Transcription factor CUX2 and post-transcriptional factor CELF4 in neurons of synapse-enriched layers during human fetal corticogenesis

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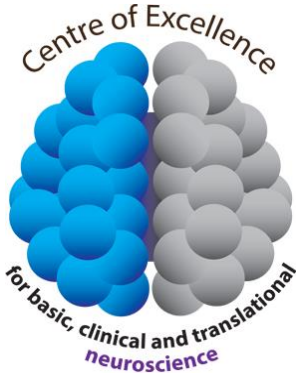

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Abbreviations:

PCW- Post conceptional weeks

PFC- Prefrontal cortex

DPFC- Dorsal prefrontal cortex

GABA- Gamma Aminobutyric Acid

PBS- Phosphate buffered saline

PB- Phosphate buffer

BSA- Bovine serum albumine

HRP- Horseradish peroxidase

DAB- 3,3'-diaminobenzidine

DAPI- 4′,6-diamidino-2-phenylindole

PCR- Polymerase chain reaction

1. Introduction

The neural mechanisms of a fully developed adult human neocortex are generally recognized as a complex marvel of biological evolution. However, the genetic processes and mechanics that enable cortical morphological formation following conception are certainly of equal complexity if not orders of magnitude more so.

In comparison to when the first documented observations of the six layers of the common human neocortex where made, it's only relatively recently that the gene expression regulating steps of transcription and post-transcription have been recognized as fundamental to the emergence of those layers within the growing fetal neocortex. Of particular consequence is the diversity and complexity of the resulting cellular populations and their developmental durations and behaviors. Essential to understanding precisely how transcriptional and post-transcriptional steps direct fetal neocortical development, and more importantly when they've gone awry, is identification and spatiotemporal patterning of expressed regulating genes, and their expected affects as either transcription or post-transcription factors.

This thesis takes a step forward in increasing this field knowledge, by analyzing spatiotemporal expression of the transcription factor CUX2 and post-transcriptional factor CELF4 within post-migratory neurons of synapse-enriched layers during early and midfetal cortical development, suggesting their possible role in cellular fate determination and synaptic profiling.

1.1. Human cortical development

The human cerebral cortex is the most sophisticated part of neuronal systems, integrating sensory, motor, cognitive, behavioral, and social functions into a unified conscious being. The neocortex in a fully developed adult brain is organized into six laminae, historically described by classical neuroanatomists (1,2). Neuronal genesis, differentiation, migration, maturation, axonal pathfinding, and synaptogenesis are fundamental processes underlying cortical development, choreographed in myriad ways necessary for the proper morphological form and function of a cerebral cortex (3,4). The complex laminar, areal, and columnar organization of the cerebral cortex occurring during prenatal and perinatal periods, can be delineated by a series of neurogenetic and histogenetic events (4–6). In particular, during early fetal and midfetal development, histogenetic events crucial for proper growth and development of the cortex take place (Figure 1.1) (4). Furthermore, a close correlation among cellular neurogenetic events and transient cortical lamination is considered to be a major feature of the embryonic and fetal telencephalic wall development (5,7).

Main neurogenetic and histogenetic events involved in corticogenesis occur in the transient embryonic and fetal zones (4). Cortical lamination is generated in the 'inside-out' manner, so the newly generated, immature neurons migrate from the proliferative zones to settle in the CP, while the consecutive neurons migrate and settle superior to them, closer to pial surface (5). Neuronal differentiation unfolds mostly when the postmitotic neurons are already in their final laminar position where they start to grow their dendrites and dendritic spines, protract their axons to fully achieve their phenotype. Elaborated axons establish primal pathways in the process of axonal pathfinding and allow the formation of the first, transient synapses, while eventually the first neural circuits are formed. Series of protracted, important events influences neocortex in a dynamic and swift way (8).

Neocortical wall organization evolves throughout fetal development. Transient layers of the fetal neocortex form and reorganize over the course of development. Anatomical features of the cerebral wall throughout development are described in section *1.4.1. Synapses in human fetal cortex.* Early on, crucial histogenetic events occur in two of the transient zones; the marginal zone (MZ) and the subplate zone (SP) (9). The MZ and SP zone show resemblance in their organization and circuitry (9,10), in terms of concomitant generation (11–14), synapse development (15,16), and

the early differentiation of Cajal-Retzius neurons (CRNs) of the MZ, and the subplate neurons (SPNs) (17). Moreover, both CRNs and SPNs compose the earliest cortical synapses, and receive inputs from different presynaptic sources (18–25). SPN inputs come from modulatory monoaminergic pathways of the brainstem (26), cholinergic afferents from the basal forebrain (27,28), glutamatergic inputs of the thalamus (29,30), as well as local presynaptic glutamatergic or GABAergic inputs from other SPNs or cortical plate (CP) neurons (24,30,31). These first transient, pioneer neurons are critical for the formation of primary cortical circuits that provide the foundation for later acquired neocortical functions (5,6,9,16).

It is generally understood that relative to other species, including those closest in evolutionary terms to *Homo sapiens*, the length of time required to complete cortical development is rather unique to humans, where it begins late within the embryonic development and lasts several years up to young adulthood (6). Given the magnitude of events that concentrate in the transient zones over the long course of cortical development, it is not surprising that disrupted development of either or both neocortical projection neurons and SP neurons are associated with several neurodevelopmental disorders, including autism spectrum disorder (ASD), epilepsy, and schizophrenia (6,32,33). Failed differentiation of CRNs is evidenced in lissencephalic brain malformations, a well-known migration disorder (34). This thesis provides additional insights into the molecular mechanisms of typical fetal cortical developmental processes, intended to further the understanding of associated disorders and their potential therapies.

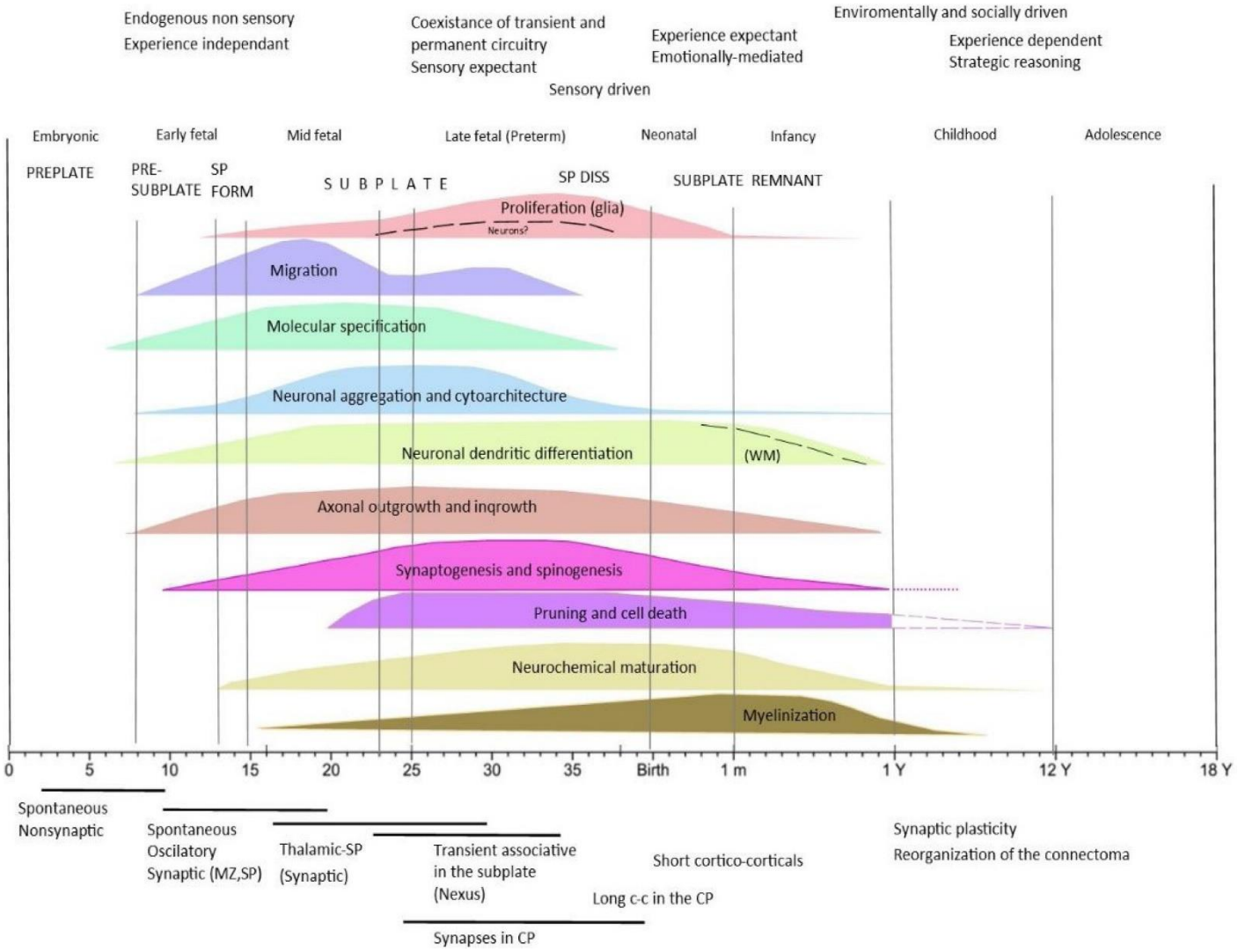


Figure 1.1. Histogenetic and molecular events outlined throughout human development from conception to the end of adolescence emphasized on the development of SP zone (top) and synapses (bottom). Taken with permission from Kostovic (6).

1.2. Transcription and Translation

The central DNA-RNA-protein dogma is the simplified explanation of the complex molecular and cellular diversity within the developing cortex. It is now generally accepted that observed complexity arises from the sophisticated regulating steps of transcription and translation, fundamental to the basic processes of gene expression and protein synthesis (3,35–37). The diverse heterogeneity of the cellular environment within the developing cortex has been verified by single-cell RNA sequencing studies resulting in gene expression variations (34-37). Differential gene expression is regulated by specific transcriptional factors (TFs) resulting in specific subtypes of cortical cells (38,39). Another group of regulatory proteins, RNA-binding proteins (RBPs) are the most prominent post-transcriptional factors, regulating translation, colluding in alternative splicing, and localizing or even degrading any kind of RNA (Figure 1.2) (40–44) RBPs can act in promotional and/or repressive way (45,46). Together, the steps of transcription and translation orchestrated by elaborate combinations of transcription and post-transcriptional factors ultimately correlate with the astounding degree of observed cellular diversity.

Both transcriptional and post-transcriptional mechanisms have been emphasized as critical influencers of cortical development. The processes of neuronal genesis, migration, and maturation are heavily dependent on transcriptional and post-transcriptional mechanisms (40)(3). Deficiencies of particular TFs (e.g. Tbr1, Satb2, Sox5, Brn1, Brn2) have been shown to disrupt neuronal migration resulting in laminar positioning defects of specific neuronal populations (35,41–47). These types of cortical abnormalities become clinical etiologies and are identified as the underlying cause of severe brain malformations and disorders (48–52). Post-transcriptional regulators have also been shown essential to proper neuronal migration and differentiation. RBP Nova2 is known to affect the correct placement of projection neurons within the cortex (53), while HuD (Hu antigen D), a general neuronal phenotype marker, is known to influence post-mitotic differentiation, (54). One of the largest human transcriptomic studies showed that the majority of substantial global spatio-temporal transcriptional differences occur during prenatal development (55), further evidencing the broad role TFs play in regulating and directing cortical development. Lastly, progenitor proliferation (56), differentiation (57), neuronal migration (58), and axonal pathfinding (59) are principal cortical developmental processes that have also been shown dependent on post-transcriptional regulating mechanisms.

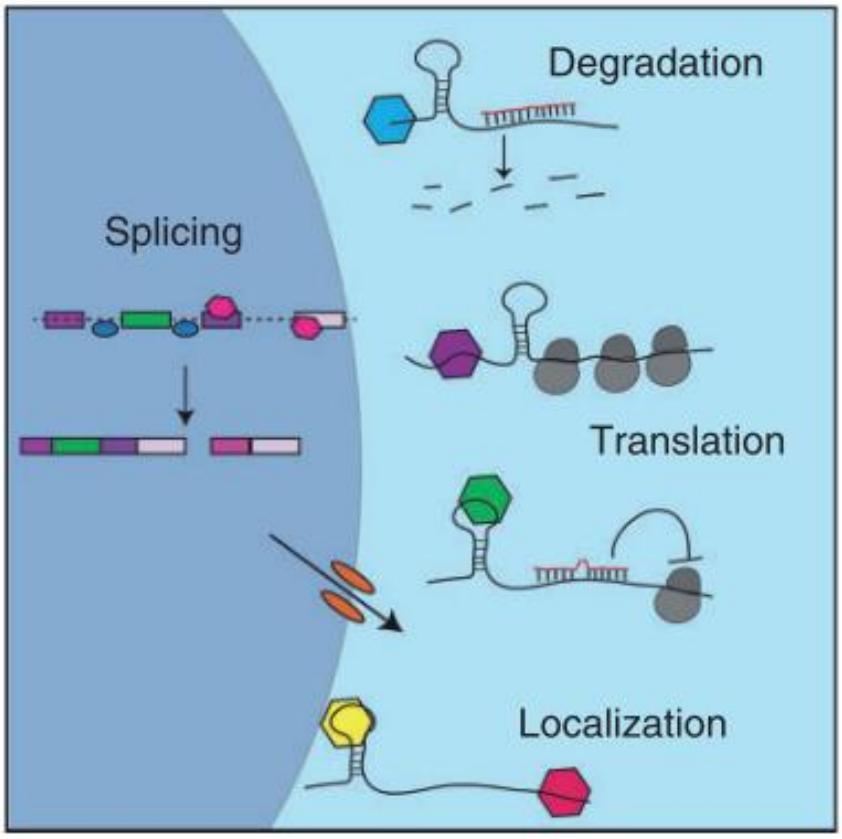


Figure 1.2. RNA-binding proteins have different functions as part of post-transcriptional mechanisms: splicing occurrs in nucleus, while degradation, translation, and localization of RNA occurs in cytoplasm. Hexagons depict different RBPs in action (Taken with permission from Lennox et al 2018, (37)).

1.2.2. Cellular fate determination / Specification of cortical neurons

One of the most prominent histogenetic events in the human cortical development is cellular fate determination, achieved by the process of molecular specification of cortical neurons (3,4,48,60). Molecular specification can be driven by cell-type specific alternative splicing , and other post-transcriptional mechanisms (36), or by selective expression of TFs. Cellular fate determination is characterized by the commitment to a neuronal or glial fate, specification of positional identity, and differentiation into a laminar-specific neuronal phenotype. Neuronal precursor cells give rise to neurons in the process of neurogenesis (61,62), with the neuron birth date initially determining potential molecular identity and eventual fate as a distinct neocortical neuronal subtype (5,63). Newly born neurons migrate to their predetermined positions in the neocortex to finalize their differentiation into laminar-specific cellular phenotypes (64). Transcription factors, Pax6 and Emx2, which are major regulators of dorsal cortical regionalization and in charge of primitive morphogenetic processes of the primordial cortical organization (65), are additional examples of the role of TFs in cellular fate determination in the early embryonic cortical patterning (66).

TFs are key regulators involved in the molecular specification and final positioning of cells within the cortical laminae (35,38,44,67). The cortical laminae can be divided into an upper layer (UL: laminae 2-4) and a deep layer (DL: laminae 5-6), where particular TFs can direct cellular fate into either of the two. While an abundance of TFs has been well studied and known to influence cortical cell fate, the ones specifically relevant to this thesis are those expressing molecular markers indicative of cortical projection neurons (Table 1.1). These molecular markers can effectively be separated into UL markers (SVET1, MEF2C, RORB, CUX1/2) and DL markers (TLE4, FOXP1/2, ER81, CTIP2, FEZF2, OTX1, SOX5, TBR1), with a subset of them (TLE4, FOXP1, CUX1/2, CTIP2, FEZF2, TBR1, BHLHB5, SATB2, LMO4, NURR1, BACH2) additionally being transiently expressed in the SP. Interestingly, a recent study suggests the SP to be the site of fate selection for cortical deep projection glutamatergic neurons (68,69). Some of the more unique genes are expressed in cells of both the UL and DL (BHLHB5, FOXP1, LMO4, SATB2, BRN1/2), particularly SATB2 which holds a role as an influencer of callosal development (41,42). Most of the aforementioned molecular markers are being expressed over the course of development which suggest their developmental role in the neocortex, and some of them deviate their expression pattern postnatally.

Table 1.1. Neocortical projection neuron markers. Gene annotations are based upon the species of discovery.

GENE	LAYER	NEURONS	SPECIES	REF
BHLHB5	Layers 2-5 Midgestation frontal cortex: UL of CP Occipital cortex: UL CP, SP	Postmitotic neurons Co-localized with Ctip2	MOUSE HUMAN	(70) (68) (IHC)
Svet1	Early embryonic: SVZ Later: migrating in IZ Upper layers of CP	Dividing precursors of SVZ Postmitotic cells of layers 2-4	MOUSE	(71) (ISH)
SATB2	Developmental cortex: The strongest expression in L2-3, 5 Weaker in L4, 6 and SP	Marker of upper and deep layers Postmitotic neurons Callosal projection neurons of UL	MOUSE HUMAN	(42) (41) (68) (IHC) (72) (transcriptomics)
MEF2C	Postnatal: Layers 2-4		HUMAN RAT	(73) (Northern Blot and ISH)
LMO4	Postnatal: Layers 2-4, 6a Human midgestation: CP, sSP L2-3	Excitatory neurons Callosal projection neurons marker	MOUSE HUMAN	(74) (75) (ISH) (72) (transcriptomics)
RORB	Prenatal (E15, P0, P15) and adult: Layer 4		RAT HUMAN	(76) (67)
CUX1	Prenatal: E14- VZ and SVZ, MZ E16- VZ, SVZ, IZ and CP Adult: Layers 2-4 (IHC) Human Midgestation and newborn: IZ, SP, CP	Marker of upper cortical layers	MOUSE HUMAN	(77,78) (79)

CUX2	Early prenatal: subpallium; pallium: SVZ, IZ, MZ Late prenatal: upper half of CP Postnatal and adult: mostly upper layers, with some reactivity in lower layers (ISH) Human Midgestation: SP migratory neurons (DCX+CUX2) Human Adult PFC: Layer 2-3 Human early and late development: CP and SP Human newborn: gyral white matter	Intermediate progenitors (SVZ) Postmitotic neurons of CP Projection neurons of L2-3	MOUSE HUMAN	(77,78) (79) (80)(ISH) (81)(IHC, RNA Scope)
DTX4	E16.5 CP E18.5 Upper half of CP Layer 4		RAT	(82) (83) (ISH)
TLE4	Prenatal: Layer 5, 6 and SP Adult: Layer 5 and 6	Corticothalamic and some subcerebral projection (L5) neurons	MOUSE HUMAN	(84) (67) (72) (transcriptomics)
ER81	Pre- and neonatal (E16.5, P0): Layer 5	Cortico-cortical and subcerebral projection neurons	MOUSE	(64) (IHC)
FOXP1	Fetal development cortex Layer 3-5	Postmigratory neurons	HUMAN MOUSE	(75) (85)
FOXP2	Layer 6	Glutamatergic neurons of infragranular layers		
CTIP2	Embryonic: CP Layer 5b and 6 Midgestation frontal cortex: CP, SP	Deep layer projection neurons Corticospinal neurons	MOUSE HUMAN	(86) (39) (68) (IHC)
Fezf2	Early embryonic: VZ Layer 5, SP	Cortical VZ progenitors Large pyramidal neurons Subcerebral projections	MOUSE	(87) (ISH)
Otx1	Embryonic: VZ and CP Postnatal and adult: L5, L6	Postmitotic neurons of L5 and 6	MOUSE, RAT	(60) (ISH) (64) (IHC)
Sox5	Prenatal and early postnatal: Layer 5b, 6, SP	Postmigratory neurons	MOUSE	(46) (72) (transcriptomics)
Brn1	Early embryonic: VZ and SVZ Neonatal P0: Layer 2-4 and 5	Early- precursor cells	MOUSE	(47) (64)

Brn2	Early embryonic: VZ and SVZ Layer 2-3 and 5 Human midgestation: IZ, SP, uCP	Late- posmitotic cells UL glutamatergic pyramidal cells	HUMAN	(79)
Tbr1	Early: preplate Mid: MZ, CP Late: layer 6 and SP	Glutamatergic neurons CR and SP neurons	MOUSE	(44)
NURR1	Dorsal: E18 - postnatal SP Human 15-21 PCW SP	Corticocortical projection neurons	MOUSE HUMAN	(88) (89) (IHC) (68) (IHC)
LMO5 (CSRP2)	Midgestation human fetal tissue: CP	Glutamatergic neurons of CP	HUMAN	(72) (ISH)
ZNF354C	Midgestation human fetal tissue	Maturing excitatory neurons	HUMAN	(72) (transcriptomics)
BACH2	Midgestation human fetal tissue	Maturing excitatory neurons	HUMAN	(72) (transcriptomics)

1.2.3. RBPs roles in the cortical developmental processes

RNA-binding proteins (RBPs) are important mediators of post-transcriptional regulation with distinct roles in neurodevelopment (52,90–92). These distinct roles are herein reviewed with specific examples of RBPs acting within cortical processes (Table 1.2.). RBPs engagement in mRNA alternative splicing amplifies the transcriptome diversity and contributes to human brain complexity (36). Prenatal deletion of the RBP HuD has been shown to affect development of neocortical layers and dendrites (93), whereas another RBP, HuR (Hu antigen R), has been shown to regulate mRNA translation in prenatal neocortical development (51). More recently HuD and Celf1 (CUGBP Elav-like family member 1) RBPs were shown to be involved in a newly described mechanism of isoform-specific translational control and neocortical pathologies when translation is dysregulated (94). Another gene in the CELF family has been studied because of its implication in human cortical malformations. It has been shown that CELF2 controls NPC differentiation and in that way appoints cell fate in cortical development (95,96). FMRP (Fragile X Mental Retardation Protein) is an RBP well known for its function in synaptogenesis, encoded by a defective *FMR1* gene containing trinucleotide expansion mutations. FMRP causes severe intellectual disability clinically known as Fragile X syndrome (97). Additionally, FMRP affects development of synaptic contacts (97) by regulating mRNA transport as well as regulating expression of various pre- and post-synaptic proteins (50). Other studies further show that downregulation of RBPs PTBP1 (Polypyrimidine Tract Binding Protein 1) and PTBP2 (Polypyrimidine Tract Binding Protein 2), is required for PSD-95 (Post Synaptic Density 95) protein expression during cortical development and synaptic maturation (98). Irregularities in post-transcriptional regulation have also been shown to lead to neurodevelopmental diseases, such as schizophrenia and ASD, and other neurological conditions (Table 1.2) (49–52). Altogether these findings reflect RBPs importance to neocortical and synaptic development.

Table 1.2. RNA-binding proteins (RBPs) in cortical development processes.

RBP	CORTICAL (BIOLOGICAL) PROCESS	TISSUE LOCATION	NEURODEVELOPMENTAL DISORDERS	REF
Celf 1	Regulates development of glutamatergic neurons in developing neocortices Post transcriptional mechanisms	Human ventral RG (VZ, SVZ)	Myotonic dystrophy (DM), Alzheimer's disease (AD),	(94,99,100)
Celf 2	Regulates neuronal progenitor differantiation Regulates mRNA encoding cell fate and disease-related factors	Cerebral cortex (VZ, SVZ, IZ), hippocampus, and amygdala	Developmental and epileptic encephalopathy	(95,96,100)
Celf 4	Neuronal differentiation and excitation, corticothalamic development, synaptic transmission and function, synaptic plasticity	Hippocampus, amygdala and cortex	Epilepsy, Autism spectrum disease (ASD)	(100–104)
Celf 6	Splice enhancement and repression	Embryonic and postnatal brain expression	ASD, miotonic dystrophy (DM1)	(100,101)
HuR	Progenitor proliferation, neuron lamination, RNA stability and translation, regulates the position, identity and maturation of post-mitotic glutamatergic neurons	Radial glial cells, upper- and deep-layer neurons	Neurofibromatosis type 1 (NF1), Amyotrophic lateral sclerosis (ALS), Spinal muscle atrophy	(37,51,54,56)
HuC	mRNA stability, regulation of genes involved in amino acid biosynthesis and alternative splicing regulation of transcripts involved in synaptic cytoskeletal dynamics	Neuroepithelial cells, radial glia, IPs, and newborn neurons	Epilepsy, ASD	(104,105)
HuD	Postnatal development, specification and dendritic arborization of a subset of deep-layer neurons	Widely expressed in the neocortex (mostly L5), the four Cornu Amonis (CA1-4) regions of the hippocampus	AD, Parkinson's disease (PD), Spinal Muscular Atrophy (SMA), ALS, Schizophrenia, Epilepsy	(54,93)

FMRP1	Necessary for proper differentiation, migration, axon formation, refinement and stabilization, synapse formation and circuit wiring of neocortical layers	RGCs and immature neurons, layer 5 pyramidal neurons	Fragile X syndrome (FXS), ASD, affective disorders, attention-deficit hyperactivity disorder, bipolar disorder, schizophrenia	(37,52,101,105,106)
NOVA2	Migration of late-born neurons	All of the neocortical layers	Neurodevelopmental disorders	(37,59,105)
FuS	Transcription; Alternative splicing; Transport; Gene silencing	Nuclei of the hippocampal neurons	ALS, Frontotemporal lobar dementia	(105,107)
TDP-43	RNA splicing and RNA metabolism	Nucleus	early embryonic lethality, ALS, Paget's disease, AD, PD	(107)
Rbfox1	Neuronal migration and axon growth: alternative splicing, regulation of microRNA processing, synaptic function	Neurons	ASD, Hydrocephalus, Epilepsy, Schizophrenia, Attention deficit hyperactivity disorder (ADHD)	(37,58,108)
QUAKING (QKI)	Regulates differentiation of myelin-forming oligodendrocyte and Schwann cells; mRNA stability; Translation; Alternative splicing; Localization	highly expressed in glial cells, including astrocytes and oligodendrocytes	Psychiatric diseases; Schizophrenia; Ataxia	(105,109)
APC	Neuron migration and RGC polarity; localization	Nucleus, cytoplasm	Not discovered yet	(37,92)
PTBP1	Progenitor proliferation and differentiation	Radial glia cells (RGC)	Postnatal hydrocephalus, Timothy syndrome (TS)	(37,110)
PTBP2	Progenitor proliferation and differentiation	RGCs and differentiated neurons	TS	(37,110)
EIF4G	Promote differentiation	Nucleus	Fragile X syndrome (FXS)	(37,111)
EIF4E1 - EIF4ET	Prevent differentiation	Brain tissue, neocortical VZ and CP	ASD, FXS Schizophrenia	(40)(37,111)
DDX3X	Implicated in neurogenesis	Embryonic and postnatal all cortical layers	Intellectual disability (ID), brain abnormalities, microgyria, ASD	(112,113)
PIWIL1	Regulates neuronal polarization and radial migration	Germline tissue	ASD	(114)

1.3. Transcription factor CUX2 in neocortical development

During fetal development transcription factors (TFs) play diverse roles regulating the varied processes which determine cerebral cortex patterning. One way to classify different populations of neuronal subtypes in the neocortex is to capture the specific TF's selective expression within each subtype (3,35,38,40). The first aims of this thesis are focused on the elucidation of TF Cut-Like Homeobox 2 (CUX2), a member of the CUX family shown to mediate the development and molecular specification of cells fated to the adult upper cortical layers (77,78). Cux2 is uniquely expressed in the nervous system of murine embryos and adults where it acts as a transcriptional repressor fulfilling its role in affecting specific cell fate (115–117).

