General Introduction

Well into the 21st century, the human brain remains a mystery. Although human brain development follows the same principles as that of all mammals[1,2], there are clear interspecies differences that ultimately lead towards the unique cognitive and behavioral features of humans[3,4]. Primarily the cerebral cortex is responsible for the higher cognitive, abstract thinking and language capacities humans contain[3,4].

Humans have an exceptionally long gestational time, childhood and adolescence[2,5–7]. Anatomically the human brain has an extended surface area and the amount of vertical columns in the cortex has increased in number, size and complexity[1,8]. This has resulted in a large change in cell number[9,10], morphology and composition of brain cells[11,12].

Genetic differences between humans and our closely related ancestors[9,13–16] and the latest humans to become extinct, Neanderthals and Denisovans[17,18], are reflected in single-nucleotide variants, insertions, deletions and structural chromosomal rearrangements[18]. The majority of alterations are found in developmental genes and their regulatory regions[18–20]. Especially the latter may have significantly contributed to human brain evolution, as regulatory genes function selectively in cell types and during specific cell cycles, adding extra layers of control of expression[13,18,21,22].

Nonetheless, human brain evolution and extended life span also appears to have given rise to susceptibility for brain diseases, such as neurodegenerative diseases[23] and psychiatric disorders[24–26]. In humans amongst others the processes of dendritic and synaptic maturation and synaptic pruning are prolonged[27]. This prolonged period links it to various neuropsychiatric disorders and intellectual disabilities[28–30]. Also many genes associated with neuropsychiatric disorders are involved in brain development and its regulation, which contains several human-specific processes[31,32]. Similarly, white matter volume in the prefrontal cortex is disproportionally larger in human brains[33,34], but progressively declines in the aging brain, linking human oligodendrocyte function to several neurodegenerative diseases[35].

To shed light on the molecular mechanisms of human brain diseases, studies are commonly performed in animal models, the mouse being highly suitable for its genetic resemblance and ease to work with[2]. Yet, the human brain is over 1000 times larger than the mouse brain[3], its cortical genesis takes roughly 20 times longer[3], its cell cycle time is 3-4 times longer[3], birth occurs during later stages of brain development and postnatal maturation takes longer before reproduction. Also, in development there is compartmentalization of the different neural progenitors and layers, such as a larger transient subplate zone and an outer subventricular zone as well as expanded superficial layers of the cortex. Also human glia are unique and distinctively different from rodent glia[36–38]. They are considerably larger in size, have more elaborate processes and physiology and form more connections.

One way to study particularly human brain development and the cells of the human brain is by using human embryonic stem (hES) cell technology. Human embryonic stem cell technology emerged in the late 1990s. It comprises the use of pluripotent stem cells from pre-implanted embryos. These cells in theory have the capacity to differentiate into the different

cell types that can be found in the human brain. A couple of commonly used hES cell lines are the H1, H9 and H11 lines[39] and protocols to tweak these cells towards the neural lineages appeared soon after their establishment in 1998. Most of these protocols are based on existing procedures to derive neural precursor cells (NPCs) from mouse stem cells[40]. Fundamental studies on human stem cell-derived neural cells though stayed surprisingly limited. A reason for this may have been the ethical and limited disease-modeling capacity of hES cells.

In 2006 Yamanaka et al. published their work on *in vitro* reprogramming of somatic cells towards induced pluripotent stem (iPS) cells[41]. With the overexpression of the four embryonic transcription factors Oct3/4, Sox2, Klf4, and c-Myc in terminally differentiated cell types, somatic cells are driven back to an induced pluripotent state. In many ways, iPS cells are morphologically and transcriptionally similar to hES cells[42]. They have the capacity to differentiate to different germ layers and terminally differentiate towards specific cell types. This has offered a less ethically controversial way to generate human brain cell types and allowed diseasemodeling in which the differentiated neural cells retain the genome of the donor.

DEVELOPMENT OF THE HUMAN CEREBRAL CORTEX

The question that emerged however is to what extent iPS technology could be applied to study human brain development and model human brain diseases.

Cortical development involves neurogenesis, differentiation, migration, synaptogenesis, and establishment and refinement of connections[4]. In humans it spans early to mid-gestational periods, although myelination takes up to the 2nd and 3rd decade of life. Human neurodevelopment starts with the formation of the neural tube from the embryonic ectoderm[7,43]. The wall of the neural tube contains a pseudostratified layer of neuroepithelial cells called the ventricular zone (VZ). These cells are the progenitors for all neurons and glial cells (astrocytes and oligodendrocytes) in the brain and spinal cord. Rounds of symmetric division of the neuroepithelial cells which give rise to two identical progenitor daughter cells, each round of replication increasing the pool of neural progenitor cells. Rounds of asymmetric division produce one progenitor cell and one post-mitotic neuron per division. To form the cortical plate, cells radially migrate from the VZ[44]. The cortex is shaped in an inside-out fashion. Neurons residing in deeper layers emerge first and newly generated neurons migrate through these layers to form the more superficial layers[44].

