неокортексе и гиппокампе: *a*, *a*' — Иммунофлуоресцентное окрашивание на NeuroD1 (зелёный канал), Tbr2 (красный канал) и Pax6 (синий канал); *b*, *b*' — увеличенные изображения областей в квадратных рамках; *c*, *c*' — увеличенные изображения областей в прямоугольных рамках; *d*, *d*' — иммунофлуоресцентное окрашивание только на NeuroD1 (зелёный канал); обращает на себя внимание отсутствие экспрессии NeuroD1 в диком типе (*d*) и эктопическая экспрессия у двойного мутанта (*d*'). VZ — вентрикулярная зона, IZ — промежуточная зона, CP — кортикальная пластинка. Белыми стрелками обозначено: *b*, *c* — область кортикальной пластинки, в которой отсутствует экспрессия NeuroD1; *b*', *c*' — аналогичная область в кортикальной пластинке двойного мутанта, в которой присутствует эктопическая экспрессия NeuroD1. Иммунофлуоресцентное окрашивание проводили на срезах головного мозга особей одного помета на 15-й день эмбрионального развития. Бар — 10 мкм.

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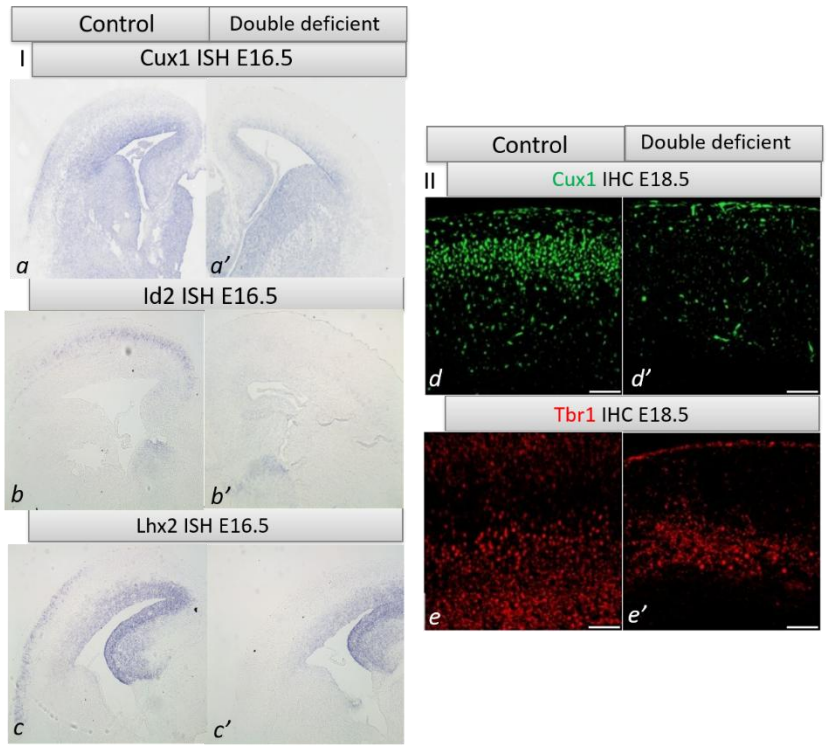

Fig. 2. NeuroD2/6 controls expression of other transcription factors in developing cerebral cortex: I — gene expression patterns in upper-layer neurons were disrupted in NeuroD2/6 double deficient cortex: *in situ* hybridization for Cux1 (*a*, *a'*), Id2 (*b*, *b'*), Lhx2 (*c*, *c'*); II — expression of Cux1 and Tbr1 is impaired in upper-layer neurons of NeuroD2/6 double deficient mice and the number of cells in the deep layer expressing Tbr1 is reduced: immunofluorescent staining of Cux1 (green, *d*, *d'*) and Tbr1 (red, *e*, *e'*); *in situ* hybridization was performed on E16.5, and immunofluorescent staining was performed on E18.5 littermate control and double deficient brains. Microscope magnification for *in situ* hybridization images — 5×. Scale bars for *in situ* hybridization images — 100 μm.