1.3.2. Neocortical CUX2 expression and functions

CUX1 and CUX2 are homeobox genes, eminently studied in murine, but only recently evidenced as being present during development of the human cortex. Their expression is found in the nucleus, an expected intra-cellular localization of transcriptional factors (116). There is an overlapping expression pattern of Cux1 and Cux2 in the cerebral cortex, suggesting related or redundant functions (117). Mouse developmental studies evidenced *Cux2* mRNA expression throughout the subventricular zone (SVZ) of the neocortex in the early embryonic period, whereas in postnatal and adult mouse neocortices *Cux2* mRNA was expressed in the upper layers, namely layers 2, 3, and 4 (77,78). Moreover, *CUX2* mRNA was shown to be predominately expressed in the cortical projection neurons of the upper layers of the adult human PFC (80). CUX2 is considered as a potential pyramidal cell marker (78,80,116), and via high-throughput molecular profiling has been recognized in the callosal projection neuronal subpopulation (84). The results on developmental mouse also revealed that Cux2 is expressed in the cortical interneurons of SVZ and MZ, that invade the pallium via tangential migration routes (77). Its expression was further evidenced in deep layers of the mouse insular cortex at P5 (118), and in layer 5 of the mouse somatosensory barrel cortex at P14 (119). Interestingly, human prenatal single cell RNA sequencing showed evidence of co-expression between the layer 5-6 markers, and CUX2 in the human embryonic and midfetal cortex (120), as also seen by qRT-PCR of FACS cells within developing mouse brain (94). Furthermore, it is proposed that Cux1 and Cux2 are regulating fundamental aspects of late neuronal differentiation and controlling intrinsic mechanisms of

dendrite development, spine formation, and synaptic function in the upper layers of the cortex (78,117,121). De novo mutations of *CUX2* were found to be a genetic cause behind epileptic encephalopathy, intellectual disability (ID), and autism spectrum disease (ASD) (32,122). Nowadays, CUX2 is widely accepted as a canonical upper layer cortical marker and is used as such in the scientific community. The aims of this thesis are to analyze CUX2 expression patterns during laminar development of the human fetal cortex, since developmental patterns and laminar shifts of CUX2 expressing neurons during human cortical development have not yet been systematically described.

1.3.3. Migratory vs post-migratory neurons

The midgestational SP serves as a waiting compartment for cortical afferents, and also as a migratory zone for postmitotic neurons on the way to their predisposed position within the CP (8). Throughout all stages of cortical development, the SP contains both migratory and post-migratory neurons (8). Until now the breadth of the CUX2 expression pattern during human fetal cortical development was not specified, except for the evidence of CUX1 and CUX2 expression in the midgestational SP (79). Kubo et al. showed CUX1 and CUX2 reactive nuclei co-labeled with the migratory cell marker doublecortin (DCX), suggesting classification as migratory neurons in the SP. The same study showed another upper layer marker, BRN2, being co-labeled with DCX in the SP (79). Otherwise, DCX was shown to be co-labeled with Cux2 in the hippocampus evidencing a neurogenic progenitor population within the adult mouse dentate gyrus (123). The rest of the CUX2 positive neurons not classified as migratory are presumed post-migratory, associative neurons. Moreover, during the period of midgestation a subset of CUX2 migratory SP neurons possibly originate from GABA-ergic populations (79) considering GABA-ergic neuronal migration has late onset (124).

1.4. RNA-binding protein CELF4 during cortical development

RBP Celf4 (CUGBP Elav-like family member 4, also known as Bruno-like 4 protein/ BRUNOL4) was first studied in adult *Celf4* deficient mice where its role in excitatory neurotransmission was emphasized (102). Celf4 was found to be expressed in soma and dendrites of the adult mouse hippocampus, in layer V of the cortex, and *in vitro* in cultured mouse primary hippocampal neurons (103). Furthermore, *CELF4* is a neuron enriched gene in comparison with other CNS cell types (125), as well as one of the highly expressed genes within the human mid-fetal SP (72,75). The activity of 15-20% of the total mRNA within the adult, mouse transcriptome is regulated by Celf4, while a notable subset of these transcripts is associated with synaptic functions (103). Celf4 protein binds mRNAs in the 3' untranslated region (3' UTR) at a (U)GU nucleotide motif that was previously known as a binding site for CELF RBPs (99,103).

While it has been shown that Celf4 acts as a regulator of translation (103), the actual mechanism behind its function is not entirely known. In the largest whole-exome sequencing study of ASD, *CELF4* was implicated as an ASD risk gene and specifically as one of the fifty genes with a high frequency of *de novo* variants in individuals with ASD (104). Consequently, while the previous version of the database for autism spectrum disorders, SFARI (Simons Foundation Autism Research Initiative), was categorizing *CELF4* as a risk gene with a score of 3, the updated SFARI 2.0 database has categorized *CELF4* as a higher-risk gene with a score of 1 (126). Another study cross-referenced adult DPFC expression data with the aforementioned ASD study, revealing that *CELF4* is among the genes enriched for cortical layer 5 and most likely its expression is decreased compared to healthy controls (127). Multiple mRNAs were found to be associated with neurodevelopmental disorders that are translationally regulated in developing neocortices by various RBPs, including the CELF4 protein (94). A phenotype associated with behavioral and developmental disorders was shown correlating to human CELF4 mutation of a patient exhibiting a borderline intellectual disability (ID) (128). CELF4's association to ASD, indications of its expression during neocortical development, and its mutated expression in relation to a cognitive disorders phenotype all substantially implicate its significant role in brain development, mandating additional studies exploring its exact developmental functions. Importantly, CELF4 protein expression and its potential function over the course of human brain development are detailed in this thesis for the first time.

1.4.2. Synaptogenesis during human fetal cortical development

Cognitive and behavioral disorders in humans are foremost the result of abnormal development of neuronal circuitry. During the early fetal and midfetal cortical development most of the neurons become fully differentiated with the establishment of their axonal outgrowths and ingrowths. At the same time, the onset of synaptic formation (synaptogenesis) occurs and is considered one of the pivotal processes of cortical development (4,128). Synaptic strata defined as synapse-enriched laminae of human fetal neocortical wall, is the localization of synapses above and below CP in the early fetal cortex, *i.e.* MZ, and SP, respectively (15,16). Two synaptic strata were observed in other species as well: fetal dog (129), monkey (31), cat (130), and rodents (131,132). However, selective markers for cells within the synaptic strata are still poorly defined. The next chapter outlines the expansion and development of synapses and axonal ingrowths, and their features during human cortical development.

1.4.3. Synapse development

Synapses are points of contact between two neurons (133) specialized to convey neuronal inter-communication. Their development as early as in utero enables the possibility of fetal sensations, and fetal reactions to sensory stimuli. Two essential categories of synapses are electrical and chemical. Electrical synapses (i.e. gap junctions) are considered to form the earliest functional cortical networks. They provide rapid communication in the way that ions flow through the channels that couple cytoplasm of the pre- and postsynaptic neurons, ultimately generating electrical signals. Chemical synapses are synaptic clefts where neurotransmitters are released by exocytosis from vesicles of the presynaptic terminals. To generate nerve signals (i.e. action potentials), neurotransmitters bind to specific receptors on the postsynaptic cell membrane, thus causing a limited ion flow through the channel. These neurotransmitter receptors differ in function and synaptic response and can be ionotropic or metabotropic, while being gated either directly by the neurotransmitter itself or indirectly by a second messenger. Neurotransmitters can interact with either excitatory or inhibitory associated receptors, respectively eliciting or repressing action potentials.

The two main types of synapses in the CNS and neocortex are GABAergic and glutamatergic. Synapses are generally differentiated by their morphology being either asymmetric and usually excitatory, or symmetric and usually inhibitory. Quantitative studies estimate around 80% of excitatory, and around 20% of inhibitory synapses are present in the mammalian neocortex (31,134,135). As the ultrastructure of asymmetric and symmetric synapses differ, they can be visibly distinguished by electron microscopy and other super-resolution microscopy techniques (136). Type I synapses have asymmetric pre- and postsynaptic densities with a prominent and thick postsynaptic density (PSD), while type II synapses lack a thick PSD but exhibit comparatively more symmetry (137). Asymmetric synapses have a large number of round synaptic vesicles, compared to symmetric synapses which have flatter vesicles and a smaller active zone. Distribution of excitatory and inhibitory synapses on the neuronal elements is diverse, with most being either axosomatic or axodendritic, and the remaining being axo-axonic. Most of the excitatory synapses are exposed on the dendritic spines, with the remaining proportion located on the dendritic shafts (138).

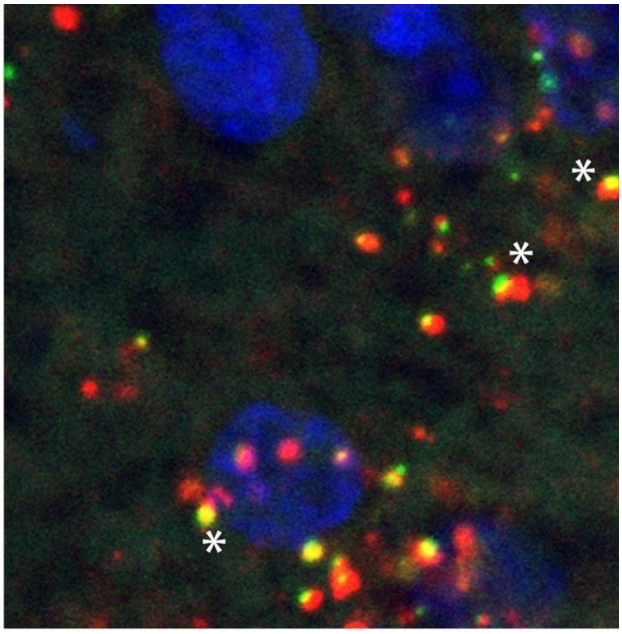


Figure 1.3. Synapses in the adult PFC imaged on confocal 60x microscope and 2.5x zoom to visualize postsynaptic (PSD95 marker, red) and presynaptic marker (synaptophysin, green). DAPI stain in blue depicts the cell nuclei. Asterisk labels presumed synapses.

Broader knowledge of synaptic organization along with modern molecular approaches of exploration enable complex research of synapses (139). Immunohistochemistry allows concurrent labeling of both pre- and postsynaptic elements thereby exposing synaptic localization and molecular specificity (Figure 1.3). While a vast number of pre- and postsynaptic proteins of numerous functions are known to be involved with synaptic formation (139,140), still missing are studies elaborating regulation of their synthesis and function in the human fetal cortex. The earliest human neocortical synapses are found in both the MZ and SP zone of the fetal cortex, subsequently spreading into the CP zone after 20 PCW (9,15,16). According to the newest study, first afferents to arrive in the pSP are thalamocortical axons showing up at 8 PCW (141), controverting the previous data indicating thalamocortical axons first innervating the pSP between 12-14 PCW (21,142). Part of this thesis is focused on the synaptic events occurring during cortical development before and during midgestation, given there is a lack of studies on the earlier stages of human synaptic development. Rapid changes in the localization and quantity of synapses occur in the early and midfetal development up to 24 PCW, when for the most part, synapses are distributed into synaptic strata, providing an ideal opportunity to analyze laminar input and distribution of postsynaptic cells to determine CELF4 involvement in synaptogenesis.

1.4.4. Synapses in human fetal cortex

This chapter will summarize features of the neocortical wall and retrospect on the contemporary location and type of synapses from early fetal until the pre-term phase of development. CP formation is the major event delineating the end of embryonic development and the onset of the early fetal development. In the **preplate phase** (6-7 PCW), before the CP is formed, the cerebral wall consists of two proliferative zones superior to the ventricle: the ventricular zone (VZ) and subventricular zone (SVZ), followed by the preplate (PP), a compartment enclosed by the pia (7,143,144). The **CP formation phase** (8 PCW) is characterized by a compact CP, and inferior to it, a narrow pre-subplate layer (5). Ultrastructural studies completed on 6-8 PCW samples observed rare, likely immature synapses within the preplate (MZ) (16,145,146). However, there is no specific data on the type or appearance of the synapses in this early stage of development.

The cerebral wall during the **pre-SP phase** (8.5-12 PCW) consists of the VZ and SVZ, the intermediate zone (IZ), the pre-SP zone, the beginning of the second CP (the so called ‘true CP’),

and the MZ (147). During the pre-SP phase, very scarce, mostly asymmetric synapses are present in the superficial IZ, pre-SP, and MZ (9,16).

At 13 PCW, the period of **SP formation** in the frontal lobe occurs. During this time, a CP packed with neurons, is an important landmark of the upper CP. On the contrary, a deep, loosely arranged CP represents an SP in formation (9). Synapses are present in the MZ, deep (second) CP and pre-SP, with the appearance of synapses in the second CP being an important feature of this developmental stage. The first synapses are visible on the proximal dendrites (asymmetric) of the second CP. At the same point of development, numerous symmetric non-synaptical junctions are present as well (unpublished human data, Kostovic; relevant monkey data in Kostovic and Rakic, 1990 (9)).

The **Stationary SP phase** is a prominent event that occurs between early and late fetal development. The cerebral wall in the regions of the dorsal frontal cortex at around 15 PCW consists of the VZ, SVZ, outer SVZ (OSVZ), IZ, the SP characterized by a superficial and deep portion, followed by the CP, MZ and the newly developed subpial granular layer (SPG) (147). Synapses are found in the two synaptic strata, through the entirety of the MZ and SP, but notably not within the CP. The SP at 15 PCW holds predominantly asymmetric synapses, where post-synaptic elements are mostly proximal dendrites of the SPN. Furthermore, the border of the CP and SP is the synapse richest site, followed by the superficial SP (sSP) (9,16).

During the late fetal period at around 20 PCW, the **stationary SP** serves as a waiting compartment for thalamocortical (TC) ingrowths before they reach the CP. The cerebral wall of the frontal lobe is structured by the following layers: the VZ, inner fibrillar layer (IFL), multilaminar axonal-cellular compartment (MACC), deep SP, sSP, CP and the MZ (142,147). At 20 PCW, synapses in the MZ and SP outbreak, with the peak in number of synapses in the superficial SP (Kostovic, unpublished), whereas only some very sporadic synapses are found in the CP (52). There is no firm data on the prevalence of the type of synapses at 20 PCW, however SPNs of the sSP are suggested being postsynaptic neurons, besides possibly basal dendrites of layer 5, 6 neurons. In the MZ, apical bouquets of the pyramidal CP neurons are depicted as postsynaptic elements of the local synapses (9,17).

At the end of midgestation in the early preterm phase, when **the SP is in the peak** of its development, the cerebral wall of the frontal lobe consists of the VZ, SVZ, the OSVZ together with the other components, the MACC, IZ, dSP, sSP, CP, MZ, and the SPG (142). An ultrastructural

study that quantified synapses at 24 PCW found the largest presence in the MZ and SP, with a notably enlarged number in the deep part of the CP. Moreover, synaptic density is the highest at the border between the CP and SP, besides in the sSP. While both asymmetric and symmetric synapses have been noted, symmetric synapses are mostly found on the cell bodies of SPNs. The other postsynaptic elements of this developmental stage include: dendrites of SPNs, basal dendrites and apical shafts of CP neurons, along with terminal bouquets in the MZ (9,16). Synapses are almost fully mature and in large counts at this stage, although throughout time most of the synapses will eventually perish (31,148,149).

1.4.5. Axonal ingrowths in human fetal cortex

At the end of embryonic development, being the start of fetal development, catecholamine (Tyrosine hydroxylase- TH expressing) axons penetrate through the lateral cerebral wall at around 6-8 PCW, directed towards the preplate (27), whereas concomitantly the earliest synapses are localized in the preplate (later on, MZ). In early fetal development, at 10 and 11 PCW, catecholamine axons are visible in the pre-SP and IZ (27). Furthermore, monoaminergic and TC axons are present in the MZ and pre-SP of the neocortex (9,21,25,141,150–154). At 13 PCW, TC and basal forebrain (BF) axons disperse through the deep CP (second plate), resulting in a loose structure (26,147,155,156) of the CP. The presence of synapses in the deep CP correlates with the localization of TC and BF axons.

In the period of midfetal development, between 15 and 20 PCW, TC and BF axons invade the whole hemisphere (except the hippocampus) through the SP (9,21,151–154). During midgestation in the period between 18 and 23 PCW, there is an accumulation of TC ingrowing axons in the sSP (9,21,151–154). Local GABA-ergic connections within the SP are present as well (Figure 2.2). TH positive axons are found at the interface of the CP and sSP (caudally) at the age of 20-24 PCW (157), which corresponds to the same place being highly-enriched with synapses. At the same time, TC afferents penetrate the mid-CP (9,21,151–154). Thus, the SP is integrally involved in the differentiation of the cerebral cortex, by interacting with and guiding the ingrowing axon systems over the course of the development (158).

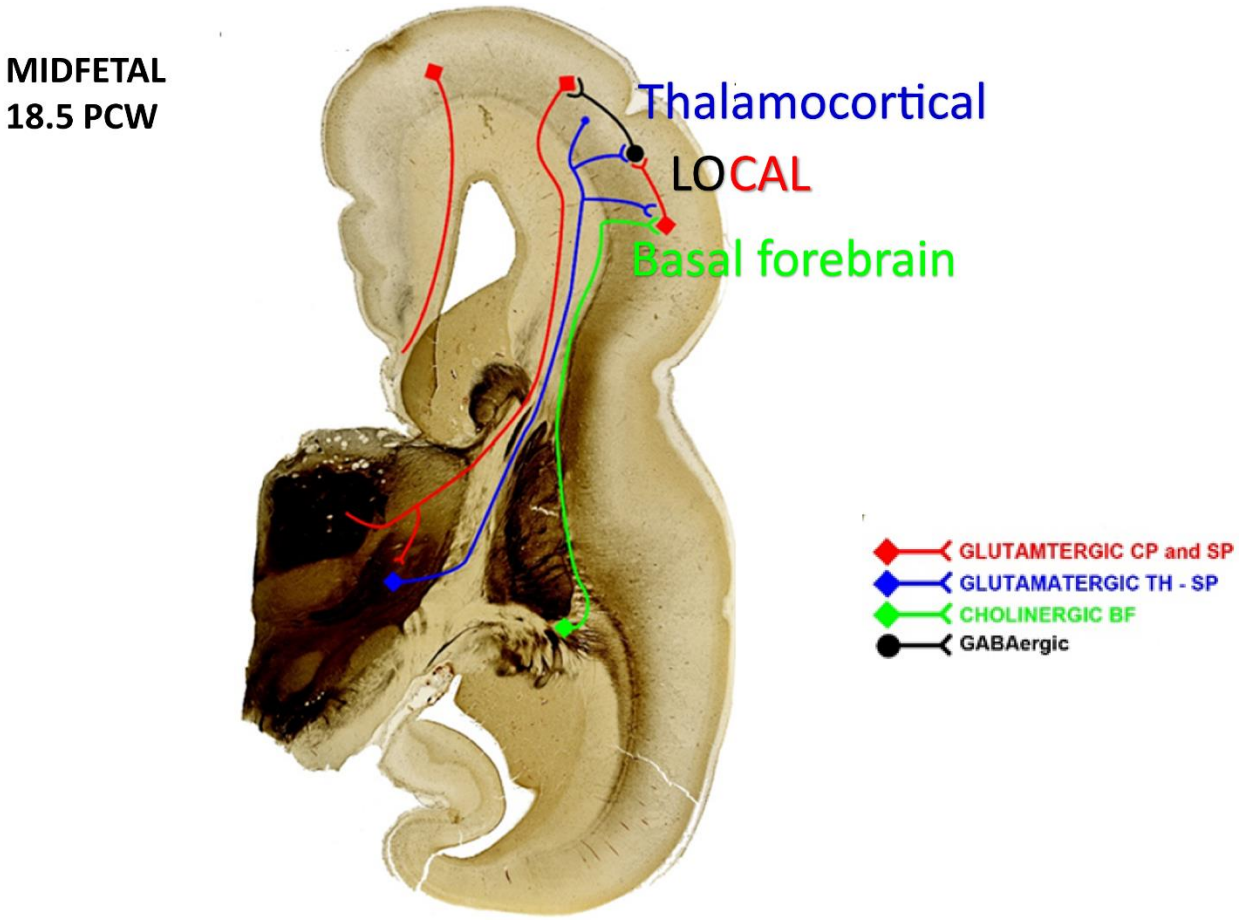


Figure 1.4. Midfetal SP circuitry of the frontal cortex includes glutamatergic, cholinergic and GABAergic connections between thalamic nuclei (TH), basal forebrain (BF) and SP, with local SP connections being made. Image was changed with permission (159).

2. Hypothesis

Transcription factor CUX2 and post-transcriptional factor CELF4 are expressed in the post-migratory neurons of synapse-enriched layers during the early and midfetal cortical development, reflecting their possible role in cellular fate selection and synaptic profiling.

3. General and specific aims

GENERAL AIM**:**

General aim of this research is to analyze a spatio-temporal patterning of transcription and post-transcriptional factors and to elucidate their prospective role in projection neuron fate determination and development of synapses in the early fetal and midfetal neocortex.

SPECIFIC AIMS:

1. Determine spatio-temporal laminar dynamics of CUX2 and CELF4 during early fetal and midfetal human cortical development
   a) Determine CUX2 and CELF4 developmental protein expression within synaptic strata

2. Identify classes of SP and MZ neurons which express CUX2 and CELF4 by using cellular markers and by specifying cellular phenotypes
   a) Specify projection neurons or interneurons, migratory or postmigratory neurons of SP, and identify Cajal-Retzius cells

3. Determine the neurotransmitter and neuromodulatory profile of CUX2 and CELF4 expressing cells

4. Determine CUX2 and CELF4 subcellular localization in postsynaptic neurons and their dendrites

5. Identify RNA binding targets of CELF4 protein during human fetal development

4. Materials and Methods

4.1. Human Brain Tissue

Human brain tissue was obtained during regular autopsies at several clinical hospitals affiliated with the School of Medicine University of Zagreb. A sampling of tissue was done following the Declaration of Helsinki 2013, previously approved by the Internal Review Board of the Ethical Committee of the School of Medicine University of Zagreb. Fetal age was determined based on crown-rump length (CRL, in millimeters) and pregnancy records, and was expressed in post-conceptional weeks (PCW). Paraffin-embedded post-mortem fetal brain tissue of the earliest age Carnagie-Stage 22 (CS) to 26 PCW of age, additionally newborn (38-40 PCW) and adult tissue samples were used (Table 4.2). Part of the human brain material was provided by the Joint MRC/Wellcome Trust grant #099175/Z/12/Z Human Developmental Biology Resource. For the analysis of protein expression during fetal cortical development, at least 2-3 brains per group were used. Samples are grouped by age and the stage of SP development (Table 4.1). Tissue was fixed in 4% paraformaldehyde (PFA) with adjusted fixation times, dissected coronally in blocks, and processed in a series of alcohols before being embedded in paraffin. Tissue was sectioned on a microtome SM2000R (Leica, Wetzlar, Germany) at 8-20 µm thick sections. Coronal sections of the frontal lobe at the level of the striatum, either in its rostral part or alternatively, the closest midlateral cortex that was available was used for immunostaining and *in situ* hybridization experiments (representative sections in Figure 4.1). The sections were selected based on Nissl stained sections that were previously stained (147) and correlated to the classic Bayer & Altman histological atlas (160). The discrepancy between somatosensory and visual cortex maturation, where the difference in the visual vs somatosensory cortex is approximately two weeks was taken into a consideration (9). Fresh frozen brains of different specimen ages from 11 - 20 PCW were dissected on dry ice and stored at -80 ºC. Dissections of the cerebral wall within the frontal lobe were used for the RNA immunoprecipitation experiments. Samples used for those experiments were grouped by age into early fetal (11 - 12 PCW), early midfetal (14, 15 PCW), and late midfetal (17, 18, 20 PCW) group.

Table 4.1. Paraffin-embedded brain tissue samples grouped by age and the stage of SP development used for immunostaining experiments.

I	PREPLATE PHASE	6 - 8 PCW
II	PRE-SP PHASE	8.5 - 12 PCW
III	SP IN FORMATION	13 - 14 PCW
IV	MIDGESTATION SP	15 - 21 PCW
V	STATIONARY SP	22 - 26 PCW

4.2. Immunohistochemistry (IHC) and Immunofluorescence (IF)

Immunohistochemistry and immunofluorescence were used to analyze the protein expression in the tissue, according to the previously published protocol was used (81,142). In brief, before proceeding IHC/IF, a standard process of section deparaffinization was performed in a series of xylol and alcohol solvents. Antigen retrieval was performed by boiling sections in citrate buffer (pH 6,0). Immunohistochemistry protocol requires an additional step of 20 min pretreatment with 0,3% hydrogen peroxide (H_2O_2) mixed with methanol and water. After three washes in PBS (Phosphate Buffered Saline), blocking solution containing 1-3% BSA (Sigma-Aldrich, St. Louis, MI, USA) and 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MI, USA) in PBS was applied on sections for 1-2 hrs. Blocking solution was replaced with primary antibodies diluted in blocking solution in the working concentrations and incubated overnight at 4 ºC. The following antibodies were used: NeuN-m (Abcam, Cambridge, UK), NeuN-rb (Abcam, Cambridge, UK), MAP2-m (Sigma-Aldrich, St. Louis, MI, USA), MAP2-rb (Sigma-Aldrich, St. Louis, MI, USA), CUX2 (Abnova, Taipei, Taiwan), CUX2 (Abcam, Cambridge, UK), Reelin (Merck Millipore, Burlington, MA, USA), Doublecortin (Merck Millipore, Burlington, MA, USA), Doublecortin (Santa Cruz Biotechnology, Dallas, Texas), FOXP1 (Abcam, Cambridge, UK), Neuroserpin (Abcam,

Cambridge, UK), CELF4 (Brunol4, Thermo Fisher Scientific, Waltham, MA, USA), CUGBP1 (Santa Cruz Biotechnology, Dallas, Texas), Ki67 (Agilent, Santa Clara, CA, USA), Synaptophysin (Agilent, Santa Clara, CA, USA), TLE4 (Santa Cruz Biotechnology, Dallas, Texas), Nurr1 (R&D systems, Minneapolis, Minnesota), SNAP25 (BioLegend, San Diego, CA, USA), Calretinin (Swant, Burgdorf, Switzerland), SST (BMA Biomedicals, Augst, Switzerland), nNOS (Merck Millipore, Burlington, MA, USA), GAD67 (Merck Millipore, Burlington, MA, USA), ChAt (Merck Millipore, Burlington, MA, USA), vGLUT1 (Merck Millipore, Burlington, MA, USA), GAD65/67 (Sigma-Aldrich, St. Louis, MI, USA), NPY (Merck Millipore, Burlington, MA, USA). After the incubation, sections were washed three times in PBS, and appropriate Alexa Fluor secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) were applied for 2 hrs at RT. Following three washes in PBS, TrueBlack quencher (Biotium, Fremont, CA, USA) was applied on sections. Finally, sections were covered using the Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Additionally, specific protocol was used for the DCX antibody double labeling with CUX2 antibody. To enhance the visualization of DCX, following the regular secondary antibody incubation used for CUX2, Tyramide Signal Amplification (TSA™) Fluorescein plus system reagent (Perkin Elmer, Waltham, MA, USA) was utilized. First, the appropriate host HRP-conjugated secondary antibody was incubated for 30 min, following the incubation with streptavidin-HRP (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 30 min, and finally, Fluorescein tyramide for 5-10 min. PBS washes were performed in between each incubation.

Following the primary antibodies incubation, IHC sections were incubated with secondary antibodies and biotin-avidin complex using Vectastain ABC kit (streptavidin-peroxidase complex, Vector Laboratories Inc., Burlingame, CA, USA). After the PBS washes, the sections were treated with DAB stain (Ni-3,3-diaminobenzidin, Sigma-Aldrich, St. Louis, MI, USA) shortly, before the dark blue color is visualized. To stop the reaction, the sections were washed with PBS, dried at RT, and finally covered with Histamount (National Diagnostics, Atlanta, GA, USA). Imaging was performed utilizing a high-resolution digital slide scanner NanoZoomer 2.0RS (Hamamatsu, Japan) and confocal microscope Olympus BX61WI, or FV3000 (Tokyo, Japan), and images were processed in Fiji (161) and Adobe Photoshop CS6.

Table 4.2. Specimens used for immunostainings and *in situ* hybridization experiments (L- left; R- right; ANT- anterior; FRONT- frontal; MID- middle).