Every cell in the different layers of the cortex has a distinct transcriptional profile related to its cellular composition and relative maturity. Neurons find their place in the cortex using somal translocation. The neuron extends one process, which is an extension of the cell body beyond the VZ into the outer region. The process then attaches to the pial surface, the outer surface of the brain. Subsequently, the nucleus then moves up the process and migrates out of the VZ. When the brain becomes larger, radial glial (RG) cells serve as guides for migrating

neurons. Their nucleus remains in the VZ and they extend their processes to the pial surface. Migrating neurons use their process as a scaffold to migrate into the brain. RGs themselves also serve as a neural progenitor pool. Next, a second proliferative zone emerges above the VZ, called the subventricular zone (SVZ). These cells give rise to the majority of the glutamatergic neurons within the telencephalon.

During development, several layers are discernable (**Figure 1**)[7,43]. The first neurons that leave the VZ form the preplate (PP). The next wave of migrating neurons splits the PP in the marginal zone (MZ) and the subplate (SP). The neurons that establish between these layers are the first cells of the cortical plate (CP). Both the MZ and the CP are transient layers, and disappear with development. The MZ moreover contains Cajal-Retzius cells, a heterogeneous population of cells that produce reelin, a secreted extracellular matrix protein responsible for migration and positioning of neurons into layers of the neocortex[45]. Subsequently, the SVZ emerges and from the VZ up to the MZ the following layers are present: VZ, SVZ, intermediate zone (IZ), SP, CP, MZ. The VZ and SVZ will eventually reduce to a one-cell-layer thick region and the IZ will develop into a white matter layer above which the 6 layers of the cortex have developed.

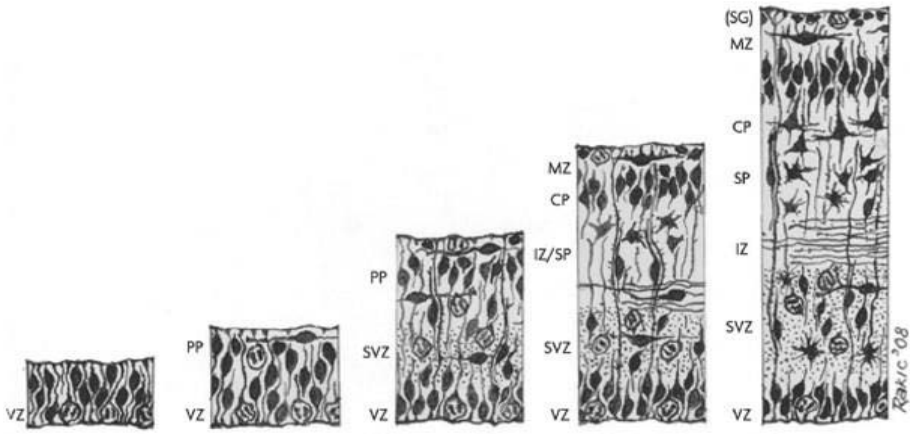

Figure 1, schematic model of human neocortical development (adapted from Bystron et al. 2008[43]). CP, cortical plate; IZ, intermediate zone; SP, subplate zone; MZ, marginal zone; SVZ, subventricular zone; (SG), subpial granular layer (part of the MZ); VZ, ventricular zone.

Another proliferative zone in the developing brain is the ganglionic eminence (GE). Here important classes of inhibitory neurons and oligodendrocytes precursor cells (OPCs) are generated. These cells migrate tangentially into the cortex[44].

Most of the knowledge regarding early brain development is derived from rodents where tracing studies with labeled virus can indicate cell progeny. Limited evidence exists on early human VZ/SVZ development. A few studies however confirm and highlight similarities and dissimilarities between rodents and human VZ development. Most knowledge is obtained by

immunostaining of primary cell cultures and slice cultures from human fetal brains. More recently, with the development of single-cell RNA sequencing technology progenitors and neurons are re- and sub-classified on the basis of their RNA expression next to their immunogenic profile[46].

THE VENTRICULAR ZONE

Several groups have described different cell types in the VZ during human brain development. The first cell types to be identified were RG and neuron-restricted progenitors[47–50]. At 4,5 gestational weeks (gw) RG are exclusively present in the VZ[47]. Immunophenotypically, RG are characterized by the expression of glia-specific antigens, such as the intermediate filament vimentin[51] or nestin, astrocyte-specific glutamate transporter (GLAST)[52] and glial fibrillary acidic protein (GFAP)[47,49]. Actively dividing RGs are visualized using the 4A4 antibody, which recognizes vimentin phosphorylated by a mitosis-specific kinase, cdc2 kinase[53]. When RG divide, their cell bodies descend to the ventricular surface to undergo mitosis (interkinetic nuclear migration)[1]. RG serve as a guide for migrating neurons, but eventually develop into neurons, astrocytes or oligodendrocyte precursor cells (OPCs). Occasionally therefore RGs in this stage are also found to express SMI-31, a marker of nonphosphorylated intermediate filament proteins, present in cells of neuronal lineage[47].