Рис. 2. NeuroD2/6 контролирует экспрессию других факторов транскрипции в развивающейся коре головного мозга: I — паттерны экспрессии генов в нейронах верхних слоёв были нарушены в коре головного мозга мышей с двойным нокаутом NeuroD2/6: гибридизация *in situ* для Cux1 (*a*, *a'*), Id2 (*b*, *b'*), Lhx2 (*c*, *c'*); II — экпрессия Cux1 и Tbr1 нарушена в нейронах верхних слоёв у мышей с двойным нокаутом NeuroD2/6, и количество клеток в глубоких слоях, экспрессирующих Tbr1, уменьшено: иммунофлуоресцентное окрашивание на Cux1 (зелёный канал, *d*, *d'*), Tbr1 (красный канал, *e*, *e'*). Гибридизацию *in situ* проводили на 16-й день эмбрионального развития, а иммунофлуоресцентное окрашивание — на 18-й день эмбрионального развития. Увеличение, при котором получены изображения гибридизации *in situ* — ×5. Бар — 100 мкм.

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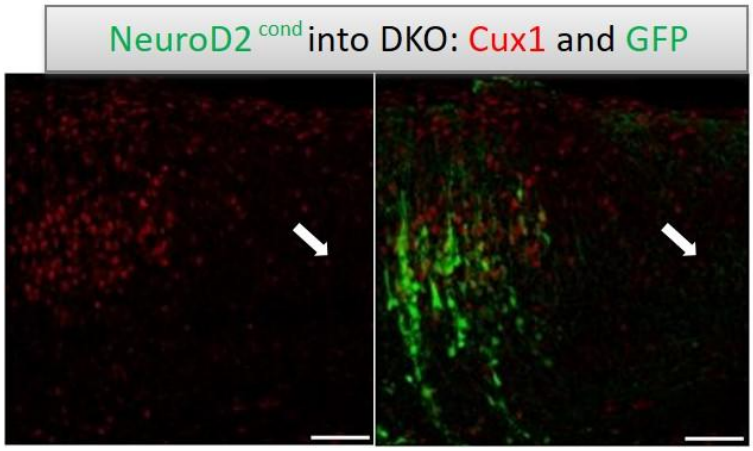

Fig. 3. NeuroD2/6 acts autonomously to control Cux1 expression. Cux1 expression is restored in NeuroD2 electroporated cells in double deficient brains: immunofluorescent staining for green fluorescent proteins (GFP) (NeuroD2, green) and Cux1 (red). Arrowheads indicate the Cux1 negative area in double deficient. *In utero* electroporation was performed at E13.5 and the brain was fixed at E18.5. Scale bars — 100 μm.

Рис. 3. NeuroD2/6 действует на клетку автономно, контролируя экспрессию Cux1. Экспрессия Cux1 восстанавливается в клетках, электропорированных NeuroD2 в мозге с двойным нокаутом: иммунофлуоресцентное окрашивание на NeuroD1 (зелёный канал) и Cux1 (красный канал). Стрелки указывают на отрицательную область Cux1 в мозге с двойным нокаутом. *In utero* электропорация была выполнена на 13-й день эмбрионального развития, а мозг зафиксирован на 18-й день эмбрионального развития. Бар — 100 мкм.

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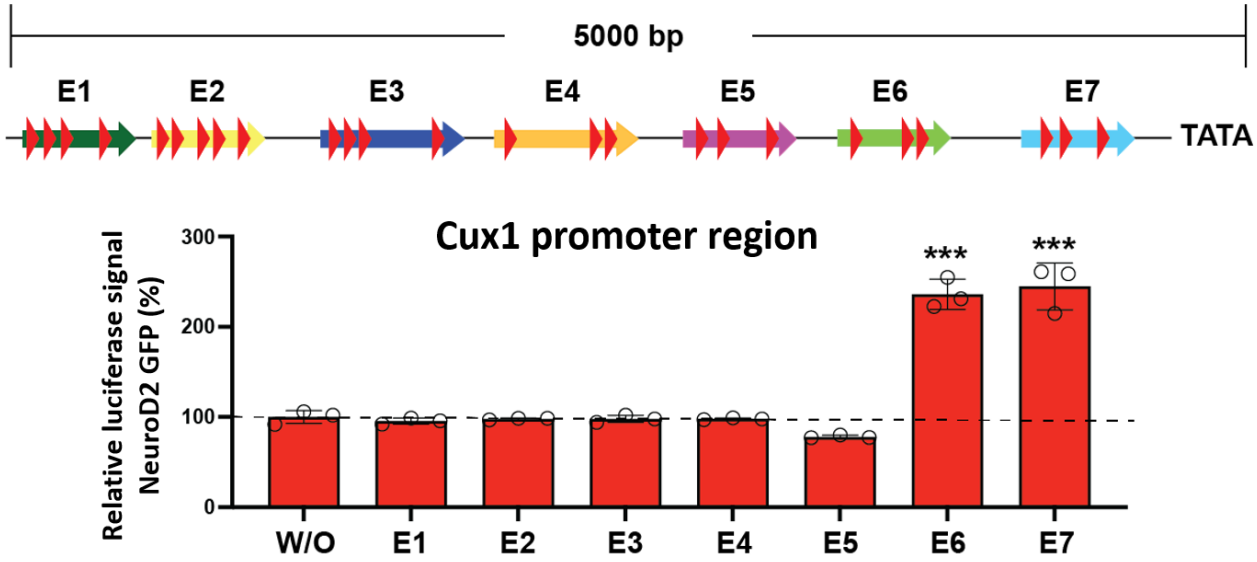