Brain tissue	Age	Block orientation	Age group by SP development
Specimen 1	CS 22		Preplate phase
Specimen 2	CS 23		Preplate phase
Specimen 3	9 PCW	L hemisphere	Pre-SP phase
Specimen 4	10 PCW	R FRONT	Pre-SP phase
Specimen 5	11 PCW	R FRONT	Pre-SP phase
Specimen 6	11 PCW		Pre-SP phase
Specimen 7	12 PCW	ANT	Pre-SP phase
Specimen 8	12 PCW	L1 ANT	Pre-SP phase
Specimen 9	13 PCW	L ANT	SP in formation
Specimen 10	13 PCW	R1 ANT	SP in formation
Specimen 11	14 PCW	L FRONT	SP in formation
Specimen 12	15 PCW	R1 ANT	Midgestation SP
Specimen 13	15 PCW	R ANT	Midgestation SP
Specimen 14	17 PCW	L1	Midgestation SP
Specimen 14	17 PCW	L2	Midgestation SP
Specimen 15	17 PCW	L ANT	Midgestation SP
Specimen 16	18 PCW	R FRONT	Midgestation SP
Specimen 17	20 PCW		Midgestation SP
Specimen 18	21 PCW	L1 ANT	Midgestation SP
Specimen 18	21 PCW	L2 MID	Midgestation SP
Specimen 19	21 PCW	L HOR SUP	Midgestation SP
Specimen 20	24 PCW	L2	Stationary SP
Specimen 21	26 PCW	L2	Stationary SP
Specimen 22	38 PCW	L2	SP resolution
Specimen 23	38 PCW	L2	SP resolution
Specimen 24	40 PCW	R4	SP resolution
Specimen 25	18 y	PFC	gWM

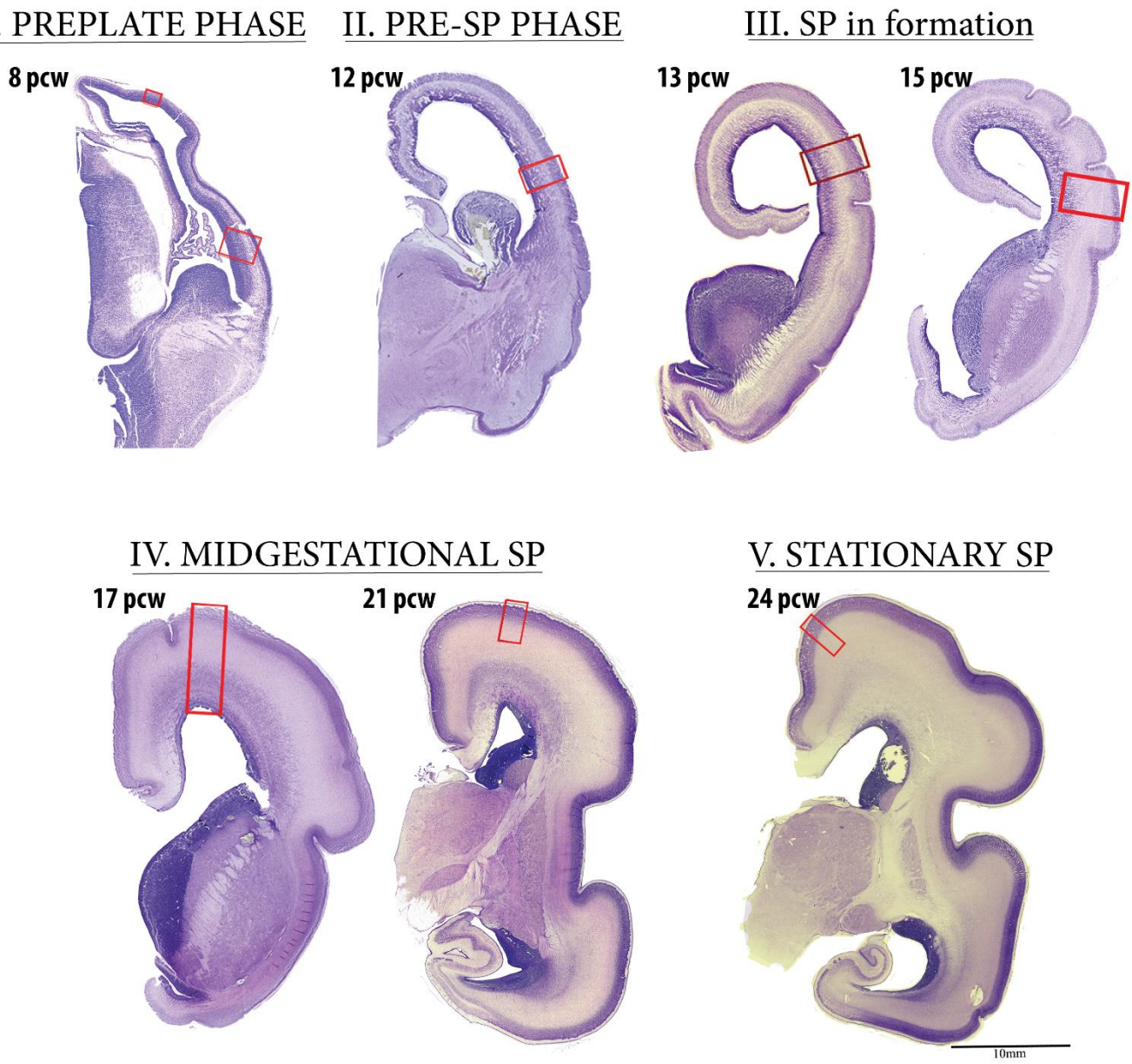


Figure 4.1. Nissl stained sections scans of representative coronal sections of the selected brain specimens. The specimens are assigned into each stage of SP development (I-V) in the early and midfetal phase of neocortical development. Age of each specimen is labeled next to the section. Red boxes indicate place of imaging, preferably through the prospective/future dorsolateral frontal cortex or the most adjacent region.

4.1. RNA Scope *in situ* hybridization

Paraffin-embedded sections were pre-processed for the fluorescent RNAscope *in situ* hybridization according to manufacturer protocol (Bio-Techne, Minneapolis, MI, USA). After deparaffinization, the sections were incubated with 3 % H_2O_2 for 10 min and washed with DEPC (Diethyl pyrocarbonate) PBS. The next step is target retrieval with the kit reagent boiling up to 10 min, following pretreatment with protease for 30 min at 40 ºC. The sections are hybridized for 2 hrs at 40 ºC in the HybEZ oven (Bio-Techne, Minneapolis, MI, USA), with the probe for *hCUX2* mRNA (custom made, Bio-Techne, Minneapolis, MI, USA). Following the hybridization, the sections were visualized using the RNA-scope Multiplex Fluorescent Reagent Kit v2 (Bio-Techne, Minneapolis, MI, USA) and the Tyramide Signal Amplification (TSA™) Plus Cyanine 3 (Perkin Elmer, Waltham, MA, USA) (162). RNAscope was coupled with immunofluorescence in such a way that the next day sections were incubated with primary antibodies used for IF: MAP2 or Doublecortin (DCX) diluted in blocking buffer, 2% BSA with 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MI, USA) in PBS overnight at 4 ºC. Secondary antibodies (Alexa Fluor, Thermo Fisher Scientific, Waltham, MA, USA) diluted in blocking buffer were applied on sections for 2 hrs at RT. Following washes in PBS, sections were covered using the mounting medium Vectashield with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

4.2. RNA Immunoprecipitation (RIP) coupled with RT-qPCR

To perform the RIP experiments, a MAGNA RIP kit (Merck Millipore, Burlington, MA, USA) protocol was followed, with minor changes and additions as shown in Figure 4.2. Fresh frozen tissue of dissected frontal lobe human fetus (approximately 100 µg) was lysed using the RIP Lysis Buffer (200 µL) prepared from the components of the kit. The tissue was gently homogenized by the pestle homogenizer, then kept for 5 min on ice, and finally stored at – 80 ºC overnight. Quick thawing of the sample by hand allows the complete cell lysis. For each brain tissue used in the experiment two reactions were ongoing: one using 2.5 µg of CELF4 antibody (rabbit, 0.2 µg/µL, Thermo Fisher Scientific, Waltham, MA, USA), and the second one with the same amount (2.5 µg) of a negative control IgG rabbit antibody (1 µg/µL, rabbit, provided in the RIP kit). Control antibody and the antibody of interest have to be derived from the same species, which in this case is rabbit. Protein A/G magnetic beads as part of the kit were used to perform immunoprecipitation of RNA-binding protein/RNA complexes. Following the protocol steps, the magnetic beads were prepared and mixed with the antibodies, eventually ending up with beads-antibody binding. A magnetic separator (Thermo Fisher Scientific, Waltham, MA, USA) was used to immobilize the magnetic bead complexes and to wash off the unbound antibody. Afterward, the lysates with the target antigen mixed in RIP immunoprecipitation buffer were added to the bead-Ab complex. What followed was the RNA immunoprecipitation (RIP) of RNA-binding Protein-RNA complexes while rotating overnight at 4 ºC. The unbound material was removed with the use of a magnet and by rewashing the beads multiple times (6X). The next part of the protocol was RNA extraction, preceded by the protein digestion. Proteinase K in 10 % SDS buffer constituted with the kit components was used for the protein digestion, and the samples were incubated at 55 ºC for 30 min with brief vortexing every 5 min. The residual product was the total RNA after RIP. Following the RNA purification process by using phenol and chloroform solvents (1:1 volume ratio), the separation of phases occurs. RNA was contained in an aqueous phase and was mixed with salt solutions and absolute ethanol before the samples were stored at – 80 ºC to precipitate overnight. The immunoprecipitated RNA pellet was re-suspended in 20 µL of RNase-free water and the RNA concentration was measured on NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer. Additionally, to prevent genomic DNA contamination of the samples, DNaseI treatment was performed using the TURBO DNA-free Kit (Thermo Fisher Scientific, Waltham, MA, USA) and following the protocol. RNA samples were incubated with 2 U of TURBO DNase

at 37 ºC, after which the reaction was stopped by the addition of DNase Inactivation Reagent. Finally, treated RNA was ready for RT-PCR and RNA-Seq analysis. A small quantity of RNA sample was used to generate the cDNA from the RNA sample (after RIP). For that purpose, M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), as an RNA-dependent DNA polymerase was used in cDNA synthesis reaction along with the accompanying components, such as Reaction Buffer, oligodT primers, and dNTPs. Reverse transcription (RT) reaction was performed at 42 ºC incubation for 2 h, 70 ºC for 15 min to stop the reaction and cooled down to 4 ºC. What followed was quantitative PCR analysis with the selected gene targets. Specific primers of each target were custom designed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) listed below. Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used to prepare the polymerase reaction. RT-qPCR was performed on the Applied Biosystems StepOne machine (Thermo Fisher Scientific, Waltham, MA, USA) as the manufacturer protocol suggests. The results were analyzed using the ΔΔCt method, while the CELF4 binding of each of the target mRNA was normalized by the Ct value of *GAPDH* mRNA as a binding control. IgG RNA after RIP was finally used as a normalization control for each target gene to discriminate the specific binding to CELF4. Analysis of the RT-qPCR data was performed in Graphpad software. Comparisons were done between negative control *Nestin* and other tested target mRNAs. Ordinary one-way ANOVA statistical test was used for multiple comparisons, while an unpaired t-test with Welch's correction or Mann-Whitney test was used for single comparisons. Additionally, a part of the isolated RNA was used for the standard next-generation, RNA sequencing and analysis by Genewiz company (South Plainfield, NJ). The list of genes generated for each sample was combined and used for the gene ontology functional analysis. The functional annotations are generated from DAVID annotation tools (https://david.ncifcrf.gov/tools.jsp).

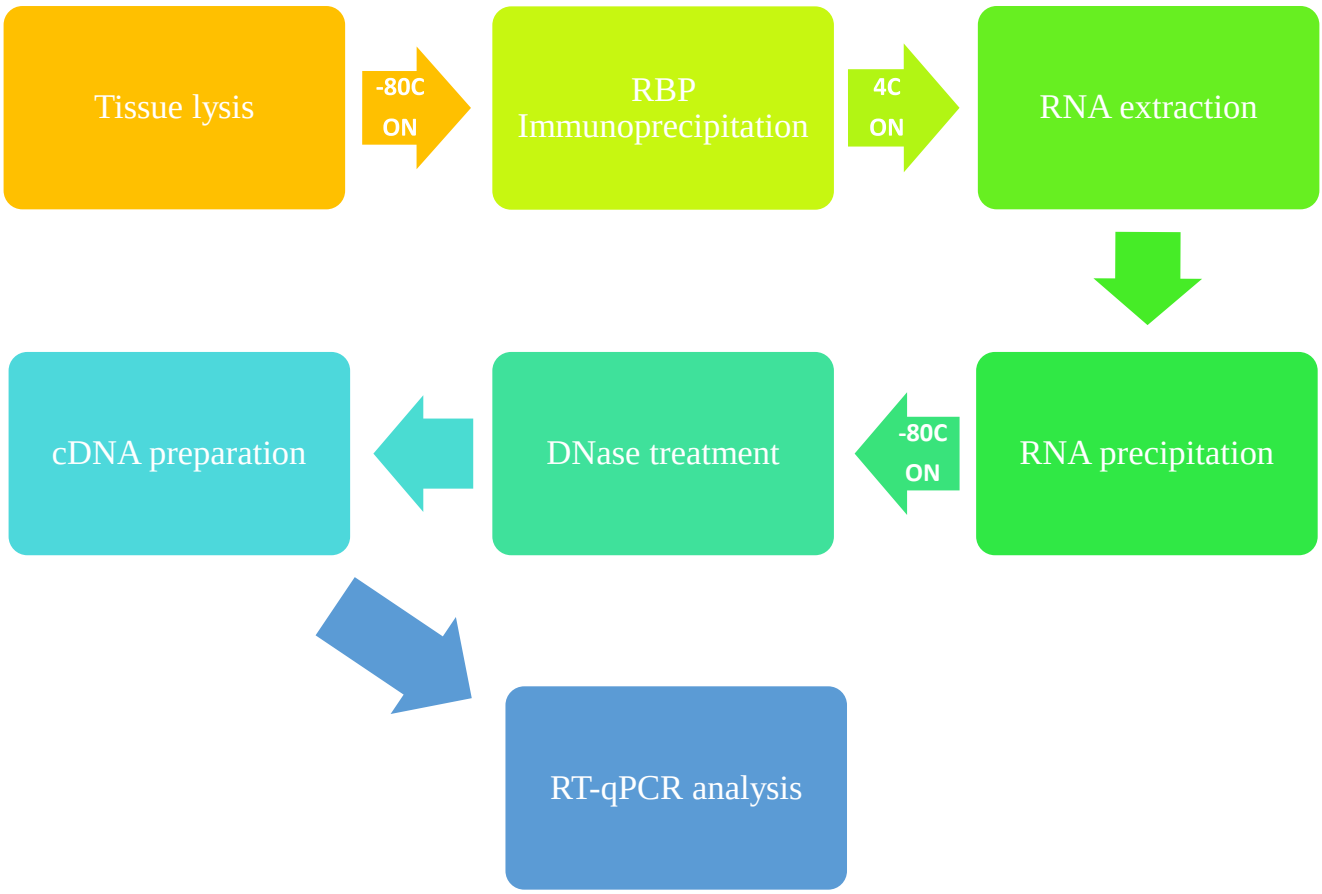


Figure 4.2. RNA-binding protein immunoprecipitation (RIP) experimental procedure coupled with RT-qPCR analysis. Steps that were followed in the procedure with the overnight (ON) treatments.

List of primers:

Human-***GAPDH***

Forward primer AAGAAGGTGGTGAAGCAGGC 20mer
Reverse primer GTCAAAGGTGGAGGAGTGGG 20mer

Human-***NESTIN***

Forward primer TCCAAGACTTCCCTCAGCTTTC 22mer
Reverse primer CAGGTGTCTCAAGGGTAGCA 20mer

Human-***Beta-ACTIN***

Forward primer GCCCTGAGGCACTCTTCCA 19mer
Reverse primer TGTGTTGGCGTACAGGTCTT 20mer

Human-***TLE4***

Forward primer AGCACTGGAAAGGACAACCT 20mer
Reverse primer TCCCCAGAGCCAGTGACAAT 20mer

Human- ***EIF4A2***

Forward primer ATGTGCAACAAGTGTCTTTGGT 22mer
Reverse primer CTCCCAAATCGACCCCCTCT 20mer

Human- ***SYNPR***

Forward primer GTTGCTGTCTTCGCCTTCCT 20mer
Reverse primer GCTGATGAACCCACCAACCA 20mer

Human- ***VGLUT1***

Forward primer TCGTGGGGGCCATGACTAAG 20mer
Reverse primer GACCCCGTAGAAGATGACACC 21mer

4.3. Electron microscopy of the human fetal tissue

Ultrastructural visualization of the human fetal tissue was carried out by deploying electron microscopy (EM). Pre-embedding immuno CELF4 labeling and EM was done to determine CELF4 subcellular localization in postsynaptic neurons of synaptic strata in human prospective frontal (17 PCW) and temporal cortex (19 PCW). The human brain samples were fixed in 4% PFA and 0.1% glutaraldehyde for a specifically determined time (up to 24hrs). The brain hemispheres were stored in 30 % sucrose at + 4 ºC. Approximately 1 cm from pial surface of the brain pieces of subplate were dissected out of the cortex, washed in PBS, and embedded in agar. First, agarose (Thermo Fisher Scientific, Waltham, MA, USA) was melted in PBS to get 3.2% liquid mixture at around 100 ºC, which is poured into plastic containers and cooled down to 60 ºC when the tissue is transferred from PBS and orientated with a spatula. Agar solidifies and finally embeds the tissue in a block. Hardened agar embedded tissue blocks were cut on vibratome at 80-100 µm slices. Slices were kept in 30 % sucrose (in glass vials) and were frozen in dry ice, afterward being quickly thawed. The process was repeated three times to be able to achieve better penetration of the antibodies, and for preserving the ultrastructure of the tissue for EM work. To block endogenous peroxidase slices were immersed 10 min in 1 % H_2O_2 in phosphate buffer (PB), which was used for the subsequent washings as well. Pre-embedding immune was done by using a primary antibody for CELF4 (Thermo Fisher Scientific, Waltham, MA, USA). First, the sections were washed in PB, after which they were blocked for 3 hrs in donkey serum on the shaker, at the RT. CELF4 Ab was diluted 1:300 in donkey serum and the sections were incubated for 2 days at +4 ºC. Subsequently, sections were washed in PB and incubated in the biotinylated secondary antibody accompanied with ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) for 2.5 hrs at RT in ½ diluted blocking buffer in PB, after which the sections were washed and finally DAB and Nickle (part of the kit) were applied in order to develop and visualize the staining. The reaction with DAB is stopped with water and lastly washing in PB. Following the protocol, tissue was dehydrated, treated with osmium, and embedded in resin (Embed 812). Finally, the tissue slices were cut on ultra-thin 1-2 µm sections which were eventually examined on a Phillips CM-12 electron microscope (Amsterdam, Netherlands) operated at 80 kV.

5. Results

Results of this thesis are conceptualized into two parts:

1) **CUX2** transcription factor protein and mRNA expression pattern throughout the fetal cortical development, and determining its role in the cortical processes
2) **CELF4** post-transcriptional factor expression pattern, subcellular localization, and mRNA binding targets and confirmation of its role in the first half of fetal cortical development

with the special emphasis on the following phases of the SP development:

- **early fetal phase** (8–14 PCW), *i.e.* before and during the initial formation of SP- preplate stage (I), pre-subplate (II), and SP in formation (III) stages, when deep layer neurons are born and first SP circuits are formed
- **midfetal phase** (15–26 PCW) when upper layer neurons are born and migrate through SP, while SP is achieving highest complexity of connections- midgestational (IV) and stationary SP (V)
- additionally, **near-term fetal development** (38 PCW) when neurogenesis ceased, SP got resolved, and upper layer neurons reached their positions
- finally in the **adult** neocortex.

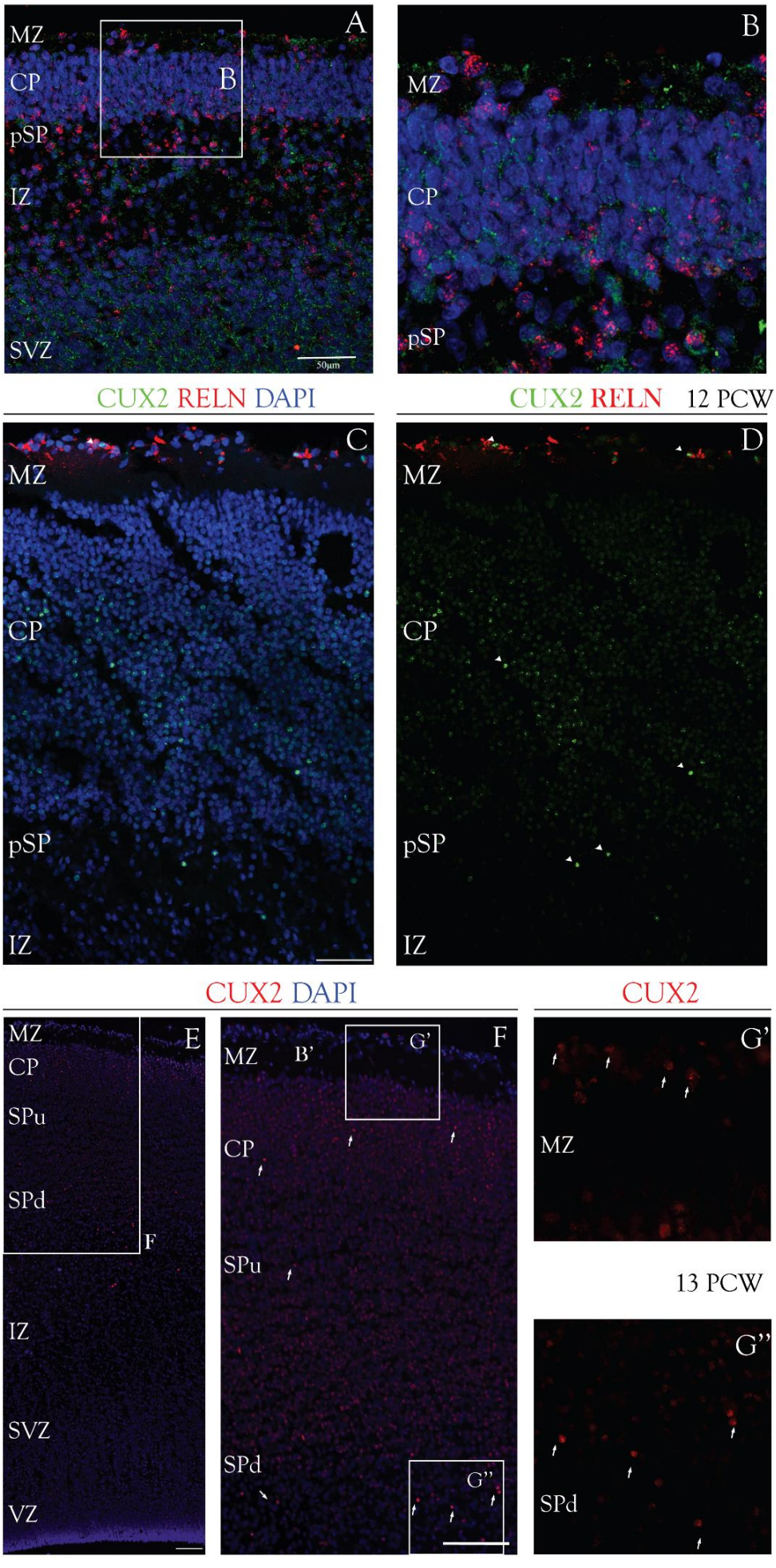
Chronological grouping of results is based on the significant events in SP development (I-V) as previously defined in human and monkey cortical development (9,147,155).

5.1. CUX2 spatio-temporal expression pattern

Spatio-temporal expression pattern of CUX2 protein in neocortex was systematically analyzed throughout the fetal cortical developmental with comparison to the adult neocortex as the first aim of this thesis. The results identified CUX2 immunoreactivity in the nucleus, as was previously described in the literature (121). At the onset of the early fetal phase (8 PCW), CUX2 immunoreactive nuclei were found during the formation and first condensation of the CP. In particular, strong CUX2 positive nuclei were depicted in the MZ, deep CP, and pre-subplate (pSP) (Figure 5.1A, B). At 12 PCW, CUX2 positive nuclei were present in the upper third of the MZ co-localizing with Reelin, a Cajal-Retzius (CR) neuron subtype marker (163,164). Heterogeneous and somewhat weaker CUX2 positive nuclei were found within the deepest portion of the CP, while strong reactive nuclei were seen within the pSP of the midlateral cortex delineating the prospective frontal cortex (Figure 1C, D). At 13 PCW, during the SP formation period, CUX2 positive nuclei were present in both the upper and deep SP, while modest immunoreactivity was observed in the CP and MZ of the prospective frontal cortex (Figure 5.1E-G). Overall, during the early fetal phase, CUX2 reactivity in the germinal proliferative zones, such as Ventricular (VZ) and Subventricular zone (SVZ) was not present.

During midfetal development, at 21 PCW in the dorsolateral cortex of the frontal lobe, CUX2 positive large nuclei were mainly depicted in the middle of the CP. CUX2 positive nuclei partially co-localized with multipolar, large MAP2 positive neurons of the SP (Figure 5.1H-K). MAP2 marker was used to label differentiated, more mature neurons. At the end of midgestation, at 24 PCW, CUX2 positive nuclei were located in the MZ, CP, and SP of the precentral gyrus (Figure 5.1L-O). At 24 PCW, Neuroserpin (NRSP) immunoreactivity co-localized with CUX2 nuclei in the SP neurons (Figure 5.1O), while NURR1 positive nuclei were not confirmed to co-localize with CUX2 (not shown). Both of these markers, NRSP and NURR1 were previously described to label SP neurons (88,165). In addition, MAP2 co-localized with CUX2 positive nuclei of large, projection pyramidal layer 5 neurons (Figure 5.1L, N). In newborn brain (38 PCW), CUX2 had the strongest expression in the upper cortical layer (L1–3) cells, and SP remnant (SPrm) in the frontal cortex, while FOXP1 expressing neuronal subtype of neocortical layers 4 and deep layer 5 had weak CUX2 positive reactivity (Figure 5.1P). Additionally, one part of the nuclei of the gyral white matter (gWM) in frontal cortex maintained CUX2 positive reactivity (Figure 5.1R). In the adult brain (18y old), CUX2 positive nuclei were found across the layers of PFC with the highest

reactivity in the upper cortical layers (magnified in Figure 5.1T), where they co-localized with MAP2 immunoreactive pyramidal neurons.