At similar ages neuron-restricted progenitors are also found[47–50]. These are dividing cells that stain positive for neuronal markers such as SMI-31, β-III-tubulin, MAP2 and doublecortin (DCX) and negative for any of the RG markers. They are also present in the pro-encephalon, where no RGs are present[47].

At 5-6 gw neurogenesis starts in humans[48]. At 5,5 gw mitotically active RG are found about 100 um above the VZ surface[47]. At 6 gw active RGs are found throughout the entire pro-encephalon. Next to this, neurogenic progenitors are found throughout the VZ and SVZ. They are dividing vertically or horizontally with respect to the VZ surface. There is also an actively dividing GLAST$^+$ and β-III-tubulin$^+$ population at the ventricular surface, perhaps indicating RG that will develop into neurons.

By 9-10 gw the cortical plate, a layer of 6 cells thick, is visible in the entire telencephalic wall[47]. RG are abundant and dividing. Many also have migrated to the SVZ and IZ. RG are reaching up into the SVZ, the IZ and CP[47]. These RG do not express neuronal and glial markers simultaneously[49].

That RG become restricted in their fate was also indicated by Mo et al[50]. They isolated RG from 14 and 20 gw VZ/SVZ using immunopanning with CD15, an extracellular matrix-associated carbohydrate[50]. Over 90% of the CD15$^+$ population co-labeled for one of the following RG markers: BLBP, vimentin or GFAP. Only less than 10% of the CD15$^+$ co-stained for β-III-tubulin. When clonal cultures of individual CD15$^+$ cells were analyzed, four types of clones

were discernable: pure GFAP$^+$ clones, pure MAP2$^+$ clones, mixed clones with a majority of GFAP$^+$ cells, and mixed clones with a majority of MAP2$^+$ clones. More glia were generated in cultures derived from the 20 gw-old VZ/SVZ than from the 14 gw-old VZ/SVZ, indicating that stage differences may play a part in their fate determination.

That the RG population itself is heterogeneous was also confirmed by Howard et al. who studied dissociated cell cultures obtained from VZ/SVZ of 19-22 gw fetuses[47]. Of the total population of dividing cells in culture roughly 30% was vimentin$^+$ or GFAP$^+$ and about 15% was GLAST$^+$. Many glial cells would simultaneously express several markers. It was unclear though if the expression of different antigens determines RGs ability to develop into either neurons, astrocytes or OPCs or that it is a function of cell differentiation.

Which factors play a part in fate-determination remains largely unknown. One however entails regional cues[50]. Mo et al. co-cultured CD15$^+$ cells with GE and cortical cells. They showed that CD15$^+$ cells co-cultured with the GE developed into calretinin$^+$ interneurons considerably more often than when CD15$^+$ cells were co-cultured with cortical cells[50]. They also found that growth factors EGF and FGF were higher in cultures containing neurogenic RGs, pointing towards which cues specifically play a role in fate-determination.

At 17-24 gw RG are still dividing but less so than at 9-10 gw[47]. In midgestation RGs are in all compartments of the telencephalon, such as the IZ and the most superficial subpial granule layer. In the VZ some calretinin$^+$ 4A4$^-$ cells are visible. They are closely apposed to the RG fibers as if using them as a guide.

By midgestation 20 gw, most of neurogenesis has taken place. RG start to transform into GFAP$^+$ astrocytes in the intermediate zone and the cortical plate[48]. Occasionally there is mitosis of the RG, but by 23 gw proliferation has finished[49]. An ependymal layer forms on the VZ. Thin GFAP$^+$ fibers cross it to attach to the VZ surface[49].

THE SUBVENTRICULAR ZONE

From 5-6 gw the VZ is the only proliferative zone. At 7-8 gw the SVZ emerges above the VZ[54,55]. Cells that are generated from the ventricular epithelium populate it. Here proliferation continues until the 40 gw-long intra-uterine period. From 10-24 gw the appearance of the SVZ changes because of tangentially incoming fibers from subcortical regions and those crossing the corpus callosum[54]. There are cell fibers visible that stretch to the subplate. The fibers divide the SVZ in the inner (iSVZ) and outer SVZ (oSVZ).