Fig. 4. **Luciferase promoter assay of Cux1 5′ regions in HEK293 cells**: **seven DNA fragments (E1-7 with 290–530 bps lengths) of the genomic region 5 kbp upstream of mouse *Cux1* gene were cloned into luciferase expression plasmids (pMCS-GL). Each fragment contains 3–5 E-boxes with the consensus sequence CANNTG (red arrow heads). The bars show normalized luciferase activity in cells (triplicates), co-transfected with pMCS-GL and NeuroD6 relative to cells co-transfected with pMCS-GL and GFP. Transfection with pMCS-GL empty backbone (w/o) was set as 100% baseline. Two most proximal fragments (E6 and E7) to Cux1 transcript start (TATA) remarkably increased the transcriptional activity of NeuroD2: E1=95.53±4.61%, *p*=0.39; E2=97.99±4.22%, *p*=0.66; E3=97.98±4.77%, *p*=0.69; E4=98.05±4.22%, *p*=0.67; E5=78.17±4.30%, *p*=0.0071; E6=236.20±10.54%, *p*=0.0002; E7=244.90±15.62%, *p*=0.0008 (two-tailed t-test); *** *p* <0.001.**

Рис. 4. Анализ люциферазной активности промотора 5'-областей Cux1 в клетках HEK293: Семь фрагментов ДНК (E1-7, длина варьируется от 290 до 530 п.о.) геномной области, находящейся на 5 т.п.н. выше мышиного гена Cux1, клонировали в плазмиды экспрессии люциферазы (pMCS-GL). Каждый фрагмент содержит 3–5 E-боксов с консенсусной последовательностью CANNTG (красные стрелки). Столбики показывают нормализованную активность люциферазы в клетках (в трёх экземплярах), которые были совместно трансфецированы pMCS-GL и NeuroD6, по сравнению с клетками, которые были подвергнуты совместной трансфекции pMCS-GL и GFP. Трансфекцию пустым вектором pMCS-GL принимали за исходный уровень 100%. Два наиболее проксимальных фрагмента (E6 и E7) относительно начала транскрипта Cux1 (TATA) могут значительно увеличивать транскрипционную активность NeuroD2: E1=95,53±4,61%, *p*=0,39; E2=97,99±4,22%, *p*=0,66; E3=97,98±4,77%, *p*=0,69; E4=98,05±4,22%, *p*=0,67; E5=78,17±4,30%, *p*=0,0071; E6=236,20±10,54%, *p*=0,0002; E7=244,90±15,62%, *p*=0,0008 (двусторонний t-критерий); * *p* <0.001.**

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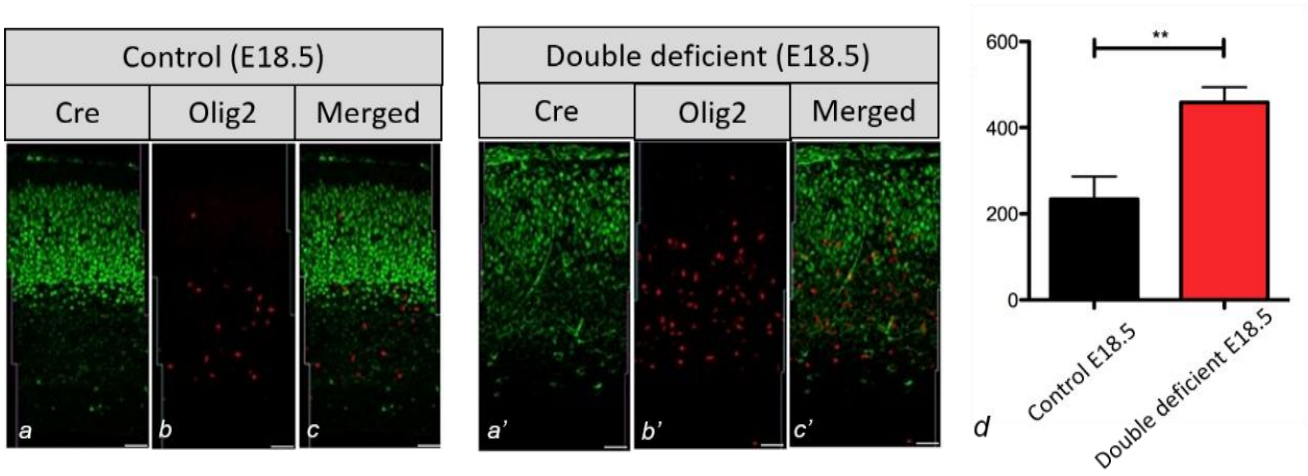