CUX2 GAD65/67
8 PCW
A
MZ
CP
B
pSP
IZ
SVZ
50µm
B
MZ
CP
pSP
CUX2 RELN DAPI
CUX2 RELN
12 PCW
C
MZ
CP
pSP
IZ
D
MZ
CP
pSP
IZ
CUX2 DAPI
CUX2
E
MZ
CP
SPu
SPd
F
IZ
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G''
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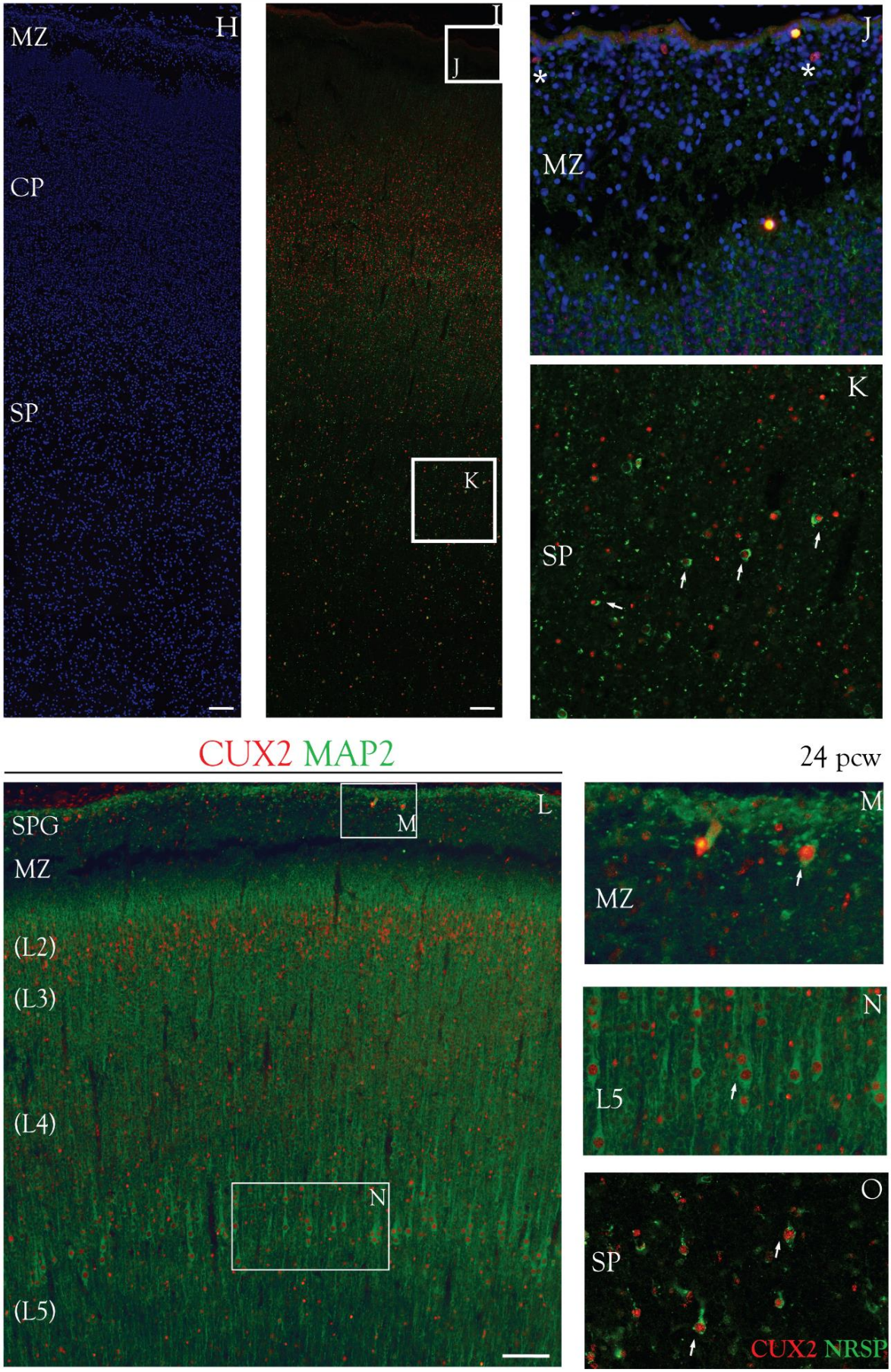
DAPI
CUX2 MAP2
21 pcw
MZ
H
CP
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I
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K
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MZ
K
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CUX2 MAP2
24 pcw
SPG
MZ
(L2)
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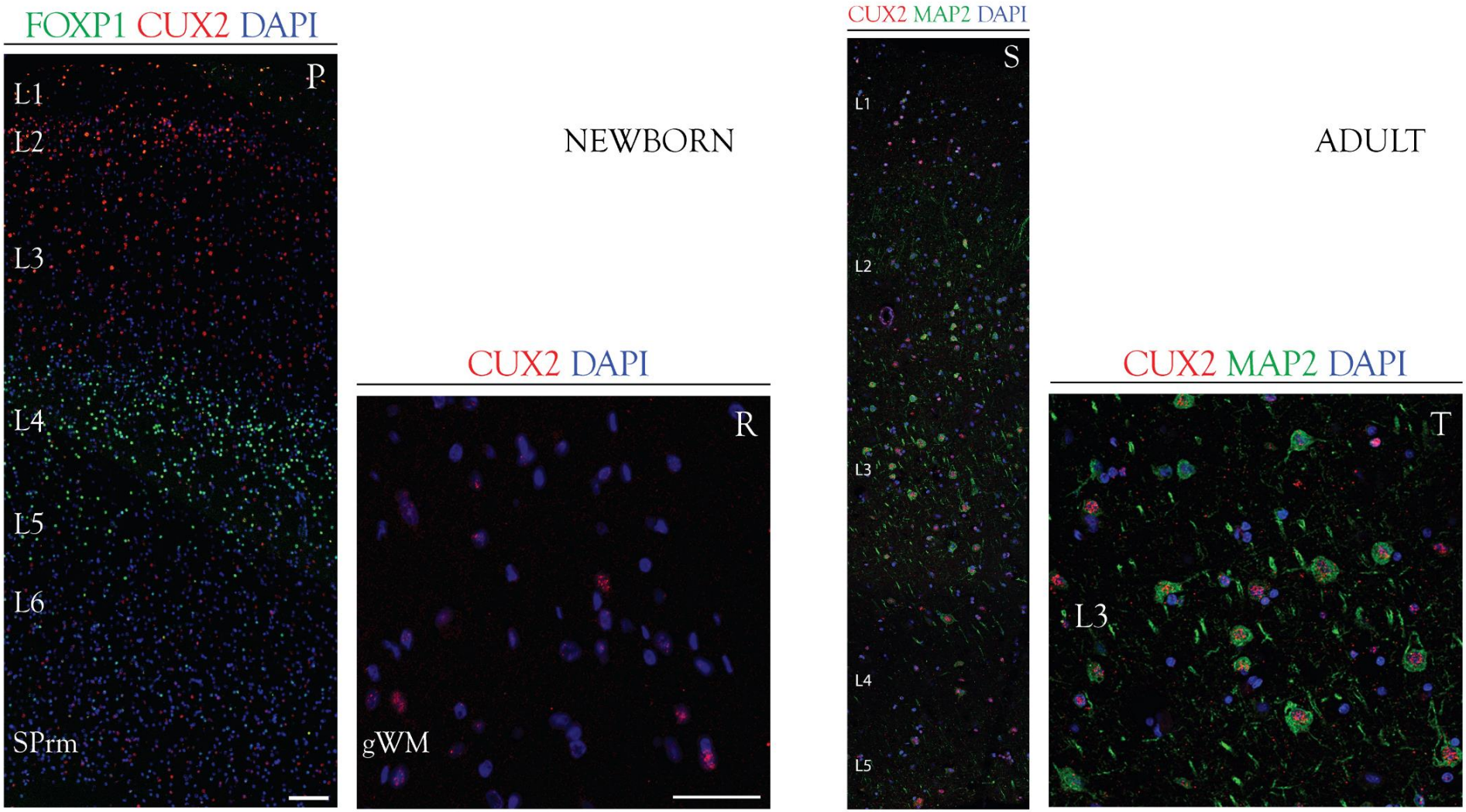
SP
CUX2 NRSP


FOXP1 CUX2 DAPI
P
L1
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L3
L4
L5
L6
SPrm
NEWBORN
CUX2 DAPI
R
gWM
CUX2 MAP2 DAPI
S
L1
L2
L3
L4
L5
ADULT
CUX2 MAP2 DAPI
T
L3

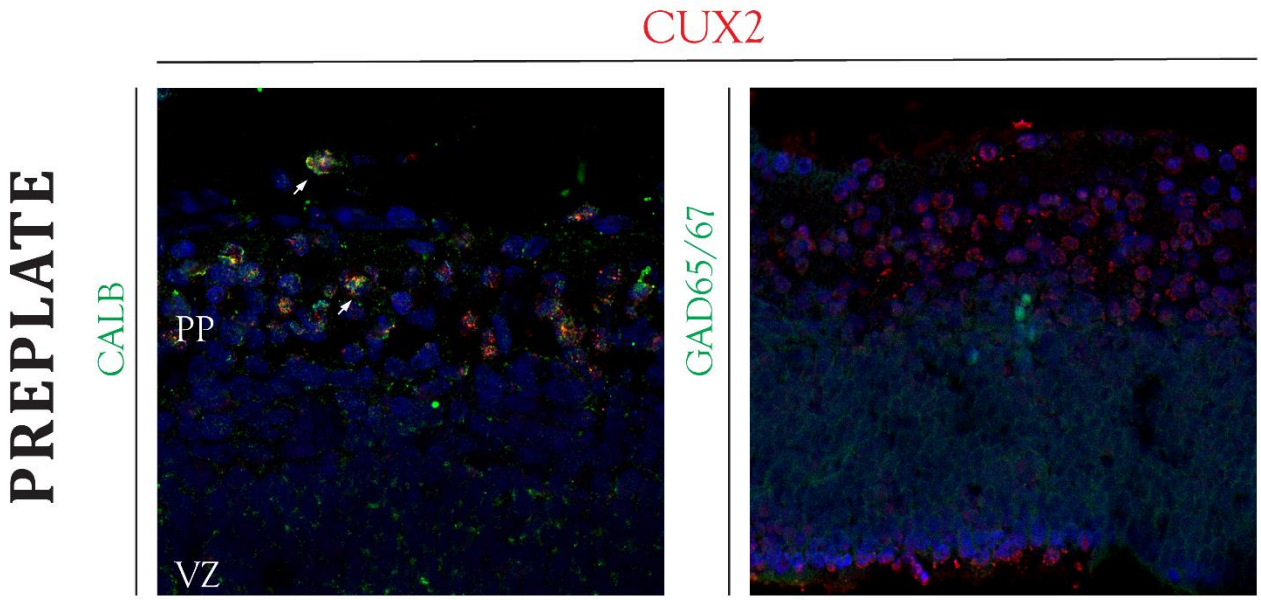
Figure 5.1. Spatio-temporal expression pattern of CUX2 protein throughout the fetal neocortical development (A-R) and adulthood (S-T). Sections are immunostained for CUX2 and GAD65/67, RELN, MAP2, NRSP, FOXP1, respectively. Highlighted in this figure is CUX2 expression within the transient zones, MZ, pre-SP and SP, additional to the expression in the adult gyral white matter (gWM). White boxes mark magnified areas of the neighboring images. Fetal zones and layers are defined and labeled on the images as previously described in Materials and Methods. Arrows and arrowheads identify colocalized markers, while asterisk remark positive CUX2 cells. Specimen age is specified along the images. Scale bar A-C= 50 μm; H-R=100 μm.

5.2. Neurotransmitter molecular profile of CUX2+ MZ and SP cells

Molecular profile of the MZ and SP CUX2+ (positive) neuronal population is shown to be heterogeneous and dynamic. Differential spatio-temporal expression of the brain neurotransmitters such as Neuropeptide-Y (NPY), neuronal nitric oxide synthase (nNOS), and the other (6,166–170) is exhibited during development. In the earliest fetal stage when the preplate (PP) features the cortical wall (at 8 PCW) there were no visible CUX2+ nuclei co-localized with glutamic acid decarboxylase (GAD65/67), a marker of inhibitory γ-aminobutyric acid (GABAergic) neurons. Moreover, a co-immunostaining with CUX2 and calcium-binding protein, calbindin (CALB) showed some co-localization in the PP (Figure 5.2). After the CP has emerged and pSP appeared, additional neurotransmitters, as somatostatin (SST) are expressed, while there was no visible expression of nNOS in the frontal or basal neocortex. Nevertheless, CUX2 expression in the early stages was somewhat weak and there was no visible overlap with the GAD65/67 expression throughout the cortical wall (Figure 5.2). In the period of CP transformation and formation of a new fetal compartment, SP, at 13 PCW, an arborized SST neuron was distinguished in the SP of the prospective frontal cortex (Figure 5.3), whereas no co-localization of CUX2 nuclei and SST or GAD65/67 markers was identified neither in MZ nor in pSP.

During the midgestation (15-24 PCW), SP is enlarged and becomes the thickest compartment of the cerebral wall with plethora of molecular markers expressed in the SP at the time (6). NPY co-localized with CUX2 nuclei both in MZ and SP in the neocortex at 21 PCW. Furthermore, a co-localization between SST and CUX2 nuclei in SP was present, but not in the MZ. nNOS neurotransmitter was expressed in some SP cells, even though it was not co-localized with CUX2 nuclei, nor it was expressed in the MZ. GAD65/67 expression was visible both in the

MZ and SP cells, yet without the overlap with CUX2 positive nuclei (Figure 5.4). On the other hand, in the stationary stage of SP development, at 24 PCW, when the SP compartment is still the most voluminous compartment of the cerebral wall and accommodates a vast diversity of neuronal subtypes most of the SP molecular markers are fully expressed, so are the neurotransmitter markers NPY, nNOS, GAD65/67, and SST (Figure 5.5). SST neurons were co-expressed with CUX2 positive nuclei in the SP of the dorsal frontal neocortex at 24 PCW, while there was no clear expression of SST positive neurons in the MZ. NPY was extensively expressed in the MZ and SP at 24 PCW, although its expression on the representative images does not overlap with CUX2. Moreover, nNOS expression was not found in the MZ of the same specimen, whereas nNOS positive cells found in the SP zone are not co-expressed with CUX2 in the SP. A significant overlap between CUX2 positive nuclei and GAD65/67 inhibitory neurons has not been found either in the MZ or SP (Figure 5.5).



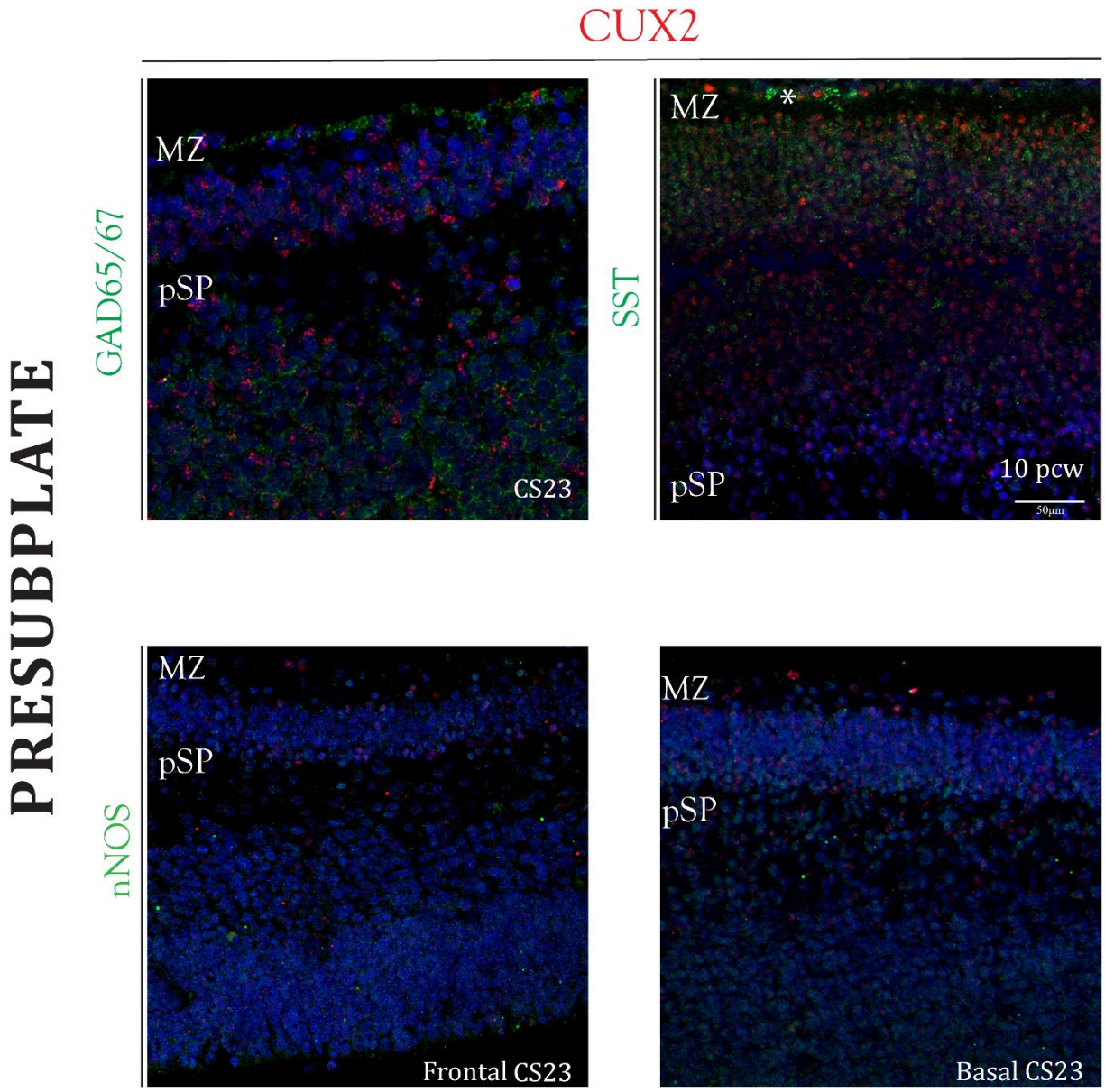


Figure 5.2. Neurotransmitter profile of the neocortex during the preplate (PP) and presubplate stage (pSP). Sections are immunostained for CUX2 and CALB, GAD65/67, SST, nNOS, respectively, highlighting the cells of the MZ and pSP. Specimen age is marked in the right bottom corner of each image. Asterisk marks a positive SST cell. DAPI stain is used to label the nuclei. Scale bar = 50 µm.

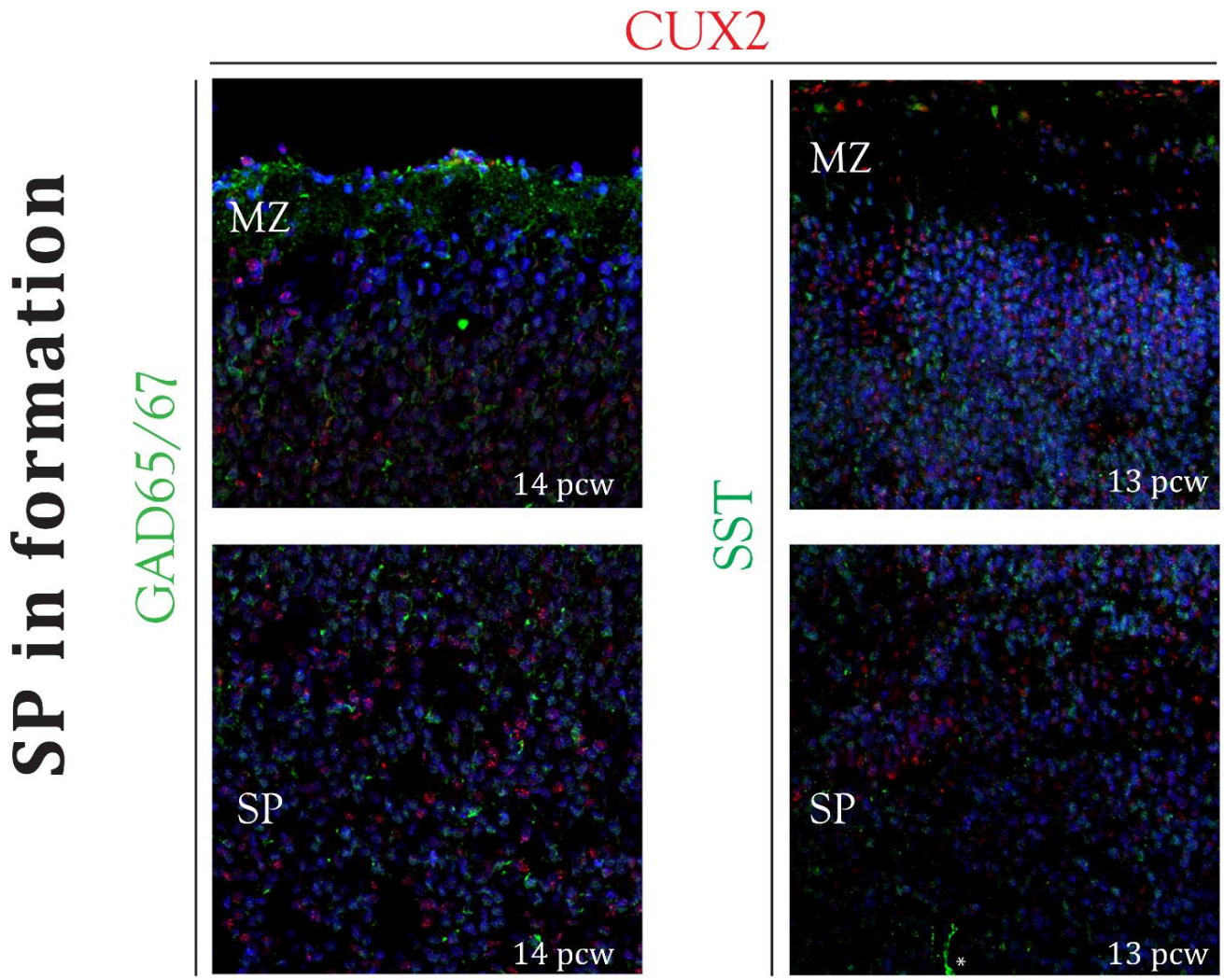


Figure 5.3. Neurotransmitter profile of the MZ and SP during the SP formation stage. Sections are immunostained for CUX2 and GAD65/67, SST, respectively, highlighting the cells of the MZ and SP in formation. Specimen age is marked in the right bottom corner of each image. Asterisk marks a positive SST cell. DAPI stain labels the nuclei. Scale bar = 50 µm.

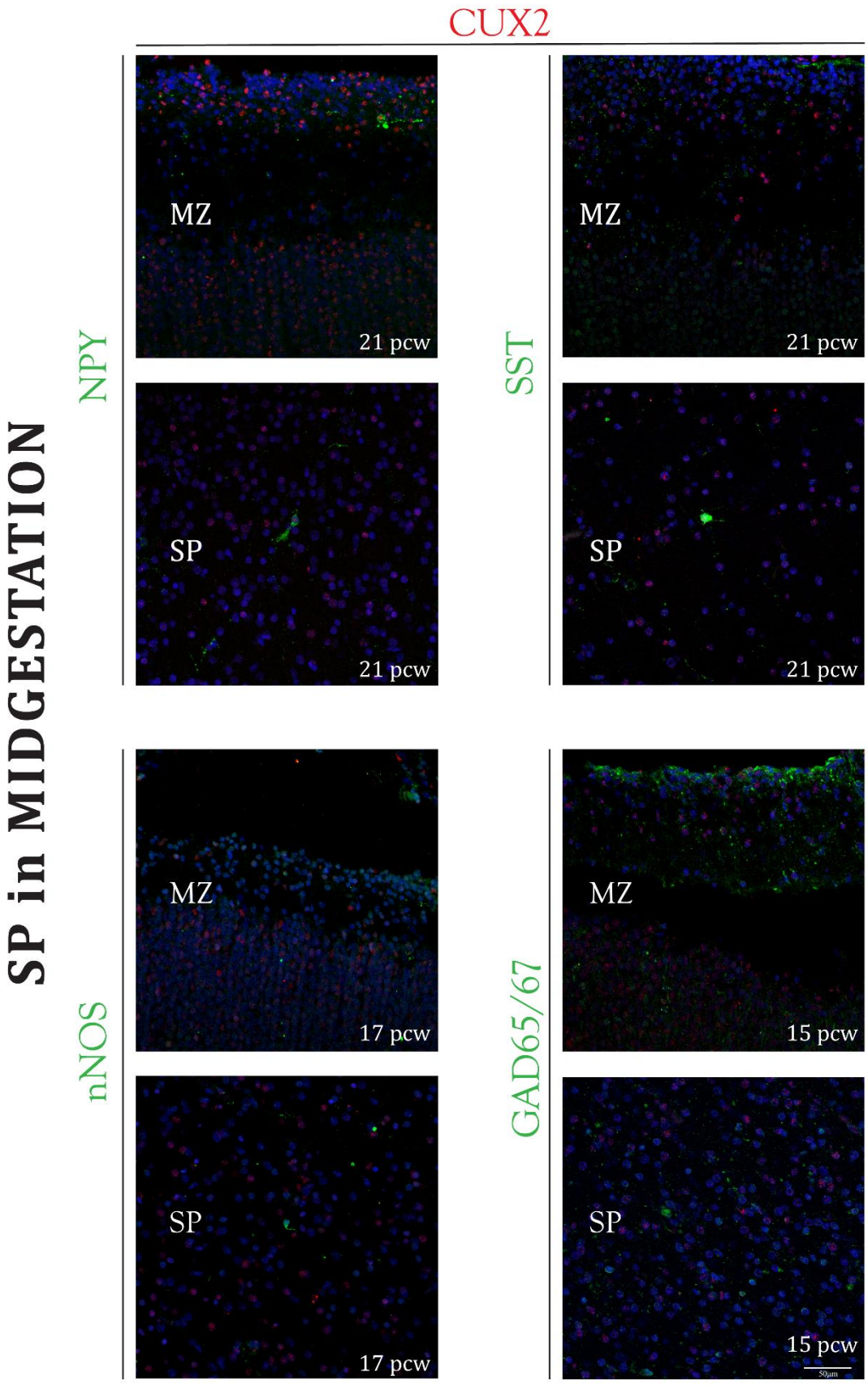


Figure 5.4. Neurotransmitter profile of the MZ and SP fetal zones during the midgestation. Sections are immunostained for CUX2 and NPY, GAD65/67, SST, nNOS, respectively. Specimen age is marked in the right bottom corner of each image. Scale bar = 50 µm.

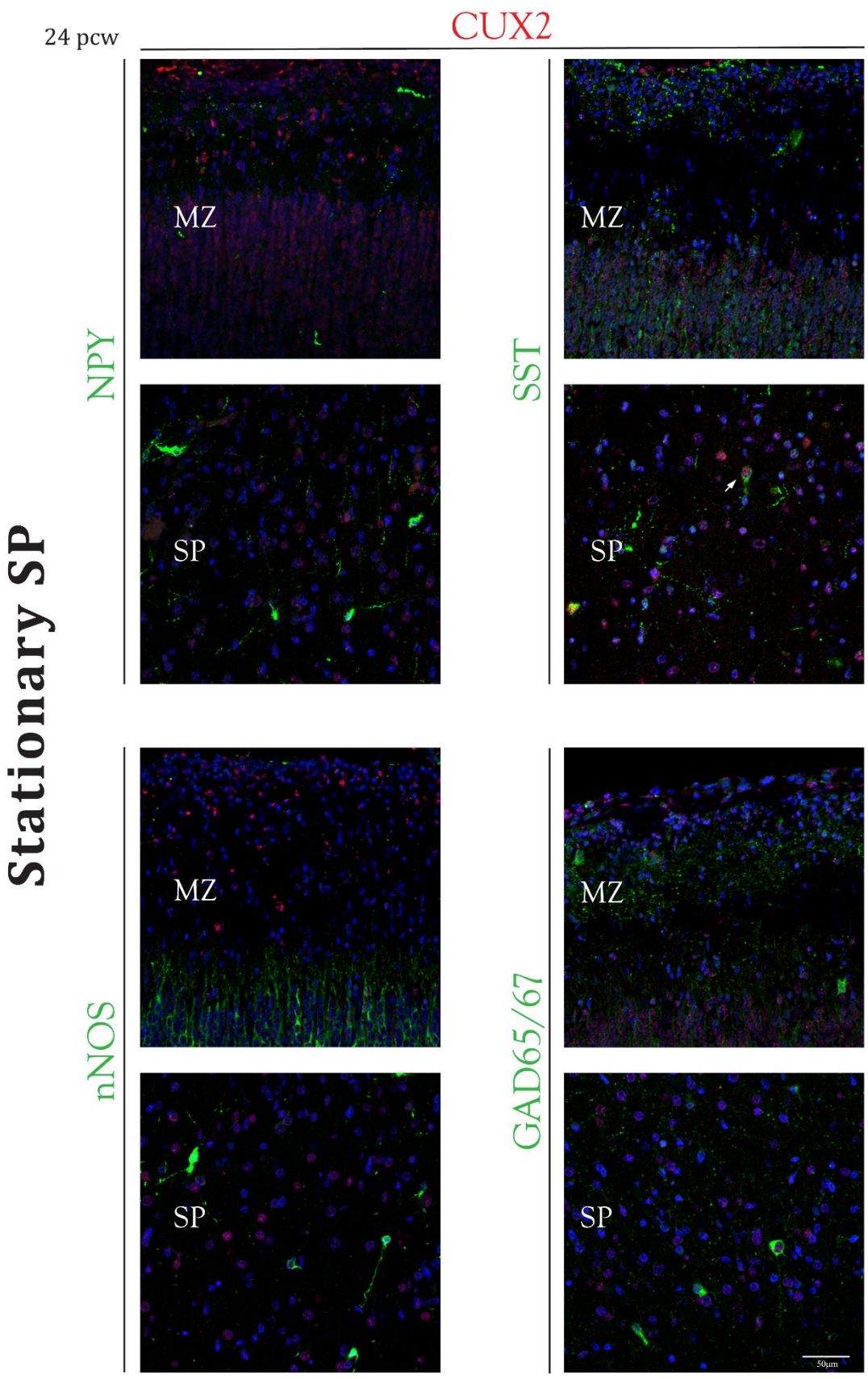
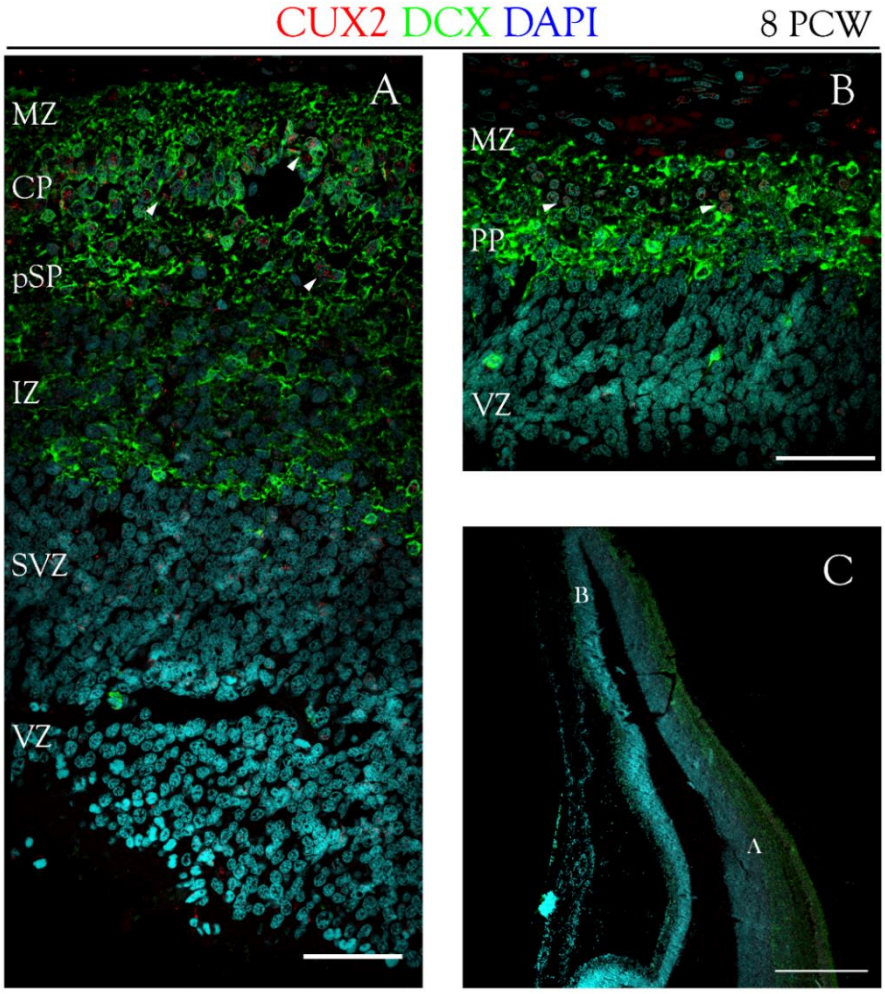


Figure 5.5. Neurotransmitter profile of the MZ and SP fetal zones during the stationary SP stage. Sections are immunostained for CUX2 and NPY, SST, nNOS, GAD65/67. Specimen age is marked in the right bottom corner of each image. Scale bar = 50 µm.

5.3. CUX2 in migratory cells

One of the aims of this thesis was to examine a migratory profile of CUX2 positive neurons in the early (Figure 5.6A-C), midfetal (D-H), and pre-term (J-I) stages of neocortical development. Migratory profile of CUX2 positive neurons during the early fetal stage (Figure 5.6A-C) showed that a part of CUX2 nuclei co-localized with Doublecortin (DCX), a marker of immature migratory neurons (34). Interestingly, only a few DCX cells were visualized in the VZ, while characteristic DCX protein expression was visualized in the preplate (PP) and pSP. In addition, CUX2 nuclei were depicted in the CP and pSP of ventrolateral cortex (Figure 5.6A) at 8 PCW, as well as in the PP of dorsolateral cortex (Figure 5.6B). The images were taken in the neocortical anlage of one hemisphere as shown in Figure 5.6C. Immunostainings in the midfetal stage of cortical development (26 PCW) have shown that large CUX2 positive nuclei in the SP mostly do not co-localize with DCX neurons (Figure 5.6G), while weak and scattered CUX2 protein expression was detected in the outer subventricular zone (OSVZ) (Figure 5.6H). Finally, in the newborn brain of neonatal period, at 38 PCW, co-localization of DCX and CUX2 positive nuclei in the frontal cortex was identified (Figure 5.6I). Interestingly, a widespread DCX expression pattern in the newborn frontal cortex (38 PCW) was noted, thus, a fraction of cortical cells maintained their DCX expression, especially in the deep cortical layers (Figure 5.6I'') (81).



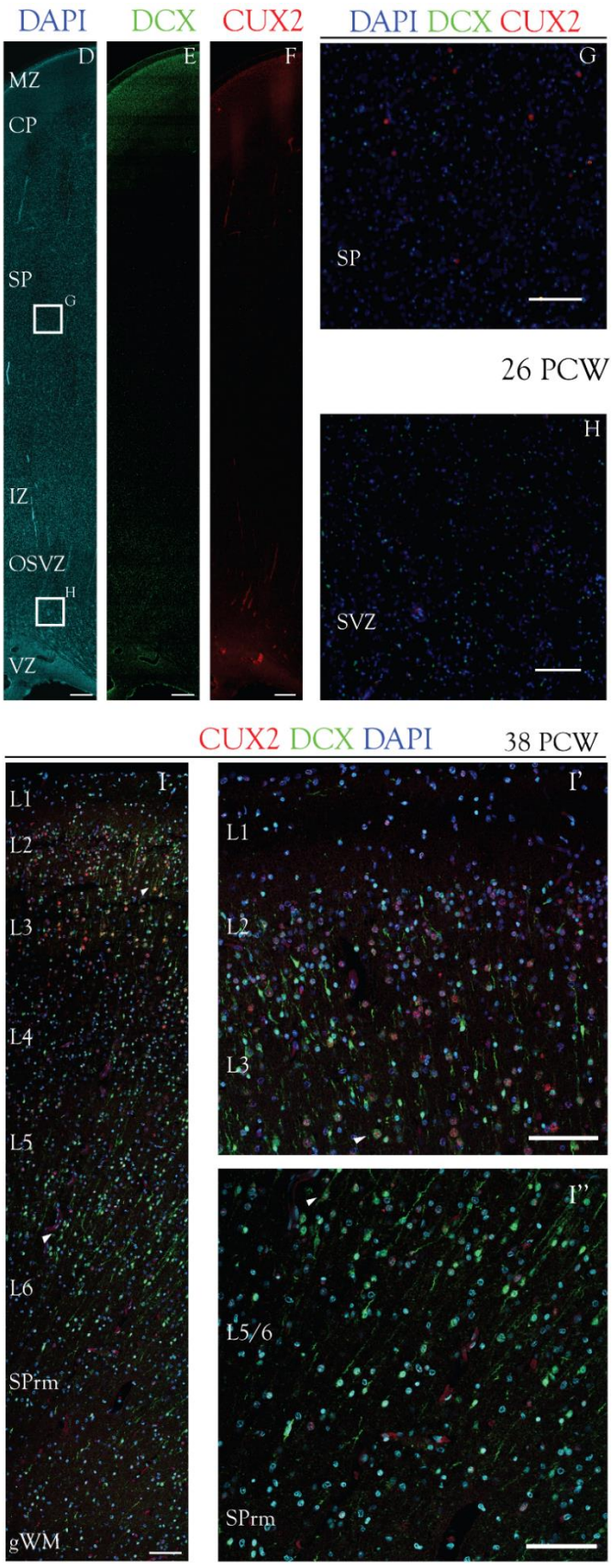


Figure 5.6. Cerebral wall of the developing frontal cortex at 8 PCW (A-C), superior frontal gyrus at 26 PCW (D-H), and frontal cortex at 38 PCW (I) were stained for CUX2 and DCX. White arrowheads mark CUX2+/DCX+ co-localization. Scale bar D-F= 500 µm; C, G-H= 100 µm; A, B, I= 50 µm.

5.4. CUX2 subcellular localization

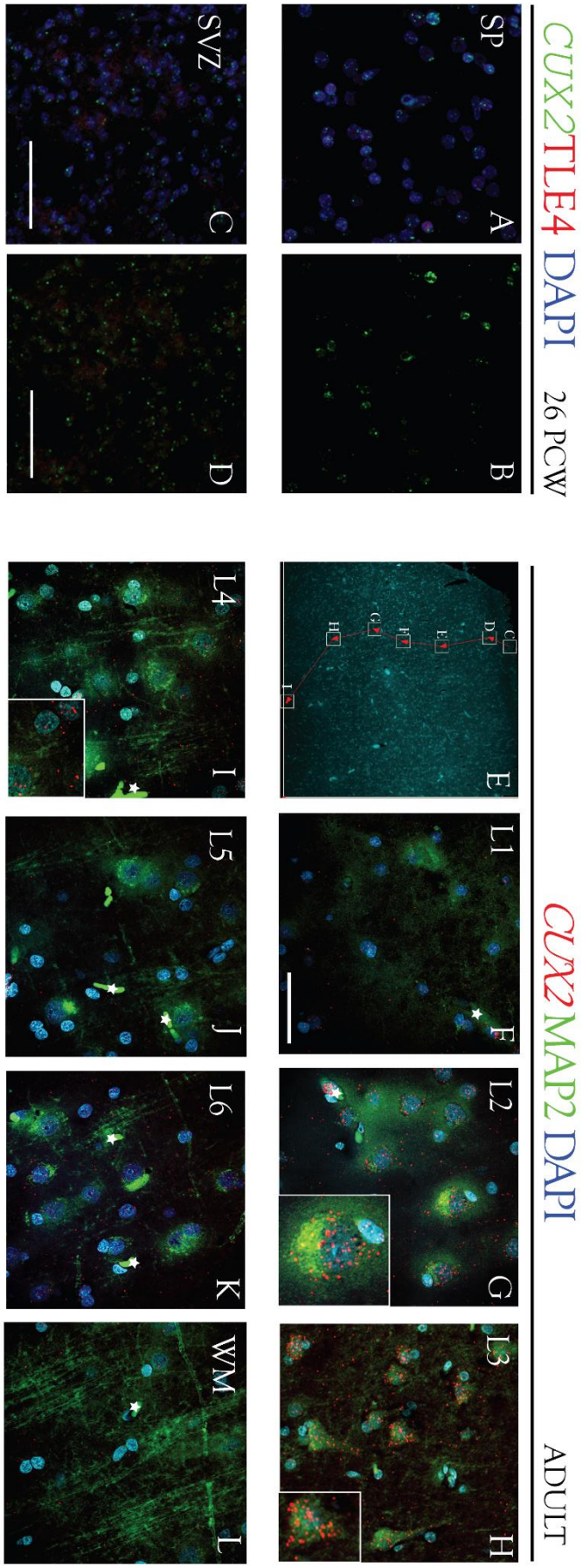


Figure 5.7. *CUX2* mRNA expression was analyzed by fluorescent RNAScope® coupled with the immunostaining for TLE4 (A, C) and MAP2 (F-L) to visualize the subcellular CUX2 localization in the midfetal and adult neocortex. *CUX2* was detected as green dots (A-D) in the fetal stage, and as red dots (G-I) in the adulthood. Confocal images were taken at 40x in the SP (A, B), SVZ (C, D) in frontal cortex at 26 PCW, as well as in each cortical layer of adult PFC (L1-L6) and WM (F-L), shown on the overview image of Brodmann area 9 (E). White stars depict blood vessels oversaturated with signal. Scale bar = 50 µm.

Utilizing fluorescent RNAScope® methodology confirmed the previous reports of *CUX2* mRNA expression in the adult PFC (80), indicating that CUX2 was exclusively present in the mature layers 2 and 3. Furthermore, during the midfetal period, when the SP reached its peak, we found *CUX2* mRNA expression in the SP, that did not co-localize with TLE4, a marker of deep layer projection pyramidal neurons (Figure 5.7A,B). Importantly, a scattered *CUX2* mRNA signal was found in the SVZ, but not as strong as in CP or SP (CP: not shown here; SP: Figure 5.7C, D). Interestingly, *CUX2* mRNA single particles (*punctae, lat*.) were visualized in the soma and neuronal processes of positive neurons, besides the nucleus (Figure 5.7G, H). *CUX2* mRNA expression was present in layers 2–4 of the adult neocortex, but the strongest signal was seen in layer 3 glutamatergic (pyramidal) neurons, co-labeled with MAP2 (Figure 5.7E-L). Furthermore, *CUX2* mRNA was not visible in gyral WM, while detected non-specific immunoreactive aggregates belong to blood vessels (Figure 5.7L) (81).

5.5. CELF4 spatio-temporal expression pattern

One of the aims of the second part of this thesis was to analyze CELF4 spatio-temporal expression specifically in the transient fetal zones, MZ, and SP throughout fetal cortical development. To analyze the MZ cellular populations, CELF4 was co-localized with Reelin, a marker of a specific population of CR cells in the MZ and SGL of the neocortex (10) (Figure 5.8 and 5.9). CELF4 protein was early on expressed in the deep CP and pSP of basal cortex (Figure 5.8A), as well as in the pSP of developing dorsolateral cortex (Figure 5.8B). At the same time (8 PCW) a weak co-localization with Reelin was present in the MZ. Reelin was expressed and co-localized with CELF4 neurons in the MZ and SGL across all of the developmental stages (Figure 5.9). Interestingly, a rare but staggering Reelin positive cells were found in layer 1 (ex. MZ) of a newborn frontal cortex. Distinct CR cell morphology was recognized in each stage of the fetal neocortical development. During midfetal development when some of the SP markers are extensively expressed, CELF4 positive neurons were found in the SP as well. Immunostaining for NURR1, a known nuclear marker of the SP cells revealed partial co-localization with CELF4 neurons in the SP (arrows, Figure 5.10), whereas a large portion of the SP cells were only NURR1 positive (asterisk, Figure 5.10). Thoroughly shown in the following figures is CELF4 widespread presence in the SP zone throughout all of the stages of SP development. CELF4 expression pattern throughout the cortical wall will be described additionally in more detail in the following sections.

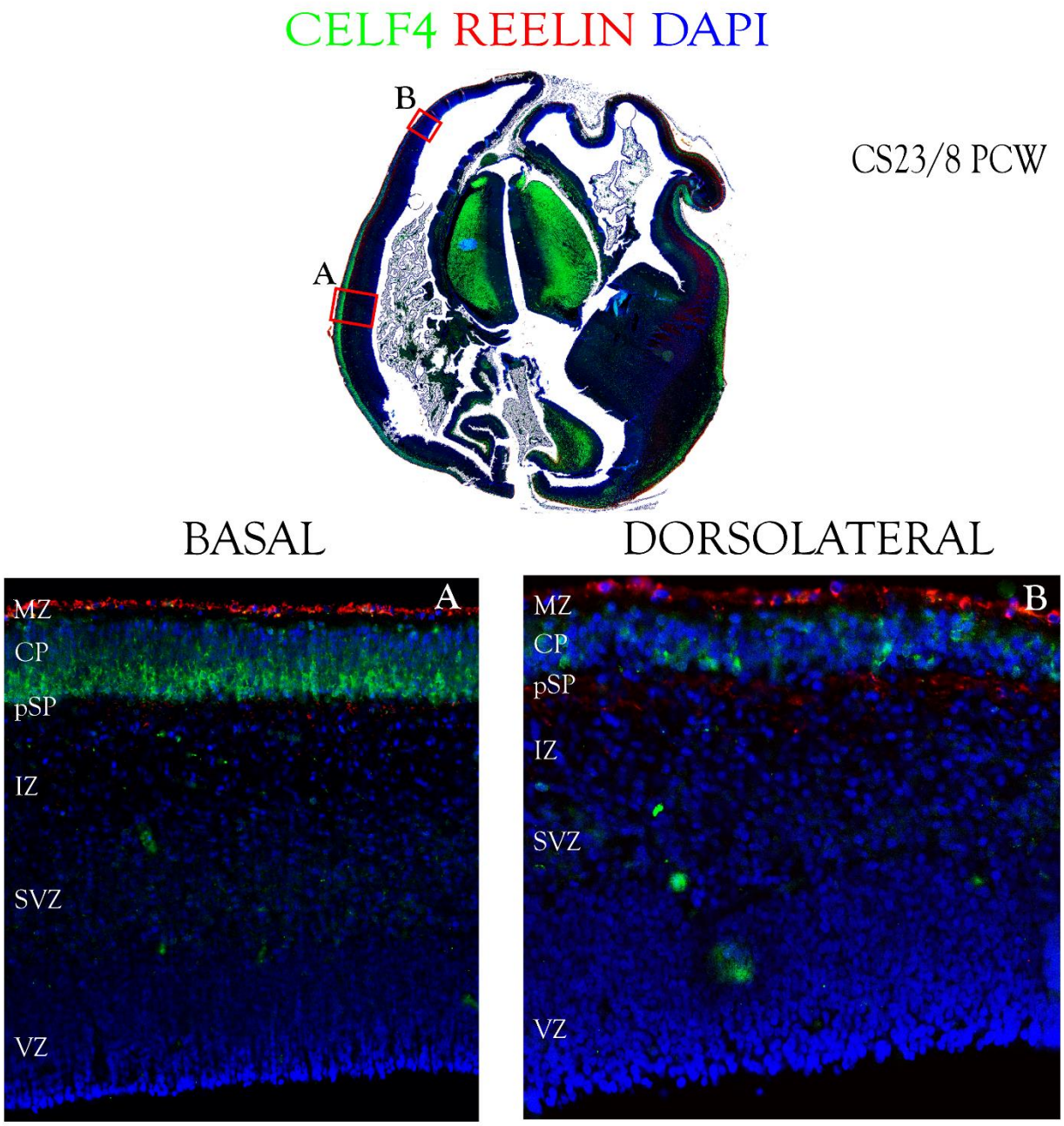


Figure 5.8. Coronal section of anterior embryonic telencephalon at 8 PCW (CS23) stained for CELF4 and Reelin. The image was taken on a high-resolution scanner to capture the whole section. Overview image shows the whole section immunostaining with the red boxes indicating the positions where the images were taken, in basal (A) and dorsolateral neocortex (B), respectively. DAPI stain is used to label the nuclei.

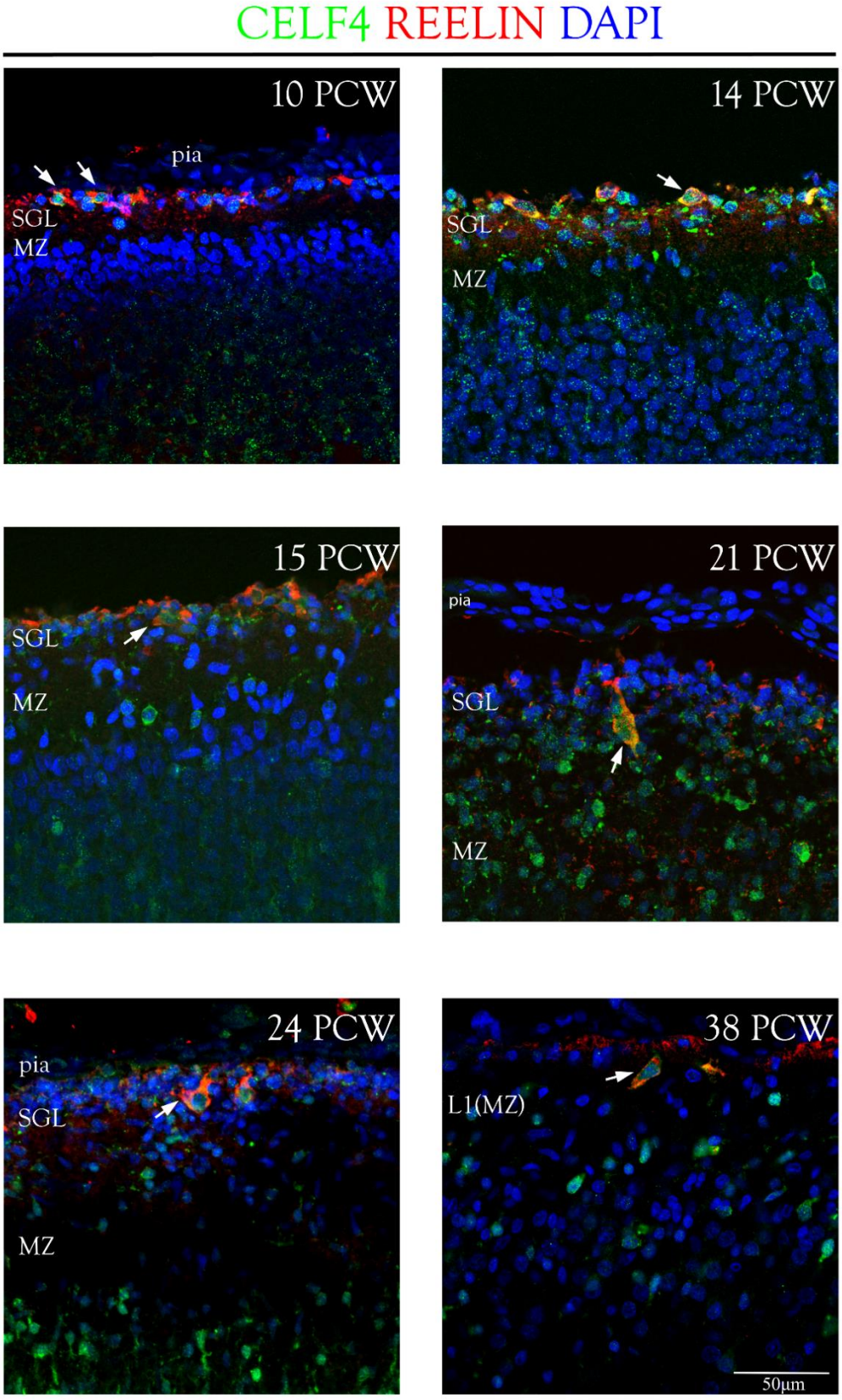


Figure 5.9. CR cells throughout human fetal development in the neocortex from 10 PCW to 38 PCW were immunostained for CELF4 and Reelin. Arrows point out double-labeled CELF4 and Reelin CR cells. Confocal images on 60x objective. DAPI stain labels the nuclei. Scale bar = 50 µm.

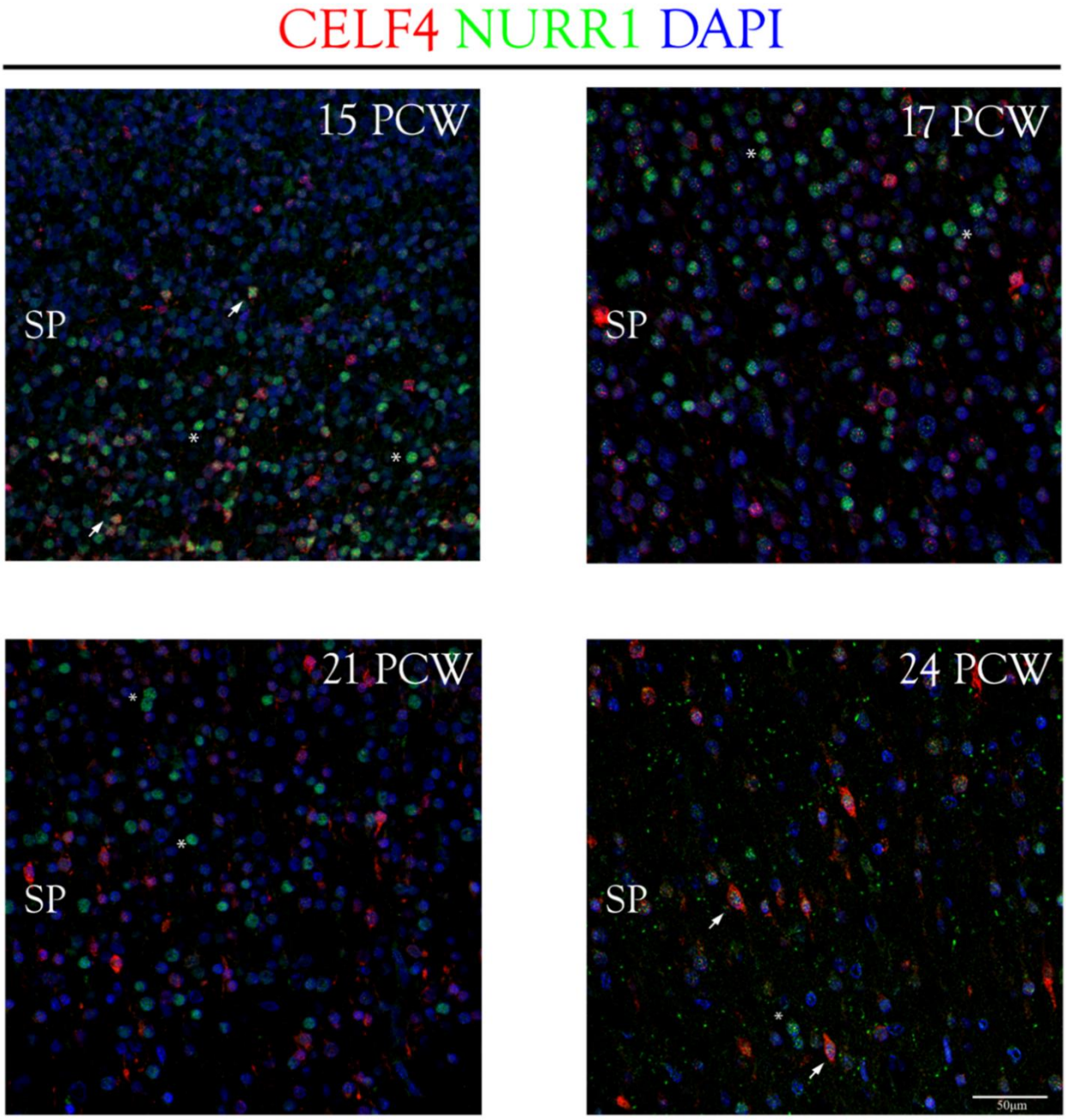


Figure 5.10. SP cells during the midfetal development positive for CELF4 co-immunostained with NURR1. Arrows point out double-labeled CELF4 and NURR1 cells. Asterisk mark NURR1 only positive cells. Images were taken on confocal microscope and high-resolution scanner by using the 40x objective. DAPI stain is used to label the nuclei. Scale bar = 50 µm.

5.6. Neurotransmitter and calcium-signaling molecular profile of CELF4+ MZ and SP cells

This section shows analysis of molecular profile of the CELF4+ (positive) MZ and SP cells with characteristic expression of neurotransmitters, GAD67, NPY, and VGLUT1, along with the calcium-signaling molecule Calretinin (CALR) (147) in the transient fetal zones, MZ and SP. In the earliest stages of fetal development of the cortex, featuring PP and pSP, at 8 and 12 PCW, CELF4 positive neurons co-localized with GAD67 and CALR (Figure 5.11). During the SP formation CELF4 positive neurons co-localized with GAD67 in the SP, besides the CELF4 and CALR co-localization in the MZ and SP neocortex at 13/14 PCW (Figure 5.12).

NPY expression was found during the midgestation in the SP zone, where it co-localized with CELF4 positive neurons. Moreover, vesicular glutamate transporter 1 (VGLUT1) a typical marker of glutamatergic neurons co-localized with CELF4 neurons at 21 PCW (171). CELF4 neurons in the SP overlap with GAD67 expression, although they do not significantly overlap with CALR positive SP neurons at the time. GAD67 and CALR positive neurons overlap with CELF4 neurons in the MZ, whereas GAD67 does not have the same expression pattern as in SP (Figure 5.13).

During the stationary SP stage, at 24 PCW, CELF4 positive neurons co-localized with the present NPY positive cells in the SP. Moreover, at the same time, CELF4 immunoreactivity was noted at the top of the MZ, on the border with the CP where it overlapped with CALR expression. Similarly, GAD67 positive neurons overlap with CELF4 neurons in the MZ, besides a typically limited fraction of GAD67 co-positive CELF4 neurons in the SP. CALR positive neurons did not significantly overlap with the expression of CELF4 in the SP (Figure 5.14).