Fig. 5. **Inactivation of NeuroD2/6 causes overproduction Olig2+ cells: immunofluorescent staining of Cre (*a*, *a*') and Olig2 (*b*, *b*') on the sections of E18.5 littermate control and double deficient brains and the overlays (*c*, *c*'). Quantification of Olig2$^+$ cells (*d*) in E18.5 control and double deficient brains (*n*=3). Scale bar — 100 μm. 0.001< *p* < 0.01.**

Рис. 5. Инактивация NeuroD2/6 вызывает перепроизводство клеток Olig2$^+$: иммунофлуоресцентное окрашивание на Cre (*a*, *a*') и Olig2 (*b*, *b*') на срезах мозга мышей контрольной группы E18.5 и головного мозга мышей с двойным нокаутом, а также наложение слоев (*c*, *c*'). Масштабная линейка: 100 мкм. Количественная оценка Olig2-экспрессирующих клеток (*d*) в контрольном мозге и мозге мышей с двойным нокаутом на 18-й день эмбрионального развития (*n*=3). Бар — 100 мкм. 0,001< *p* <0,01.

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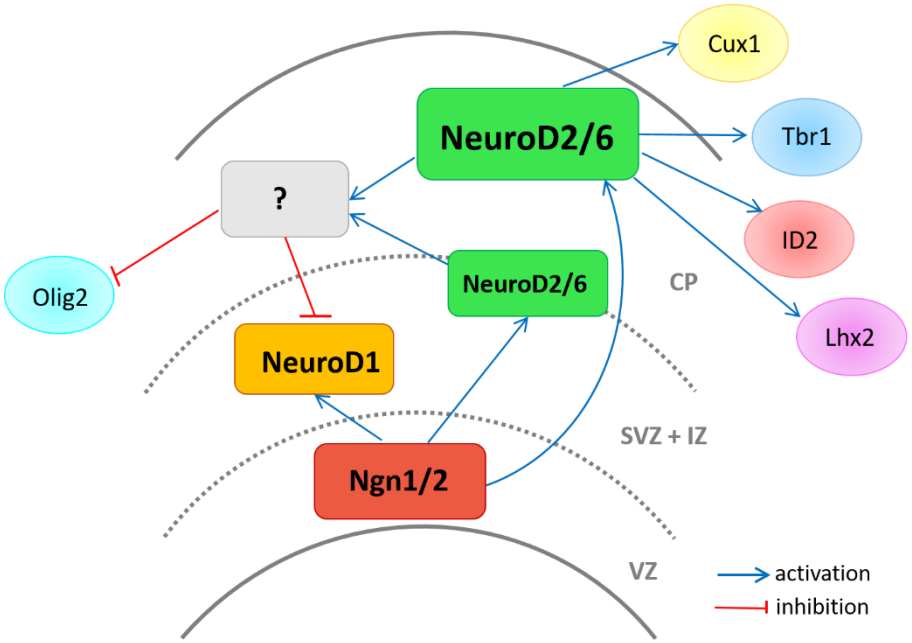

Fig. 6. Sequential expression of proneural bHLH transcriptional factors in neocortex: Ngn1/2, NeuroD1, and NeuroD2/6 were ventro-dorsally differentially expressed in developing cerebral cortex. Ngn1/2 promoted expression of later bHLH transcriptional factors; however, genetic interaction between NeuroD1 and NeuroD2/6 remains still elusive.

Рис. 6. Последовательная экспрессия пронейральных транскрипционных факторов bHLH в неокортексе: Ngn1/2, NeuroD1 и NeuroD2/6 дифференциально экспрессируются в развивающейся коре головного мозга. Ngn1/2 может способствовать экспрессии более поздних транскрипционных факторов bHLH, однако генетическое взаимодействие между NeuroD1 и NeuroD2/6 остаётся не конца изученным.