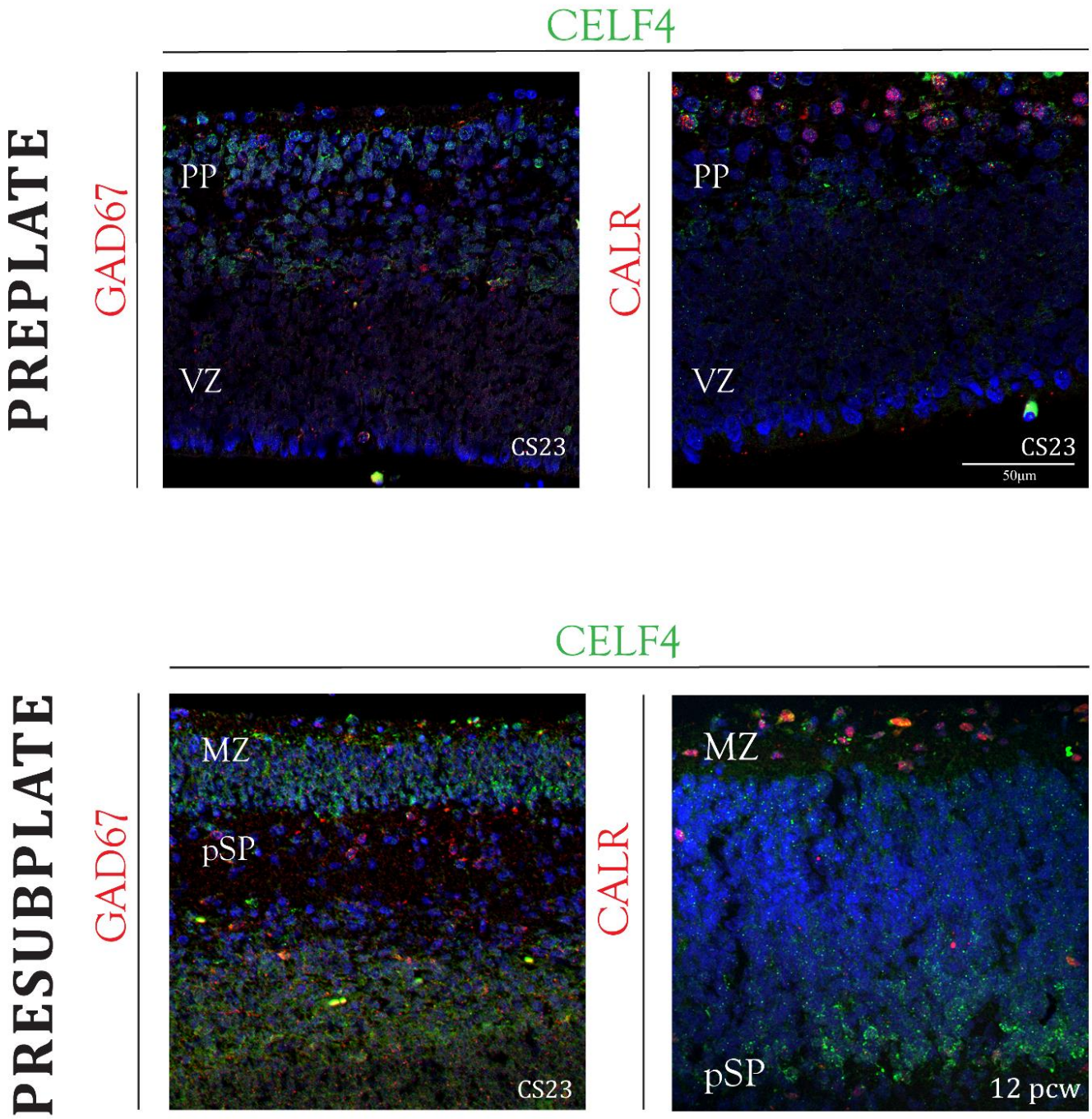


Figure 5.11. Neurotransmitter and calcium-signalling molecular profile of the early developmental stages (preplate and presubplate) in the MZ (here PP) and SP (here pSP) zones. Sections are immunostained for CELF4 and GAD67, Calretinin (CALR). Specimen age is marked in the right bottom corner of each image. DAPI stain is used to label the nuclei. Scale bar = 50 µm.

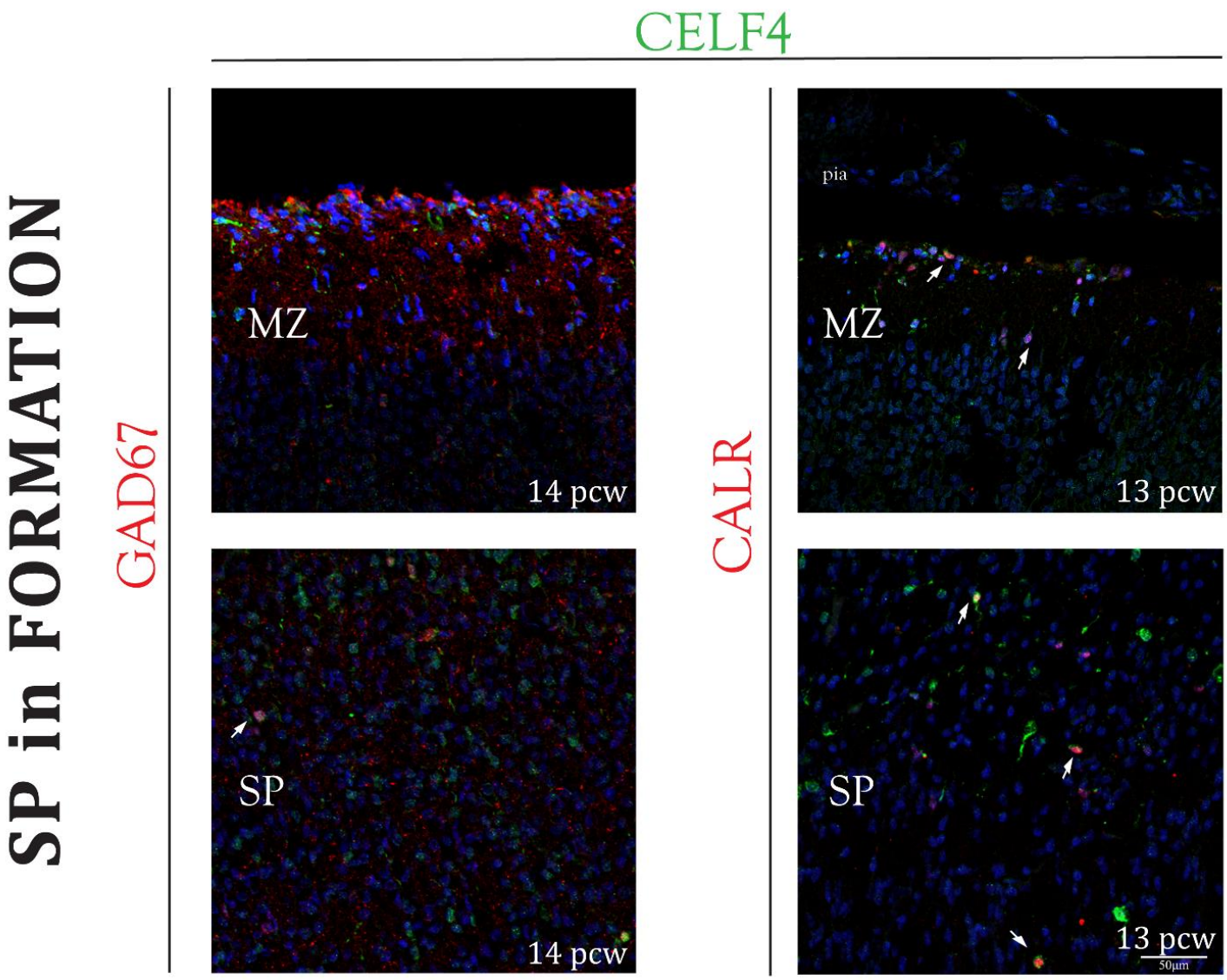


Figure 5.12. Neurotransmitter and calcium-signalling molecular profile of the MZ and SP zone in the formation stage of SP. Sections are immunostained for CELF4 and GAD67, CALR. Arrows point out co-localized CELF4 and GAD67, CALR cells, respectively. Specimen age is marked in the right bottom corner of each image. DAPI stain is used to label the nuclei. Scale bar = 50 µm.

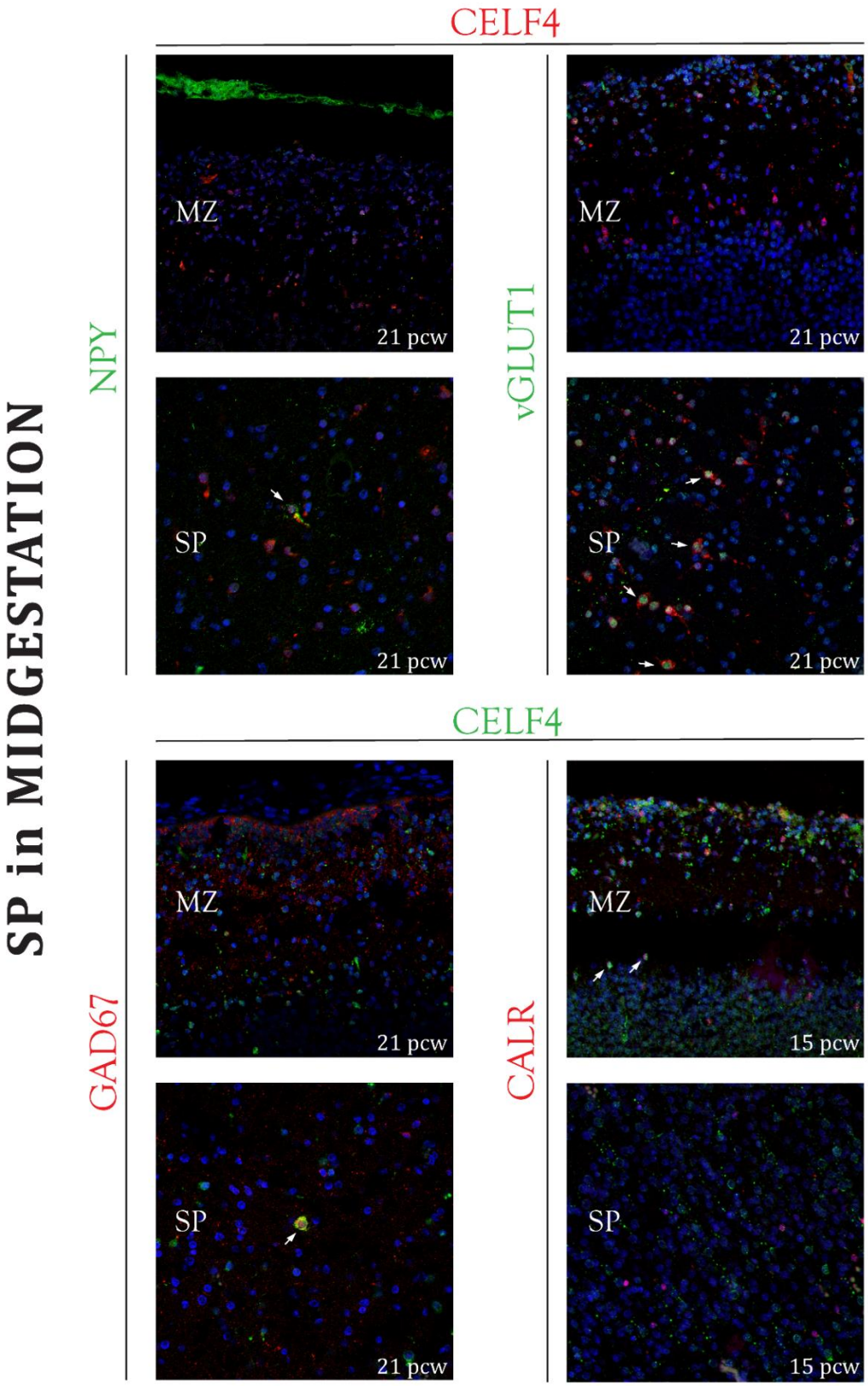


Figure 5.13. Neurotransmitter and calcium-signalling molecular profile of the MZ and SP zone during the midgestation of human fetal development. Sections are stained for CELF4 and neurotransmitters: NPY (green), vGLUT1 (green), GAD67 (red) and CALR (red). Arrows mark part of the co-localized cells. Specimen age is specified in the right bottom corner of each image. DAPI stain is used to label the nuclei. Scale bar = 50 µm.

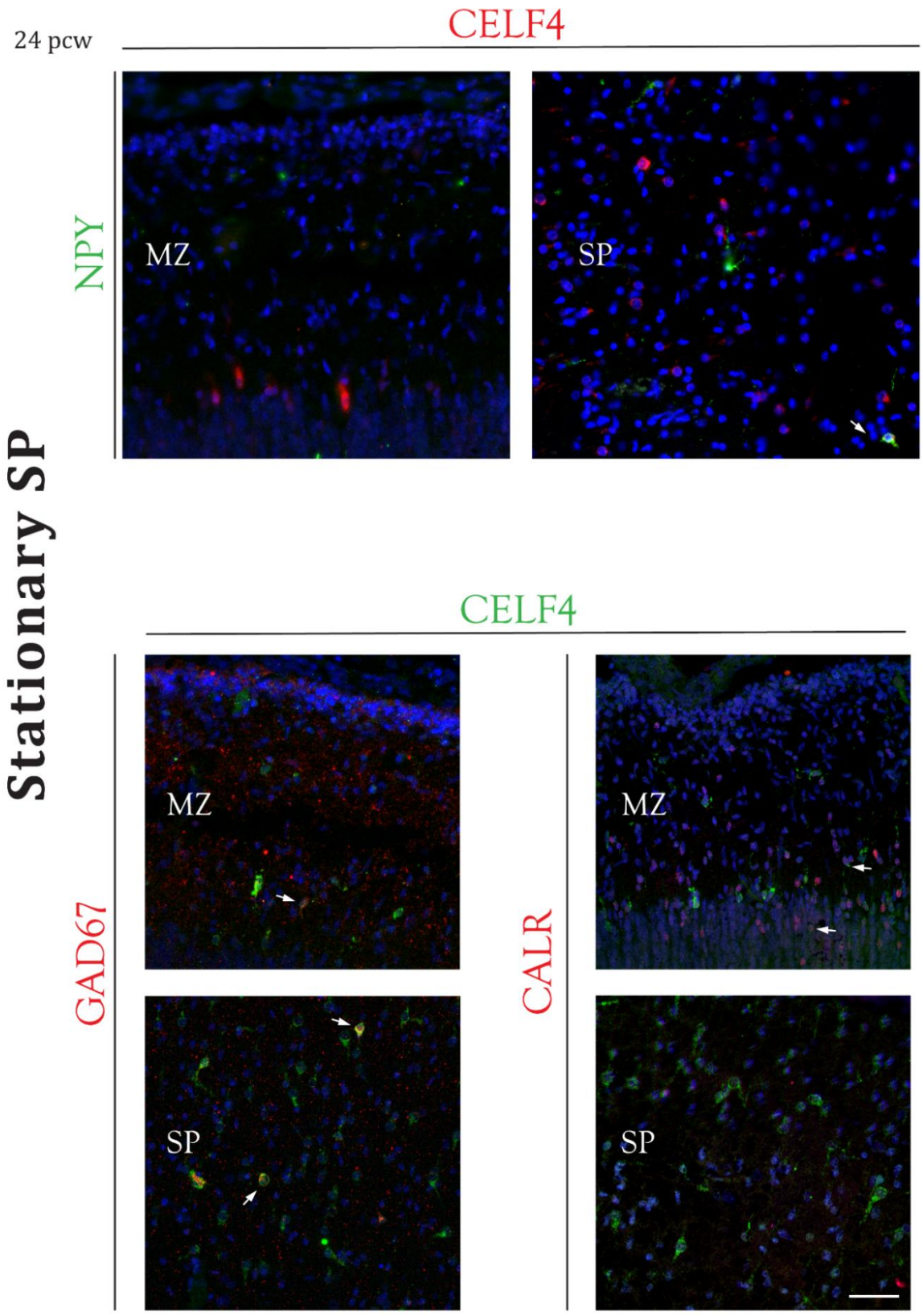
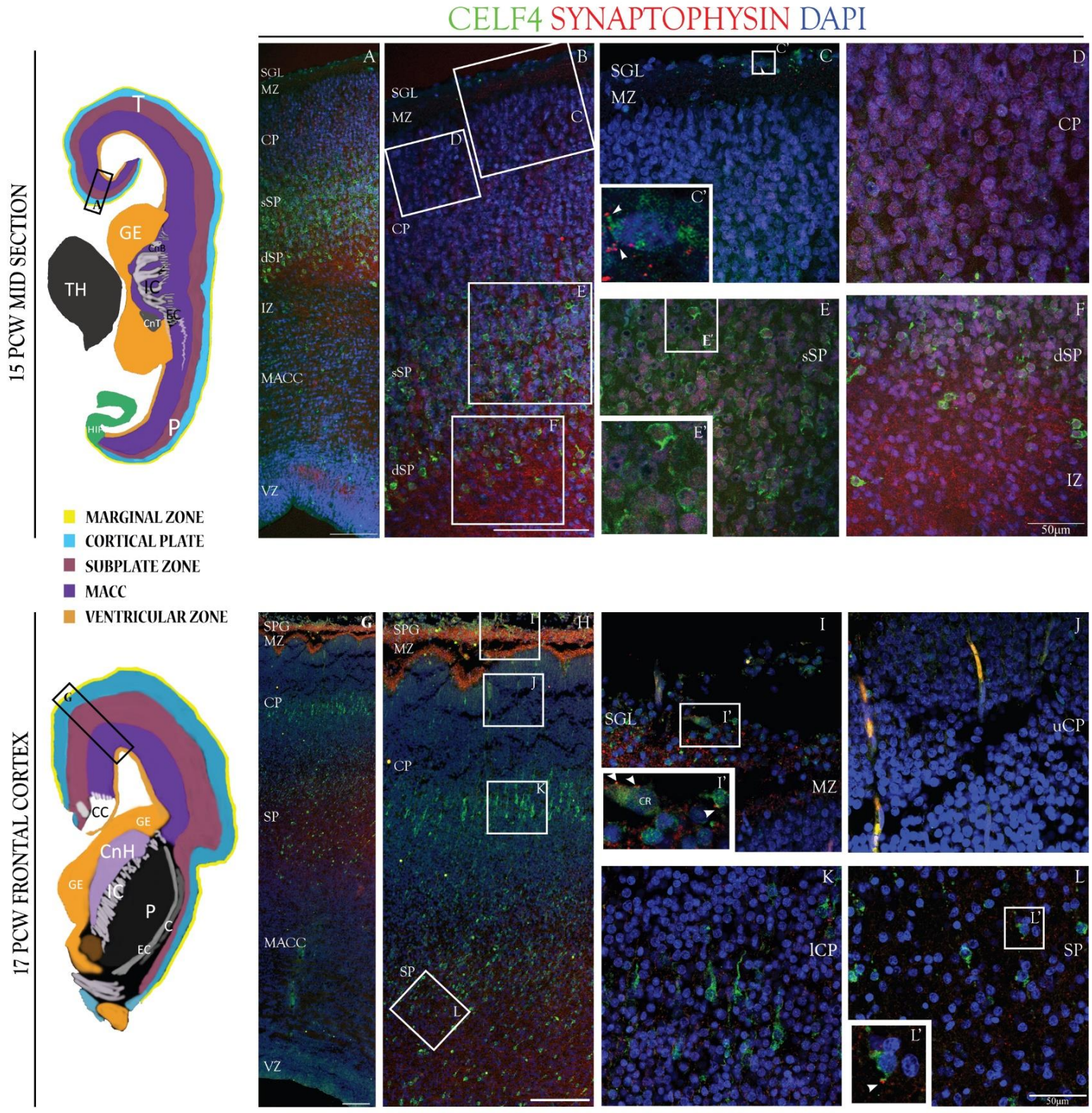
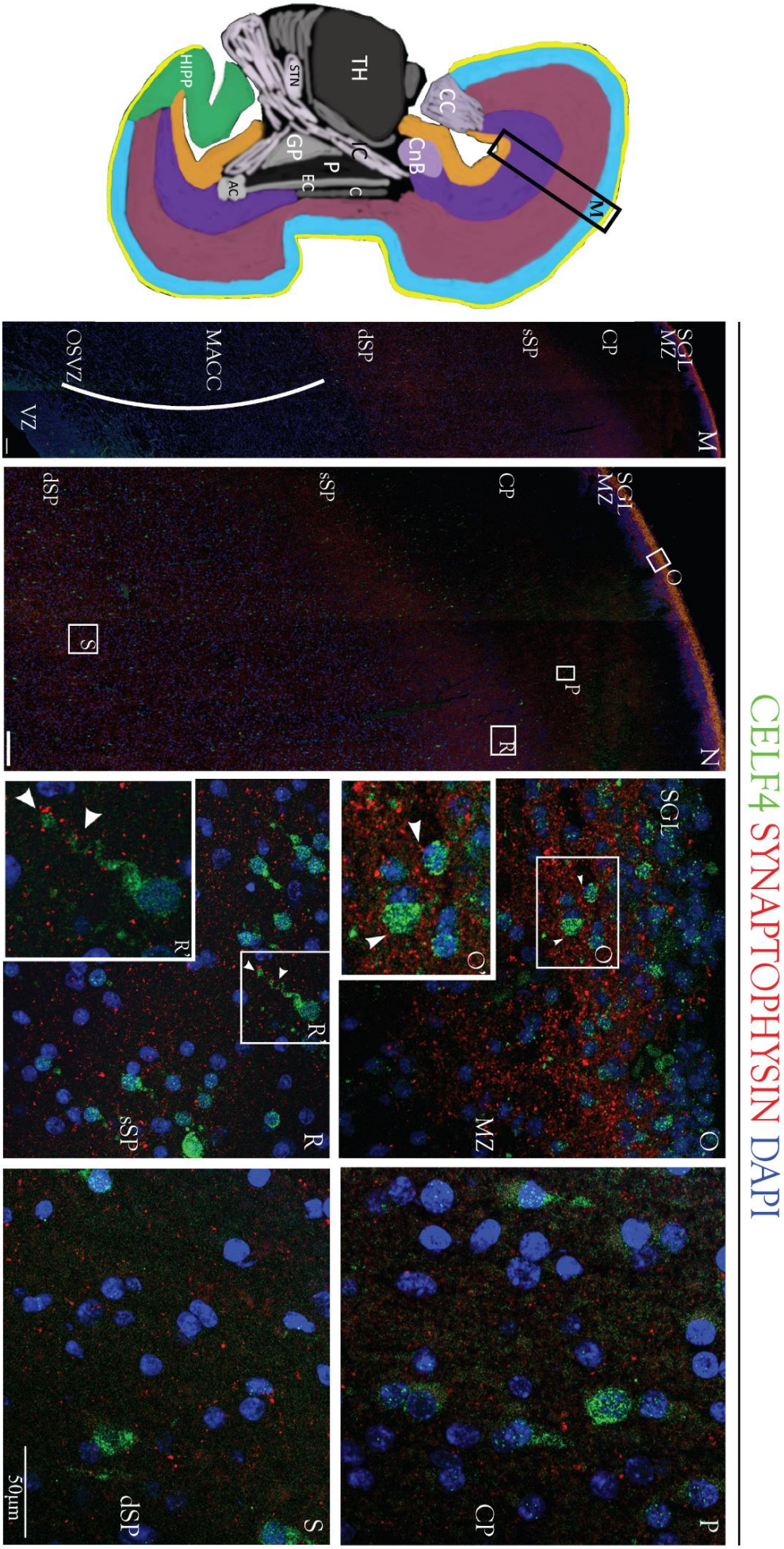


Figure 5.14. Neurotransmitter and calcium-signalling molecular profile of the MZ and SP zone during the stationary phase of SP development. Dorsal frontal cortex sections at 24 PCW are immunostained for CELF4 and neurotransmitters: NPY (green), GAD67 (red) and CALR (red). Confocal images were taken on 40x objective in the MZ and SP, respectively. Arrows mark a part of the co-localized cells. DAPI stain is used to label the nuclei. Scale bar = 50 µm.

5.7. CELF4 during synaptogenesis





CELF4 SYNAPTOPHYSIN DAPI

Figure 5.15. Graphic schematics based on Nissl staining of the near section with anatomical annotations on the far left of the figures. Boxes mark the positions of strips where the confocal images are taken in the 15 PCW mid telencephalon (A), 17 PCW dorsal frontal cortex (G), and 21 PCW frontal cortex (M) coronal sections. The cerebral wall strip images are taken on the high-resolution scanner. Confocal images, at 60x objective, showing double-labeling for CELF4 and synaptophysin throughout the cerebral wall with magnifications on synaptic strata- MZ and SP (sSP-superficial SP; dSP- deep SP), as well as CP (lCP- low CP; uCP- upper CP). Synaptophysin punctae are visualized as red dots (C') and are pointed out in the magnified boxes with arrowheads. Scale bars A, B, G, H, M, N=200 µm; C-F, I-L, and O-S=50 µm. DAPI stain is used to label the nuclei.

Immunostaining for CELF4 and a presynaptic marker, synaptophysin, throughout the cerebral wall at 15 PCW revealed the presence of CELF4 neurons within synaptic strata, *i.e.* MZ and SP (Figure 5.15C and E, F). Synaptophysin was not expressed in the CP (Figure 5.15D), where rare, scattered CELF4 positive neurons were found. Synaptophysin punctae found on the CELF4 positive neurons were smaller dimension and more dispersed, presuming they were still not fully mature (Figure 5.15C'). Moreover, in the superficial SP (or second, loosening CP) where strong positive CELF4 neurons were found, synaptophysin punctae were not visualized (Figure 5.15E). In the deep portion of the SP possibly migrating CELF4 neurons were present at 15 PCW. Moreover, synaptophysin immunoreactivity in the IZ was found in the locally present fibers (Figure 5.15F) with scattered CELF4 positive cells. Highly occasional CELF4 positive neurons were found in the SVZ and VZ of 15 PCW neocortex.

In the dorsal part of the frontal cortex at 17 PCW, a significant CELF4 expression throughout the SP, CP, and MZ was found. Same as the previous results, CELF4 and synaptophysin expression overlap exclusively in the MZ and SP (Figure 5.15I, L). On the contrary, no visible expression of synaptophysin was present in the upper and lower CP (Figure 15J, K). The middle part of CP contains specific neurons, that look differentiated with apical processes oriented towards pia, have a large pyramidal shape, and probably belong to migratory neurons. Synaptophysin punctae were distinguished on the CELF4 positive Cajal-Retzius and other types of cells that are part of the SGL and the deep MZ (pointed by arrowheads, Figure 15I'). A substantial group of strongly positive CELF4 neurons was found in the SP with the synaptophysin punctae visualized

on the dendrites of the positive neurons (Figure 5.15H, L') Furthermore, CELF4 positive neurons were found in the IZ and mostly superficial MACC (OSVZ), while there were no positive neurons in the ISVZ and VZ (Figure 5.15G).

Furthermore, in the dorsal frontal cortex at 21 PCW dense synaptophysin staining visible as red background on lower magnification was present in the MZ and sSP (Figure 5.15M, N). Synaptophysin punctae were visualized on the higher magnification as red dots, showing co-localization on CELF4 positive cell soma and inverted pyramid cell process (arrowheads, Figure 5.15O', R'). CELF4 positive neurons were visible in the SGL and lower band of MZ, on the border with CP (Figure 5.15O). Neurons of CP had a divergent expression of CELF4, such that the upper half of CP is negative, the midline is positive, and the lower half is positive with not a lot of pyramidal shape neurons. Moreover, a dense CELF4 expression was found within superficial SP with various neuronal types: polymorphic, migratory neurons with the apical process, and fusiform neurons. Likewise, lower SP contained CELF4 positive scattered cells (Figure 5.15R, S). Finally, CELF4 reactivity in the compartments inferior to SP (IZ, OSVZ, VZ) is only occasional, overall not present (Figure 5.15M).

5.8. CELF4 subcellular localization

To report on subcellular protein and RNA CELF4 localization, electron microscopy (EM) and RNAScope® methodology were utilized in this study. Ultra-thinly sliced SP dissections during midfetal development (17 and 19 PCW) were immunostained for CELF4 and prepared accordingly for EM. Unfortunately, the poor tissue fixation resulted in a mostly disrupted intra-cellular and extra-cellular environment which prevented a detailed analysis. Nevertheless, the EM has shown CELF4 protein punctae and the possible synaptic contacts (electron micrograph, Figure 5.16). CELF4 protein punctae were localized in the nucleus, as well as in the extracellular matrix (ECM). Moreover, CELF4 subcellular localization was investigated by RNAScope®, and in addition, analyzing nNOS and SST neurotransmitter expression during midgestation. *CELF4* mRNA (green punctae, Figure 5.17) were mainly visualized in the soma, both nucleus, and cytoplasm, and partially in the dendrites as well. Its co-localization with nNOS was found in SP in comparison with positive control immunostaining of striatum (Figure 5.17A, D), while only in SP at 21 PCW SST positive cell had visible CELF4 punctae (Figure 5.17C).

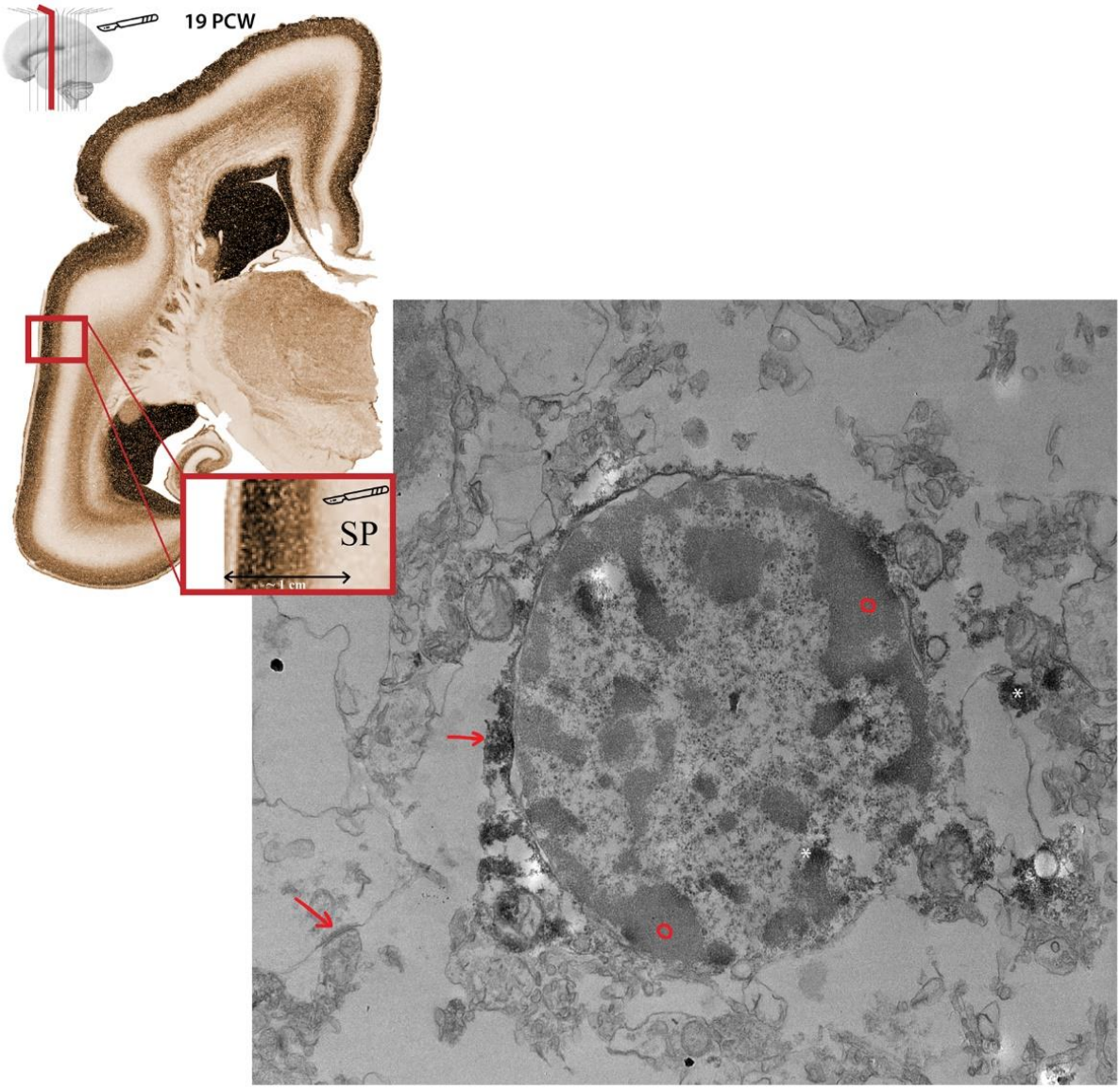


Figure 5.16. EM micrograph (bottom image)– showing a nuclear area of a small cell with the immunoreactive product (white asterisk). A typical SP neuropil with a large ECM space and possible synaptic contacts (arrow). Cell nucleus with the dispersed chromatin in the middle, and clumps of chromatin on the periphery (red circle). Top left corner images (Taken from Bayer&Altman (160)) depict the cutting spot in the 19 PCW brain (red line, knife) and the spot where SP is dissected out of the temporal cortex (SP, knife). Direct magnification of the electron micrograph is 5000x.

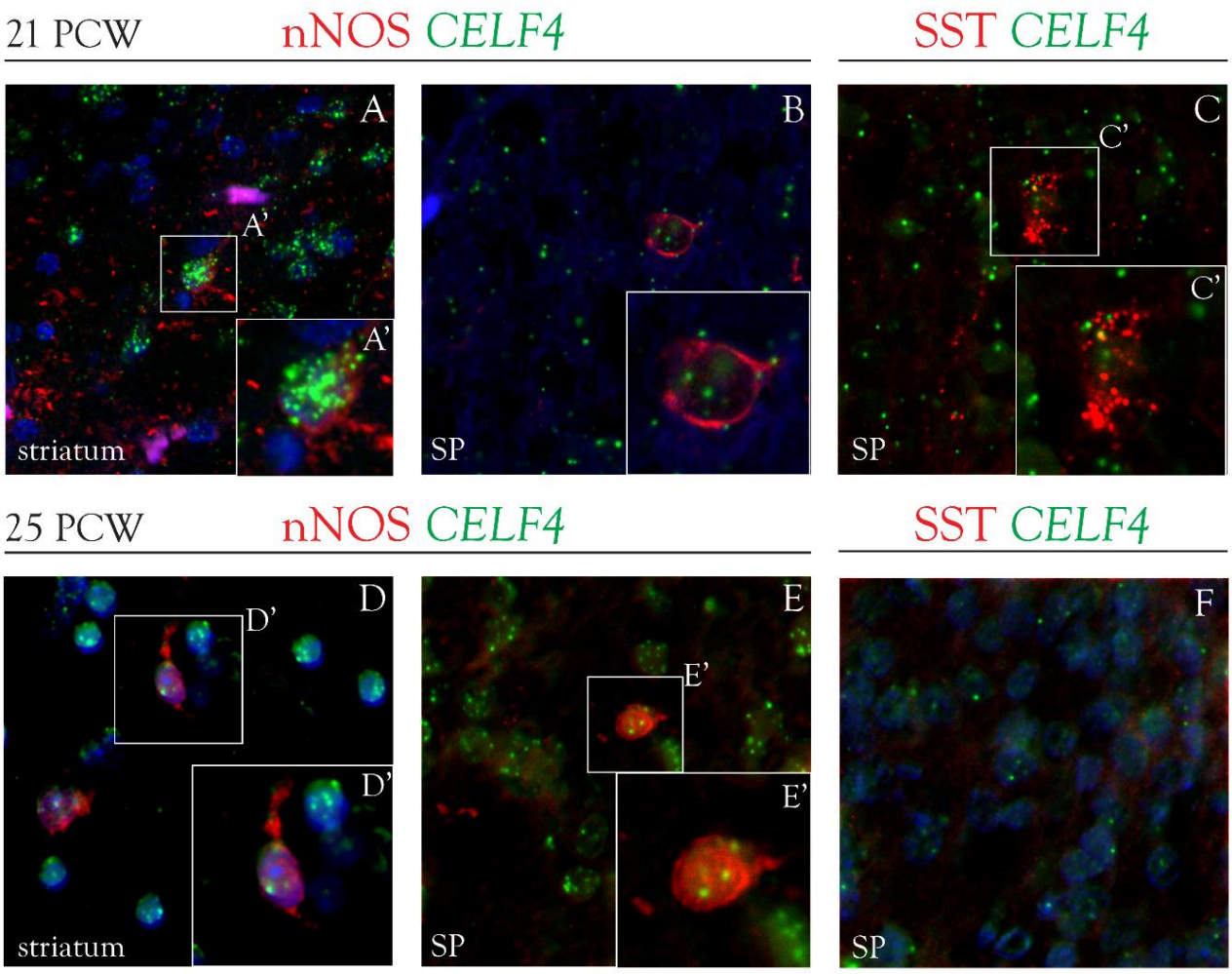


Figure 5.17. *CELF4* mRNA expression was analyzed by fluorescent RNAScope® coupled with the immunostaining for nNOS (A, B, D, E) and SST (C, F) to visualize the subcellular *CELF4* localization in the midfetal frontal cortex. *CELF4* was detected as green dots (punctae) (A-F). Confocal images were taken at 40x and 60x in the SP of frontal cortex (B, C, E, F), and in the striatum as a positive control (A, D) at 21 and 25 PCW.

5.9. mRNA binding targets of CELF4 protein

One of the goals was to determine mRNA binding targets of CELF4 protein throughout fetal neocortical development. Co-immunoprecipitated mRNAs were sequenced and analyzed to reveal the targets. Followed by RT-qPCR to confirm the targets. The samples were classified by age into early fetal (**1**), early midfetal (**2**), and late midfetal (**3**) groups. The gene list of CELF4 binding targets was further analyzed by the gene ontology (GO) functional annotation (Figure 5.18). Gene ontology results encompassed the top 10 biological processes in which CELF4 binding targets are involved. Specifically emphasized were transcription, regulation of transcription, neural development, axonal fasciculation and guidance, and importantly synapse assembly. Moreover, some of the binding targets were chosen as RT-qPCR candidates because of their previous co-incidence with CELF4 shown by IHC experiments. By comparing the target genes to negative control Nestin and house-keeping gene beta-Actin confirmed that TLE4, SYNPR, and EIF4A2 are CELF4 binding targets in all three of the midfetal period groups, while vGLUT1 only becomes a target in the late midfetal period of neocortical development (Figure 5.19-22). TLE4 and SYNPR become significantly stronger binding targets throughout the development.

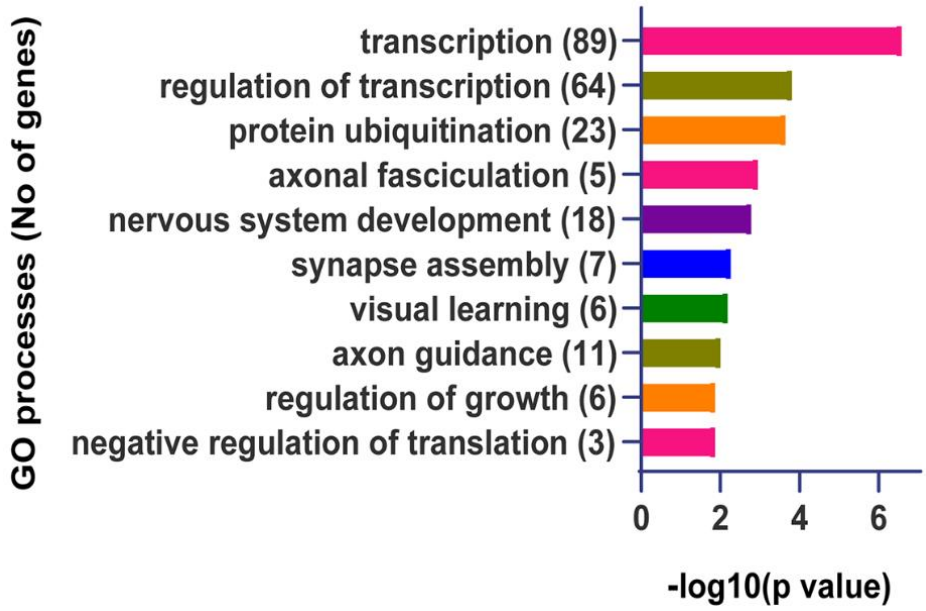


Figure 5.18. Gene ontology (GO) analysis showing the top 10 ranked terms with the number of associated genes (in parenthesis). This chart summarizes the gene list that is acquired after RNA-sequencing of CELF4 protein binding targets and the annotations from DAVID annotation tools (https://david.ncifcrf.gov/tools.jsp).

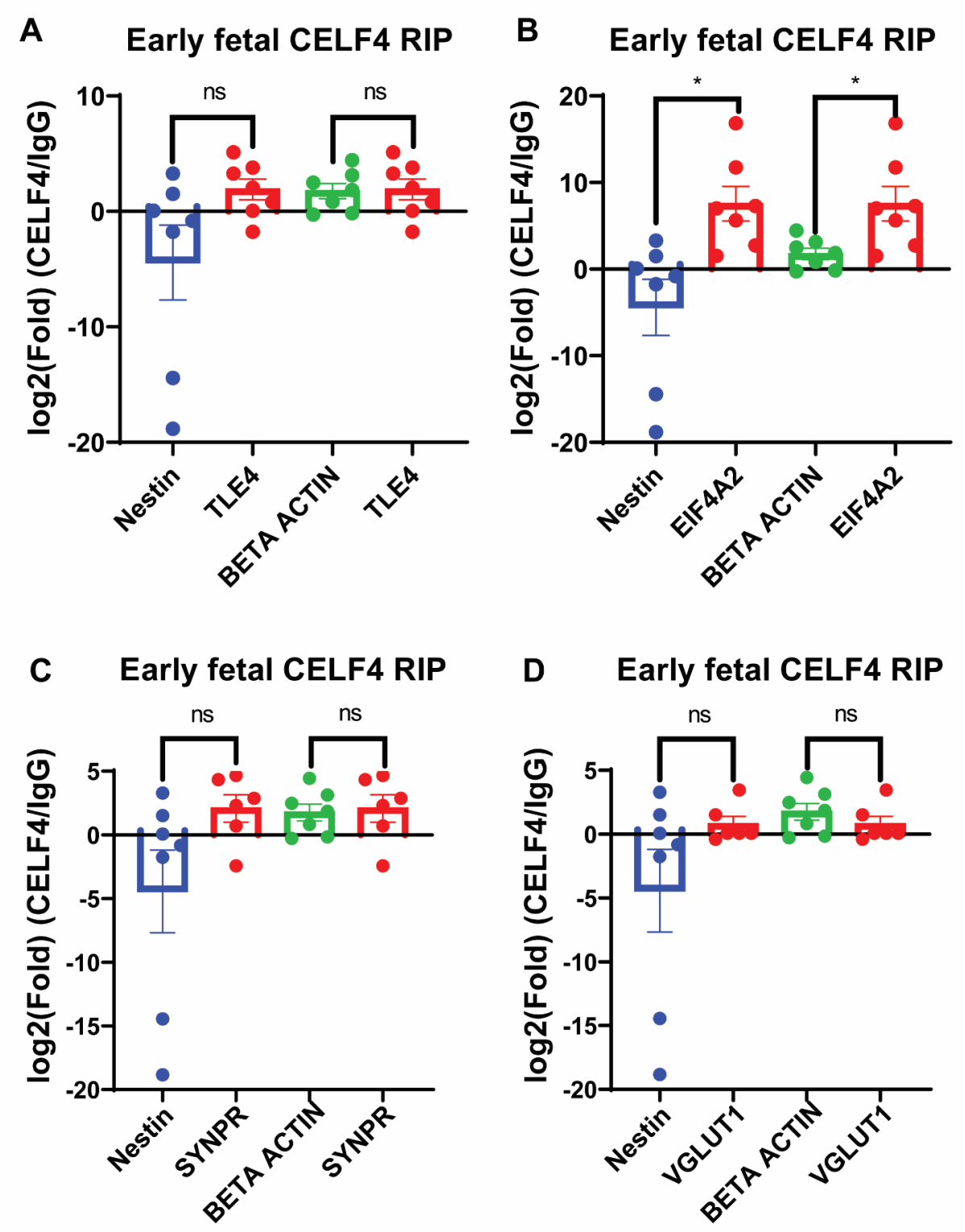


Figure 5.19. Relative mRNA levels determined by RT-qPCR from CELF4 vs. IgG RIPs is presented as a log2 of fold change. Three brain specimens of different age (11, 12 and 12 PCW) were used for individual RIP experiments. Gene expression data (A-D) was first normalized to GAPDH expression, and the RT-qPCR data was pooled and averaged for the analysis. Unpaired t-test with Welch's correction was used for the comparisons. Nestin and Beta-actin were used as negative controls for CELF4 binding. * p>0.05; ns- not-significant.

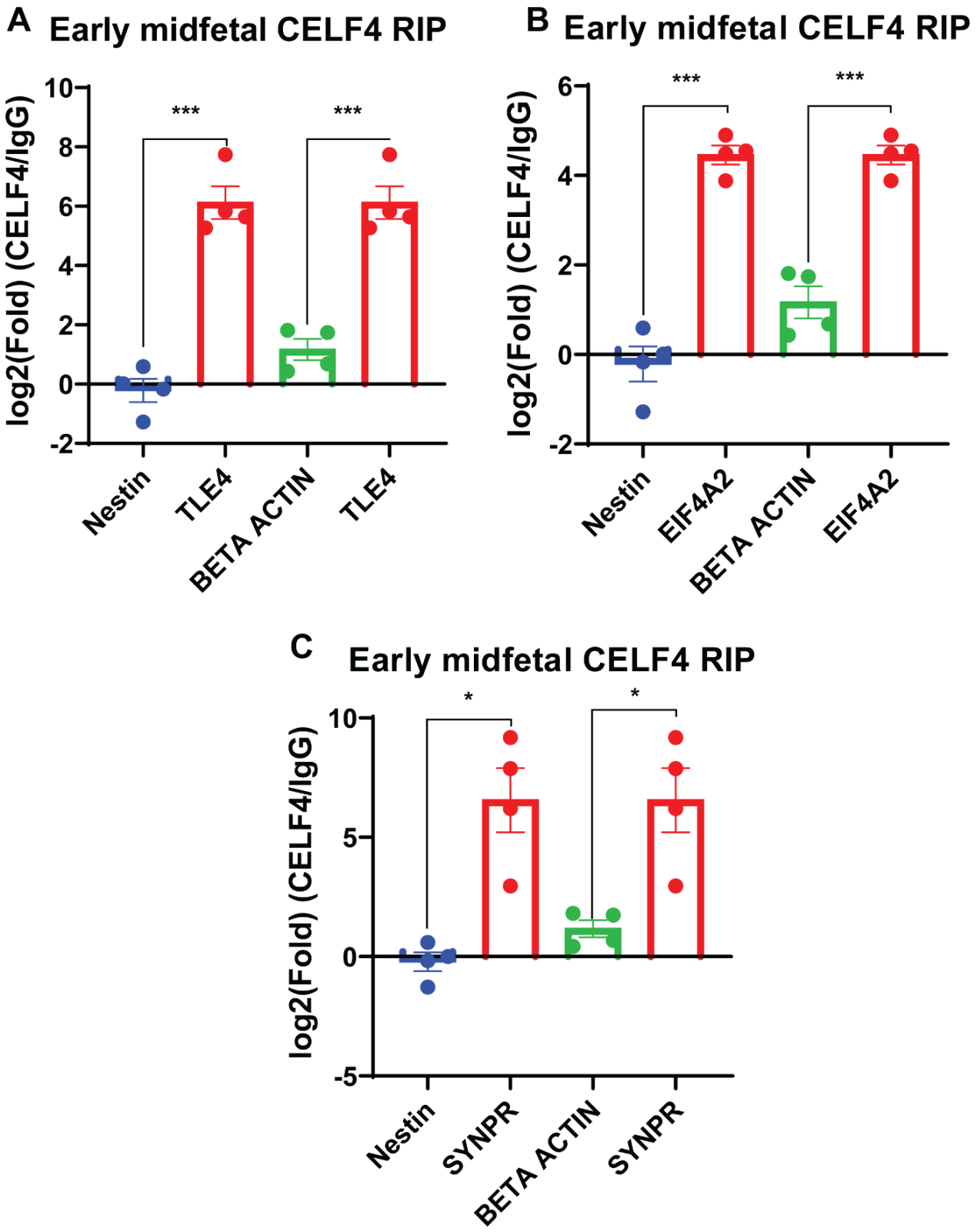


Figure 5.20. Relative mRNA levels determined by RT-qPCR from CELF4 vs. IgG RIPs is presented as a log2 of fold change. Two different brain specimens (14 and 15 PCW) were used for individual RIP experiments. Unpaired t-test with Welch's correction was used for the comparisons. Nestin and Beta-actin were used as negative controls for CELF4 binding. * p>0.05; ns- not-significant.

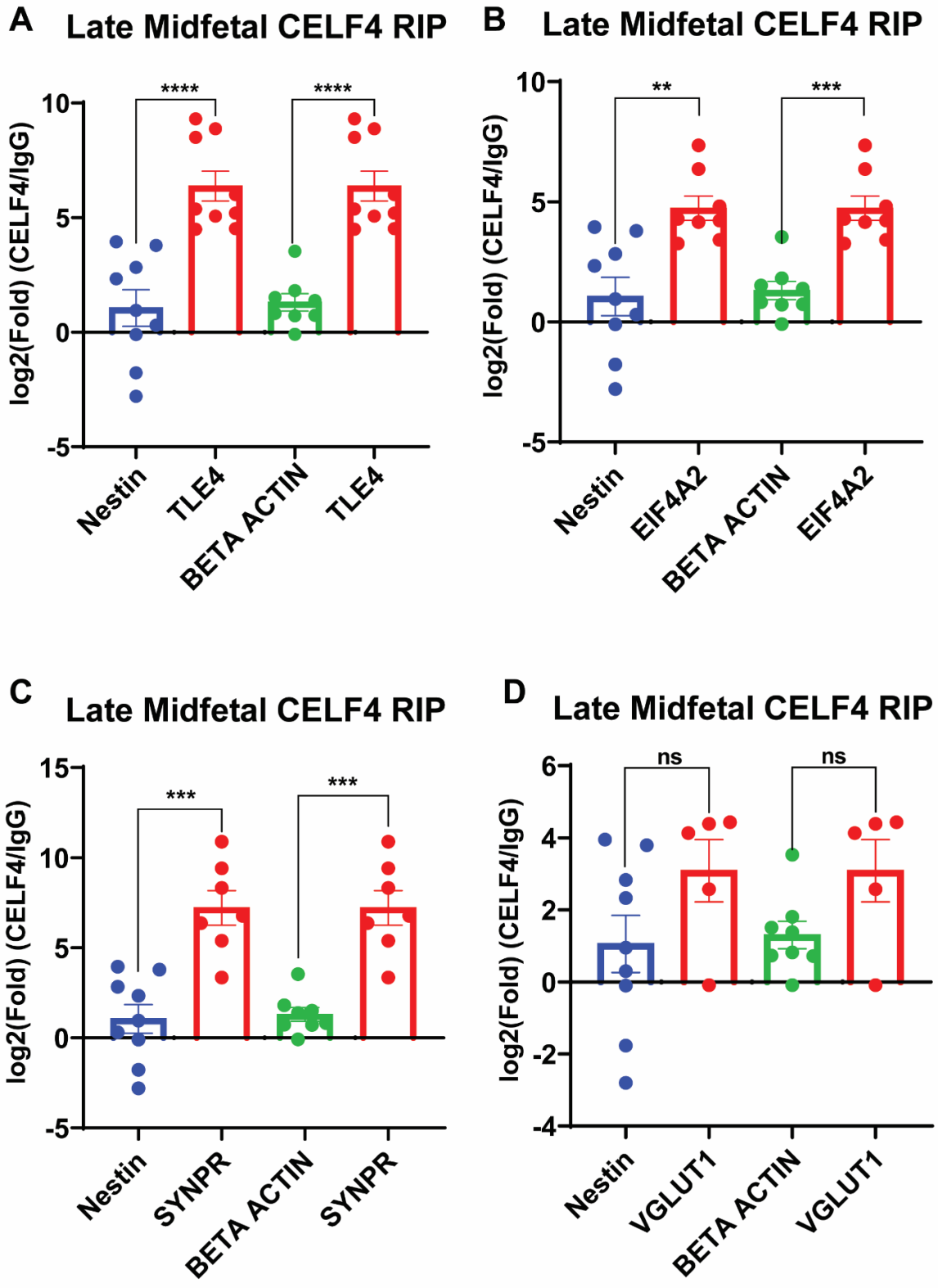


Figure 5.21. Relative mRNA levels determined by RT-qPCR from CELF4 vs. IgG RIPs is presented as a log2 of fold change. Three brain specimens of different age (17, 18 and 20 PCW) were used for individual RIP experiments. Gene expression data (A-D) was first normalized to GAPDH, and the RT-qPCR data was pooled and averaged for the analysis. Unpaired t-test with Welch's correction was used for the comparisons, except for TLE4 where Mann-Whitney test was used.

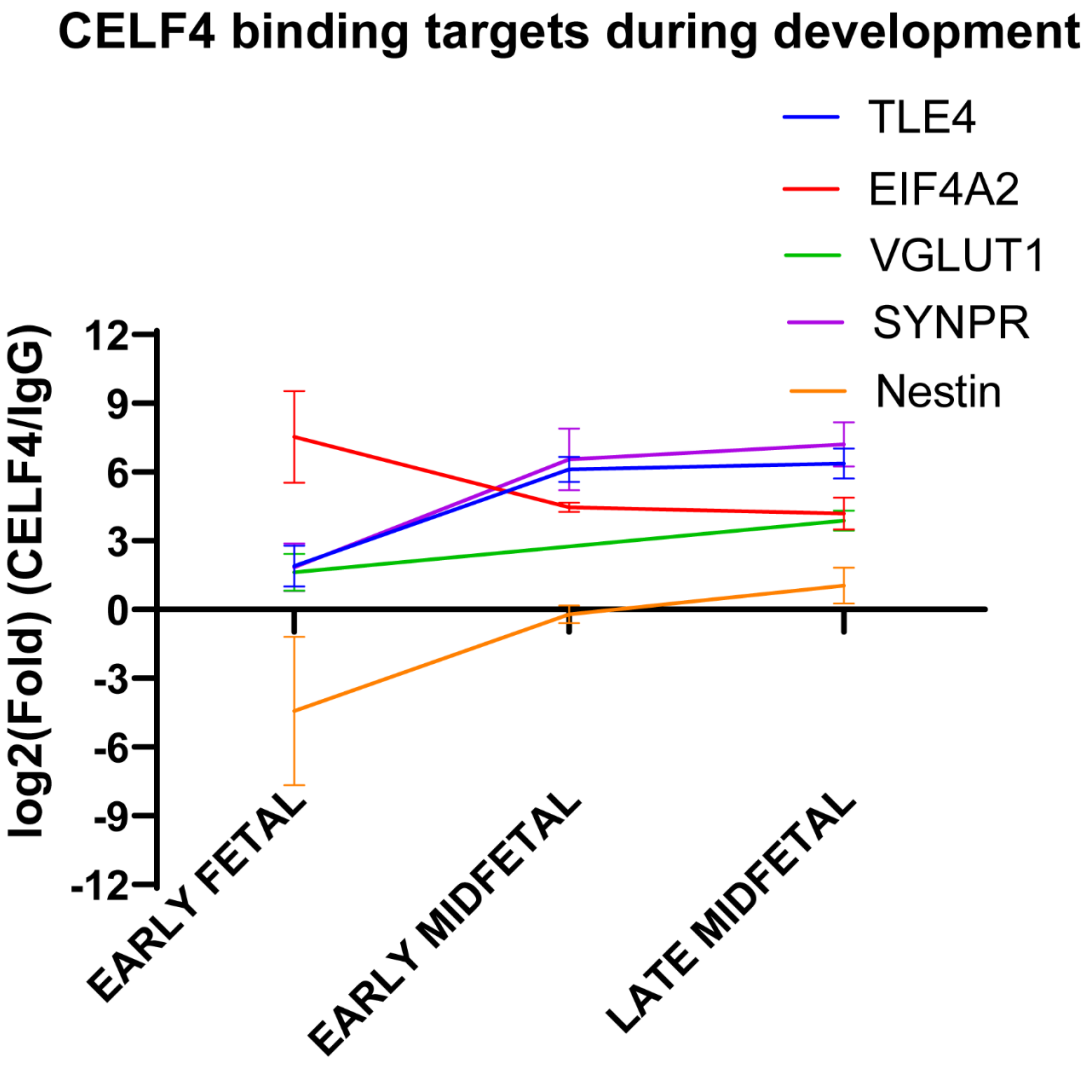


Figure 5.22. Relative mRNA levels of CELF4 binding targets, TLE4, EIF4A2, VGLUT1 and SYNPR determined by RT-qPCR out of CELF4 vs. IgG RIPs and compared between the ages by log2 of fold change. Specimens of different age were classified into early fetal, early midfetal and late midfetal groups. Gene expression data was first normalized to GAPDH expression, and the RT-qPCR data was pooled and averaged for the analysis.

6. Discussion

The results of this thesis demonstrate the differential spatio-temporal expression of CUX2 and CELF4 as one of the constituents of normal cortical development. Regulation of gene expression on the level of transcription and post-transcription is presumed as one of the determinants of morphological and functional cortical complexity (3,35–37). Early fetal and midfetal cortical development are the periods when neurons are in the burst of their proliferation and migration, whereas a timely outbreak of various histogenetic processes has a crucial impact on proper cortical growth and development (4,128). Herein, CUX2 and CELF4 are associated with the processes of migration, and respectively, molecular specification, and synaptogenesis. Importantly, neocortical neuronal identities are not completely resolved until the end of midfetal cortical development and their final identity stays plastic during early postmitotic differentiation (67,120), while synaptogenesis goes through major alterations as development progresses (15).

The unique molecular identity of developing neurons defined by the expression of transcription factors (TFs) dictates neuronal development, their specificity and connectivity, and the ultimate neocortical development. The results of this thesis showed that TF CUX2 is expressed in the migratory projection neurons destined for the upper cortical layers during the process of their migration through transient fetal compartments. Also, previously not reported CUX2 expression in the transient populations of postmigratory neurons was found in the two transient and integral compartments of the developing human fetal cortex, the SP and MZ. The novel findings of CUX2 expression in the transient neuronal populations that do not belong to the upper cortical layers suggest its multifunctional role during prenatal corticogenesis.

RBP’s multiverse roles during neocortical development place them in the spotlight as important influencers of cortical development. Previously was shown that specific RBPs have a role involved in certain developmental processes, such as progenitor proliferation (56), differentiation (57), neuronal migration (58), and axonal pathfinding (59). Research of CELF4 localization and its possible role in the human fetal neocortex as part of this thesis showed its dynamic laminar protein expression throughout all of the developmental stages. Moreover, CELF4 was previously associated with the process of synaptogenesis (102,103), which was additionally explored and confirmed by this study. CEFL4 maintains its expression even in adulthood which suggests it retains its function in the mature neocortex.

Together, transcription and post-transcriptional mechanisms accord most of the histogenetic events during neocortical development which initially manifests in the transient fetal zones MZ and SP, and later in the CP. The results of this thesis show a correlation between the expression of TF, CUX2, and RBP, CELF4 with the developmental processes occurring within the same spatio-temporal frame, while depicting their role in the cortical differentiation. However, the hypothesis that TFs have a bigger influence on the cell type specificity than the RBPs, is still valid, but findings reported in this thesis need further investigations complementing on that knowledge (172).

6.1. CUX2 in the neocortex throughout development

One part of this thesis studied CUX2 expression patterns during laminar development of the human fetal cortex. A potential novel role for CUX2 in the early neocortical circuits and cellular fate selection is proposed based on the differential spatio-temporal expression patterns in the transient cellular compartments across early and late human fetal cortical development. To date, systematic studies of the spatio-temporal expression patterns of critical modulators of cortical development during human fetal development are scarce. These studies, including our research here are essential for a comprehensive understanding of normal corticogenesis and abnormalities in the neocortex-associated disorders, such as autism and epilepsy (94,165).

6.1.2. CUX2 expression in the postmigratory neurons and early differentiated neurons of the transient cortical compartments SP and MZ

The most striking displays of CUX2 expression observed throughout fetal cortical development were featured in the large, Reelin-positive CR cells, and within the vast population of polymorphic SP neurons, besides its presence in the CP. The results of this thesis found that Reelin-positive CR cells have CUX2 positive nuclei throughout the human fetal development, which is compatible with the results already shown in the mouse cortex (173). Moreover, the results show CUX2 expression in SP postmigratory neurons in all phases of SP development: preplate (I), pSP phase (II), SP in formation (III), midgestational SP (IV), stationary SP (V), with the addition of SP resolution (6,9,147) and its expression in the adulthood. The identification of CUX2 positive nuclei in SP neurons was based on co-existence with MAP2 and Neuroserpin, previously determined markers of fetal SP neurons (165,174). Interestingly, CUX2 nuclei also co-localized with some of the brain neurotransmitters and neuromodulators expressed in the neocortex at a specific time and location. The results show CUX2 co-localized with SST, NPY, and calcium-signaling molecule calbindin, revealing a specific interneuronal CUX2 population.

During the period of midgestation, a part of CUX2 positive SP neurons may belong to tangentially migratory GABA-ergic neurons (79), even though most of them probably belong to migrating upper layer neurons. The original study on the mouse embryonic development showed Cux2 co-expression with DLX2, a marker of the cortical interneurons that originate from subpallium and tangentially migrate into the cortex (77). Three persistent subpopulations of

GABAergic SP neurons are grouped by their expression of somatostatin (30% of total GABAergic SP neurons), serotonin receptors (60%), and parvalbumin (10%) (175), whereas the somatostatin subgroup additionally co-expresses nNOS, calbindin, and NPY (176). GABA-ergic neuronal migration has a late-onset (124) and GABA-ergic SP neurons may have long projections (33,177,178). The rest of CUX2 positive SP population may belong to the glutamatergic population of SP projection neurons. These neurons exhibit transient widespread projections (179). Indeed, SP neurons project subcortically to the thalamus (180,181), and through the corpus callosum to the contralateral hemisphere (182). Similar to SP neurons, CR cells, have well-protracted subcortical and intracortical axons and are also considered transient projection neurons (1,6,183). Thus, herein was found that besides previously reported CUX2 expression in the adult upper layer cortical projection neurons, CUX2 is also expressed in the fetal transient projection neurons, and somatostatin SP neuronal population. Together these findings suggest a role for CUX2 in the formation of the first neuronal circuits.

Derived from the timing of the CUX2 expression in the SP is further and indirect evidence that CUX2 expression is associated to the migratory upper layer neurons, and with the residing postmigratory SP projection neurons. CUX2 expression was visualized already in PP and pSP phase, while it was present during the SP formation when projection neurons of upper cortical layers are not born yet (63). Additionally, CUX2 was expressed in SPrm white matter (WM) neurons in the newborn neocortex when all neuronal bodies of upper layers are supposed to be in their final laminar, postmigratory position. A noted timed decrease of CUX2 positive nuclei in CR cells, and SPrm-WM neurons (81) could be explained by the fact that both of the populations, CR and gyral WM neurons, gradually transform into different morphology and position themselves in WM (9,30,184,185), possibly losing their projection traits, their specialized features in the fetal transient networks. Thus, partial loss of CUX2 expression in the gWM neurons during postnatal development may be an important indicator of the projection profile of gWM neurons. Due to the extremely rapid growth of WM in the human cerebrum during early postnatal development (186), it is normal to expect changes in the long axonal projection of gWM neurons. However, gWM neurons maintain their local connectivity, serving as ‘gatekeepers’ for afferent inputs reaching superficial gWM (8).

Furthermore, a weaker, CUX2 reactivity was observed in the pyramidal neurons of prospective layer 5 during midgestation. This finding suggests that the CUX2 achieves its role

during the formation of transient widespread circuits and axonal collateralizations of the deep neocortical layers during development (38,46). Previously, Cux2 expression was found postnatally in the deep layers of mouse insular cortex (118), and partially in layer 5 of the mouse somatosensory barrel cortex (119). Interestingly, a recent human prenatal scRNA-seq study showed evidence of co-expression between layer 5-6 markers and CUX2 in embryonic and mid-fetal tissue (120). In summary, CUX2 laminar distribution throughout fetal development shows dynamic changes in protein expression while it is predominantly expressed in differentiated layers of the cortex, and later on in the superficial layers (81). The findings of this thesis indicate a heterogeneous CUX2 neuronal population in the neocortex during fetal development, where a portion of cells is migrating toward the CP, another population is projecting their axons to the specific brain targets, with a specific, interneuronal population present in the neocortex as well.

6.1.3. CUX2 in the developing migratory population of the upper cortical layer projection neurons

The results of thesis show that during fetal development projection neurons predominantly express CUX2 while migrating through SP and deep CP. These results are consistent with the previous experimental data on rodents (77,78,118) and with results on the post-mortem human brain (79,81), which also showed that CUX2, a marker of upper layer projection neurons is expressed during fetal development. Even though our results are showing scarce reactive nuclei in the superficial IZ, it is surprising that CUX2 protein or its mRNA expression was not present in the subventricular proliferative zone during the neurogenesis, as described in rodents (77,78).

However, a limited, weak *CUX2* mRNA and protein expression was visualized in the SVZ of 26 PCW preterm stage neocortex. The results found that *CUX2* mRNA seems to be localized apart of neuronal soma in the dendrites as well. Importantly, the timing of *CUX2* mRNA expression in the human SVZ roughly corresponds to the developmental period in the monkey brain where layer 3 neurons of PFC are born between E70 and E85 (187), and in visual cortex around E90 (63). Therefore, it is possible that at least some of the reactivity differences may be attributed to the species difference (mouse vs human), and the timing of developmental events. Previously, CUX2, but also CUX1 and BRN2 upper layer markers, were found to be co-expressed with Doublecortin (DCX), a migratory cells marker, suggesting that majority of the CUX2 neurons belong to the

migratory neurons in SP (79). The results of this thesis found DCX/CUX2 co-localization in the early fetal stage, while a fraction of cortical cells in the newborn frontal cortex maintained their DCX expression. The reports from the literature are in line with this thesis findings, collectively suggesting that CUX2 has a role in the process of migration of human neurons.

6.1.4. Diverse roles of CUX2

CUX2 expression in multiple neuronal classes at different phases of differentiation suggests its diverse roles in the neocortical development. Indeed, prior studies in animal models found CUX2 primarily as a layer-specific regulator playing a role in the process of spine formation, dendritic branching, and synapse regulation (78,117,121). The results of this thesis revealed CUX2 presence in different neuronal populations that are either migrating or are forming first cortical synapses and circuits in transient zones SP and MZ. Collectively, these findings support the idea that CUX2 participates in the molecular specification, dendritic development, circuit formation and synaptogenesis of fetal transient SP and MZ projection neurons as it does in permanent projection neurons of upper layers.

Finally, an imminent, far-reaching role for CUX2 in the telencephalon, related especially to its homeobox gene properties can be suggested (78). More specifically, the findings of this thesis imply that projection neurons have a special role in large-scale organization of the telencephalon during development, and the presence of CUX2 in transient and permanent projection neurons may be related to this general role. Transient projections across different telencephalic domains provide important signaling for molecular specification of the whole neuronal system, which is necessary in establishing the complex telencephalic organization. This conforms to the prediction that transient projection networks provide both synaptic and non-synaptic signals between remote cerebral regions before the final connectivity is established (6). Finally, CUX2 expression is maintained during adulthood in the upper cortical layers, which suggests its importance for both normal cortical development and the maintenance of predominantly associative and integrative cortical neuronal populations.

This thesis analyzed molecular and histological hallmarks of CUX2 neuronal populations within the neocortex during the extensive period of human fetal neocortical development and in the adulthood. However, being limited to human postmortem tissue, this thesis lacks axonal tracing

or similar experimental approaches to determine the possible projection character of CUX2 neurons. Nevertheless, the finding of CUX2 expression in the transient but critical SP neurons of neocortical development further stresses the importance of CUX2 deficiencies associated with neurodevelopmental disorders, such as autism and epilepsy (32).

6.2. CELF4 in the neocortex throughout development

Somewhat fresh research field of post-transcriptional mechanisms became a recent hotspot for research of their influence on the neocortical development. Moreover, RNA-binding proteins (RBPs) are the most prominent mediators of post-transcriptional regulation that hold distinct roles in neurodevelopment (52,90–92). Their function in alternative splicing, mRNA stability, translocation, and repression or derepression of translation is crucial for regular neuronal development. CELF4 is a post-transcriptional factor associated with regulation of translation (102,103). The thesis aimed to determine unknown CELF4 spatio-temporal expression pattern, subcellular localization, and mRNA binding targets to confirm its role in the first half of fetal cortical development. Finally, RBPs engagement in the mRNA alternative splicing amplifies the transcriptome diversity and likely in that way contributes to the complexity of human brain functions (36).

6.2.2. CELF4 expression in the postmigratory neurons and early differentiated neurons of the transient cortical compartments SP and MZ

Fluid dynamics of the CELF4 protein throughout the neocortical development of the human fetus are displayed as part of this thesis for the first time. Early on, at the end of embryonic and the start of fetal development CELF4 is present in the PP which transforms in MZ and pSP where it stays expressed. CELF4 during the development is mainly not present in the proliferative zones, but positive CELF4 neurons are occupying the IZ and MACC of the neocortex. Remarkable Reelin-expressing cells are co-localized with CELF4 in the SGL/MZ throughout the fetal development, starting from 8PCW to 38 PCW neocortex. The morphology of Reelin-positive cells indicates that co-localized CELF4 neurons belong to the CR class of neurons (1,168). SGL is a human and

primate specific compartment of the MZ which contains small granular cells that are tangentially migrating during fetal development, what is more, this layer disappears in the pre-term fetus (5,168,188–190). The rest of the CELF4 positive cells that are a part of the MZ may belong to various Reelin negative interneurons typically found there (168). Furthermore, the results reveal CELF4 expression in SP neurons in all phases of SP development: preplate (I), pSP phase (II), SP in formation (III), midgestational SP (IV), stationary SP (V), with the addition of SP resolution and its expression in the adulthood. Co-localization of CELF4 with NURR1 unveils a specific subplate neuronal population, while the rest of the CELF4 positivity in the SP could belong to the other SP neurons since NURR1 does not label all of the SP neurons (191), or else to the neurons migrating towards the CP and its final place in the future cortex. The results of this thesis confirm that CELF4 expression is part of the primed cells- CR and SP neurons which are the first neuronal populations to differentiate in the neocortex (1,2,180).

Up to now, CELF4 neuronal identity has only been related to excitatory neurons (102,192), while its molecular profile was not studied in depth. One of the aims of this thesis was to determine the CELF4 neurotransmitter/neuromodulator profile, in addition to the expression of calcium-signaling molecule, calretinin. As previously discussed, neurotransmitters are expressed promptly in the neocortex and that is how they were analyzed here. In the earliest stages of fetal development of the cortex, CELF4 positive neurons co-localized with GAD67 and CALR in the PP, and later on in the transformed MZ and pSP of the neocortex. Moreover, at the time when SP compartment is shaping (13/14 PCW), GAD67 and CALR were co-localized with CELF4 positive neurons in both the MZ and SP. Notably, CALR cells were co-expressed with CELF4 at the top of the CP, on the border with the MZ, throughout the development, which could represent the deep granular layer (DGL) of the MZ (10,163). During the midgestation (15-25 PCW), the expression of NPY starts in the SP and the results showed co-expression with CELF4 in the prospective frontal cortex. At 21 PCW the results showed VGLUT1 co-expressed with large CELF4 positive SP neurons, which could be postmigratory, projection neurons of the SP. Moreover, SST and nNOS positive neurons of the midgestational SP and striatum were co-localized with *CELF4* mRNA. The results of this thesis explicitly found CELF4 neurons being a part of the population of interneurons, co-localized with different markers, Reelin, GAD67, NPY, CALR, SST, and nNOS throughout the SP development.

6.2.3. CELF4 during fetal synaptogenesis

Previously, CELF4 role was associated with synaptic function, where the annotations for CELF4 binding targets in the adult mouse were found highly enriched within the processes related to synapse regulation (103). From the results of the CELF4 expression in human fetal cortex it was evident that CELF4 might have a role in synaptogenesis during fetal development. To further pursue this findings electron microscopy (EM) and RNAScope® experiments were performed. In the case of EM, the poor tissue fixation resulted in a mostly disrupted tissue and cellular architecture, which prevented a detailed analysis. Despite this, the EM revealed CELF4 protein punctae localized in the nucleus, and in the extracellular matrix (ECM), indicating possible synaptic contacts. Although by the literature CELF4 was not present in synaptosomes but was shown in dendrites, our results confirm its expression within neuronal dendrites (103).

Immunostainings with the presynaptic marker, synaptophysin (167,193) revealed synaptic strata (128), *i.e.* MZ and SP, in the neocortex from 15-21 PCW. Synaptophysin punctae were localized on the CELF4 positive neurons in the SG layer of the MZ, in the deep MZ, and the SP. CELF4 positive neurons co-localized with synaptophysin punctae at the top of the cerebral wall were identified as large CR cells, and smaller, CELF4 positive interneurons. In addition, synaptic punctae were identified on the cell soma and inverted pyramid cell process, along with the dendrites of CELF4 expressed neurons. Notably, synaptophysin punctae were smaller in the younger specimens immunostainings which correspond to immature synapses (148). Although the sensitivity of visualization of immunoreactive presynaptic punctae is restricting the full comprehension of synapse display, it is obvious that synaptophysin and CELF4 are co-expressed in the previously described synaptically active laminae during fetal development (9).

The results of this thesis verified synaptoporin (SYNPR) as one of the CELF4 binding targets in early and midfetal human cortical development. SYNPR acts in synapse formation (194) and is highly homologous to synaptophysin (195). Consequently, the occurrence of synaptophysin punctae on part of the CELF4 neurons in the synaptic strata in correlation with SYNPR (CELF4 binding target) indicates its role in synaptic formation. Synaptic function has been strongly associated with neurodevelopmental disorders, like in ASD where a handful of risk genes have an impact on synaptogenesis (104,140,196,197). Coexisting results in mice and humans implicate CELF4 in synapse function and formation during development.

6.2.4. CELF4 developmental role

As pioneers in the human fetal studies with the CELF4 RBP, the goal of this study was to determine its role in human cortical development. CELF4, a presumed translational regulator was previously associated with the role in synaptic function (103). The results of gene ontology (GO) uncover that human developmental CELF4 binding targets are abundant within the GO terms for the processes of transcription, regulation of transcription, neural development, axonal fasciculation, axonal guidance, and synapse assembly. To be more specific, genes of CELF4 mRNA binding targets affirmed here are TLE4, a marker of deep cortical layers (67), EIF4A2, translation factor (198), VGLUT1, glutamate transporter (171), and SYNPR. Altogether, the results imply CELF4 has numerous targets and roles in cortical development, specifically in regulating mRNAs involved in the processes of axonal pathfinding, transcription regulation, and synapse formation.

CELF4 association with the regulation of synaptogenesis during fetal development and its early and continuous expression in the synaptically primed compartments, MZ and SP, demonstrates its role in synapse formation. Strong CELF4 immunoreactivity within the midfetal SP identified various neuronal types: polymorphic, migratory neurons with the apical process, and fusiform neurons. This argues that CELF4 has a multiverse role within the SP, as it encompasses all of the SP neuronal subtypes. On the other hand, its identity as an RBP may contribute to a more general role that is retained even during human adulthood, whereas its wide expression in different brain regions, such as the thalamus, striatum, hippocampus, and cerebral cortex adds to this theory.

Predominant CELF4 expression in excitatory neurons of cortical layers suggests its role in projection neurons of the cortex, as seen in the mouse cortex (103) and throughout the human fetal development (192), as confirmed in this thesis. CELF4 expression in the pre-SP and in the mature SP throughout the SP development, while later on in the deep layers of the cortex implies its role in the projections that are maintained during adulthood. However, we demonstrated that CELF4 has a role in the interneurons of the MZ and SP throughout human fetal development. By co-expression with the typical neurotransmitter markers, it was mostly visualized as part of the somatostatin subgroup. As neuropeptides NPY, and somatostatin were almost exclusively present in the residing SP neurons of the perinatal stages (199) indicates that part of the CELF4 neurons at the time are SP neurons. GABA SP neurons misplacement and misconnection may be the cause of epileptic syndromes (8,200), since CELF4 has already been implicated with epileptic seizures (102,201).

To conclude, a disrupted transcriptional and/or post-transcriptional regulation leads to neurodevelopmental disorders, such as schizophrenia, ASD, and others (49–52). Importantly, the vulnerability of the SP was also associated with the same disorders (159,200,202). Our results indicate the spatio-temporal expression pattern of TF, CUX2, and RBP, CELF4 in correlation with the dynamics of cortical processes taking place in the same laminae, which indicates their important roles in the differentiation of the cortex. CELF4 has already been categorized as an ASD risk gene (104,126), and also is contributing to epilepsy (102,201). Nonetheless, it is essential to research the functional mechanisms in the cortex during development. Future studies should address alterations in synaptic activity during the periods of synaptogenesis and include patients screened for CELF4 mutations to determine the peculiar pos-transcriptional mechanisms involved and its phenotype when CELF4 is defective.

7. Conclusions

1) CUX2 is expressed in neurons of the transient compartments from 8 PCW to 38 PCW of the human fetal prospective frontal cortex, specifically in the CR cells of the MZ and in the subpopulation of neurons of the SP zone.

2) A heterogeneous CUX2 neuronal population is present in the neocortex during fetal development, including a subpopulation of migratory cells, interneurons, and a projection neuronal subpopulation, suggesting its important role in fetal cortical development.

3) Transcription factor CUX2 is involved in the molecular specification, circuit formation, and synaptogenesis of fetal transient SP and MZ projection neurons, as it is in permanent projection neurons of upper cortical layers.

4) CELF4 is expressed in the postmigratory neurons and early differentiated neurons of the transient cortical compartments SP and MZ throughout fetal cortical development.

5) CELF4 has broad, multiple roles in cortical development by regulating mRNAs involved in the processes of axonal pathfinding, transcription regulation, and synapse formation during early and midfetal cortical development.

6) Our results indicate that the spatio-temporal expression pattern of both CUX2 and CELF4 is in correlation with the dynamics of cortical processes taking place in the same laminae, which suggests their role in the differentiation of the cerebral cortex.

8. Sažetak

Razvoj moždane kore fetusa čovjeka je kompleksan proces, ispunjen ključnim histogenetskim zbivanjima koji se ostvaraju u prolaznim fetalnim zonama, a čiji učinci se vide u konačnoj složenosti funkcija ljudskoga mozga. Transkripcijski i post-transkripcijski mehanizmi su u pozadini neurogenetskih i histogenetskih događaja, te imaju izravan utjecaj na razvoj mozga. Tema ovog doktorskog istraživanja je analiza vremensko-prostornog obrasca ekspresije transkripcijskog faktora CUX2 i post-transkripcijskog faktora CELF4 tijekom fetalnog razvoja ljudskog mozga. Da bi istražili njihovu ulogu u razvoju moždane kore, korišteni su postmortalni uzorci mozga starosti od 8 PCW do 38 PCW, te dodatno uzorci odraslog mozga. Rezultati ovog istraživanja pokazuju da je CUX2 eksprimiran u jezgrama neurona prolaznih fetalnih zona u dobi od 8 do 38 PCW moždane kore čeonog režnja, a posebno unutar jezgara CR stanica marginalne zone (MZ) i neurona subplate (SP) zone. CUX2 i CELF4 su eksprimirani u migratornim stanicama, projekcijskim neuronima i u interneuronima moždane kore. CELF4 pozitivni neuroni su pronađeni unutar sinaptičkih slojeva koji su imunoreaktivni na sinaptofizin, tijekom midgestacije. Tijekom ranog i srednjeg fetalnog razdbolja CELF4 veže mRNA *TLE4*, *EIF4A2*, *VGLUT1* i *SYNPR*. Zaključno, CUX2 sudjeluje u procesima molekularne specifikacije, organizacije neuralnih veza, i sinaptogeneze u fetalnim prolaznim MZ i SP projekcijskim neuronima, te u stalnim projekcijskim neuronima površnih slojeva moždane kore. Uloga CELF4 proteina u razvoju moždane kore je u regulaciji mRNA uključenih u procese regulacije transkripcije, navođenja aksona i formacije sinapsi. Rezultati ove disertacije prikazuju vremensko-prostorni ekspresijski obrazac transkripcijskog faktora CUX2 i RNA-vezujućeg proteina CELF4 u korelaciji sa dinamikom zbivanja u moždanoj kori koji se događaju u isto vrijeme u istim slojevima, te time odražavaju njihovu ulogu u diferencijaciji moždane kore.

9. Summary

Cortical development of the human fetus is a protracted process filled with critical histogenetic events which give rise to the brain complexities differentiating in the transient neocortical compartments. Transcriptional and post-transcriptional mechanisms are the fundament of neurogenetic and histogenetic events, with the transcription and post-transcriptional factors acting as crucial impactors of neural development. In this thesis, spatio-temporal dynamics of CUX2 transcription factor and post-transcriptional factor, CELF4, in human fetal development are studied in depth. To explore their role in cortical development, human post-mortem fetal tissue from 8 PCW to 38 PCW, in addition to adult tissue was used for the experiments. The results show CUX2 nuclear expression in neurons of the transient compartments from 8 PCW to 38 PCW of the human fetal prospective frontal cortex, specifically in the CR cells of the MZ and in the neurons of the SP zone. Both CUX2 and CELF4 are expressed in the migratory cells, projection neuronal subpopulation, and in the subpopulation of interneurons. CELF4 positive neurons were found in the synaptophysin-immunoreactive synaptic strata during midgestation. We have also identified human early and midfetal mRNA binding targets of CELF4: *TLE4*, *EIF4A2*, *VGLUT1*, and *SYNPR*. In conclusion, CUX2 participates in the molecular specification, circuit formation, and synaptogenesis of fetal transient SP and MZ projection neurons as it does in permanent projection neurons of upper layers. CELF4 attains a role in cortical development, by regulating mRNAs involved in the processes of transcription regulation, axonal pathfinding, and synapse formation. The results of this thesis show the spatio-temporal expression pattern of TF, CUX2, and RBP, CELF4 in correlation with the dynamics of cortical processes taking place in the same laminae, which indicates their important roles in the differentiation of the cortex.

10. References

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11. Curriculum vitae

Terezija Miškić was born on December 7th, 1992 in Zagreb. She graduated with a Bachelor's degree in Nutrition, in 2014 from the University of Zagreb. Afterward, she pursued a double Master's degree in Molecular biotechnology, where in the last year of her Master's degree she did in the joint program of the University of Zagreb and Universite de Orleans (France). Part of the program was her internship in Lyon, where she started studying cortical development for the first time. She carried out an interdisciplinary project between the Laboratory of therapeutic applications of ultrasound and Stem-Cell and Brain Research Institute, INSERM, and graduated in July 2016 with the thesis on: 'Bio-Acoustic Levitation for Engineering Three Dimensional Cortex-like Constructs'. In 2017 she started a government funded fellowship at the Croatian Institute for Brain Research where she studied molecular mechanisms of Alzheimer's disease in Professor Goran Šimić's laboratory and co-authored her first publication. Finally, in 2018 she started her PhD studies and a fellowship under the Scientific Centre of Excellence for Basic, Clinical, and Translational Neuroscience (funded by the EU), with the mentorship of Professor Željka Krsnik, PhD. Throughout her studies, she visited Rutgers University (New Jersey, USA) multiple times to undertake her research work as part of co-mentorship with Professor Mladen Roko Rašin, MD, PhD. Together with her mentors and scientific advisor Professor Ivica Kostović, MD, PhD she published her first-author publication. She has two publications and has attended six scientific conferences while presenting her work.

She is a member of the Croatian Society for Neuroscience, and the ALBA Network. Notably, she is an active futsal player with multiple awards during her University sports career. She speaks fluent English, conversational French, and beginner Spanish.