Several classes of progenitors are found in the SVZ[56]. One resembles RG in phospho-vimentin, nestin and GFAP expression and is also Pax6$^+$ and Sox2$^+$ [56]. In contrast to RG though these cells have basal processes extending to the pia, but lack an apical process that is connected to the surface of the VZ. They are termed outer radial glia cells (oRG). In contrast to RG that show interkinetic nuclear migration, these cells show mitotic somal translocation

where the nucleus moves up the basal fiber before cell division. As the cell divides the upper cell inherits the basal process, whereas the lower cell becomes bipolar, generating an oRG and an oSVZ progenitor. This is an example of asymmetric self-renewing division. Both oRG and oSVZ progenitors are able to divide again. The oRG is able to yet again asymmetrically divide, whereas the oSVZ generates two similar daughter cells. This process ensures rapid expansion of the progenitor pool. Hansen et al. also found that daughter oSVZ cells can readapt oRG morphology[56].

Outer RG develop into excitatory neurons[54–56]. From 7-27 gw β-III-tubulin$^+$, PSA-NCAM$^+$ and MAP2$^+$ immature neurons are present in the SVZ[56]. TBR-1$^+$ and glutamate$^+$ cells are present, labeling projection neurons, which were migrating radially to the upper cortical layers[54]. NeuN$^+$ and NSE$^+$ cells are mostly visible away from the SVZ in the subplate, the cortical plate and layer I[54].

However, from 7-22 gw Zecevic et al. also found GABA$^+$, calretinin$^+$, and calbindin$^+$ inhibitory neurons[54]. They had unipolar or bipolar morphology, suggestive of their migration. In slice cultures of 22 gw-old VZ/SVZ a BrdU-incorporation proliferation assay showed that 25% of the BrdU$^+$ cells expressed Dlx and19% expressed Nkx2.1, indicating these cells were progenitors to interneurons. Yet, 55% of the Dlx$^+$ cells and 80% of the Nkx2.1$^+$ were also PDGRFα$^+$, an early oligodendrocyte progenitor marker, signifying that in the SVZ progenitors to both interneurons and OPCs are present.

Hansen et al. similarly found progenitors of interneurons. By following division of oRG in real-time and determining daughter cell fate by immunostaining, they showed that daughter cells can start to express TBR-2, an indicator of commitment to the neuronal lineage and newly-born neurons of the excitatory lineage, or ASCL1, a transcription factor to indicate GABAergic fate.

At 25-27 gw the VZ becomes a one-cell-layer thick ependymal layer whereas the SVZ is still present around the lateral ventricle[54]. The subependymal zone contains neural stem cells, which then remain throughout adulthood for repair processes[57].

INTERNEURONS

In contrast to rodents, in humans two-thirds of the interneurons are generated in the SVZ[58–64].

The first-born GABAergic interneurons are generated in the GE in the basal ganglia and migrate tangentially into the CP. The first wave of migration contains pioneer neurons that make up the early PP. These contain different types of cells, including Cajal-Retzius cells. Production of interneurons in the GE is followed by generation of interneurons in the SVZ. In the mature brain several classes of interneurons are found. They are roughly divided by their expression of the neurochemical markers parvalbumin (PV), somatostatin (STT) and serotonin receptor 3A (Htr3a) and are further subdivided based on morphological features,

cellular and subcellular targeting, electrophysiological and synaptic properties as well as expression of other markers[65–67]. This classification is largely based on studies in mice and serves as a starting point for understanding the interneuron diversity in humans.

Several studies shed light on the development of interneurons in the human brain. The GE is the main source of interneurons in early brain development (6-15 gw)[42,50–52]. In mice a regulatory network of the transcription factors Dlx1, Dlx2, Ascl1, Gsx1 and Gsx2 is required for the generation of interneurons in the subpallium[70,71]. In humans Dlx^+ and $Nkx2.1^+$ progenitors for interneurons are also found and migrate tangentially to the developing neocortex. They develop into $calretinin^+$ and $calbindin^+$ interneurons in the deeper layers V and VI of the neocortex[58]. At 15 gw the GE is still the main source of cortical interneurons, as indicated by calretinin labeling[58]. From 16-24 gw however, Dlx^+ [63], $Nkx2.1^+$ [63], $Ascl1^+$ [60] and $Gsx2^+$ [64] populations are also discernable in the VZ/SVZ. These cells regularly co-localize with markers GABA, GAD2 or calbindin. VZ/SVZ RG that are $Pax6^+$ and $BLBP^+$ are also able to produce interneurons[64]. Yu et al. also confirmed the presence of RG that are $GABA^+$ and $calretinin^+$ [63].

At midgestatin $Ascl1^+$ cells are also found in the GE. There they co-label with Dlx[59,62]. In the VZ however, $Ascl1^+$ and Dlx^+ cells do not co-localize, nor do $Ascl1^+$ and $Nkx2.1^+$ cells, indicating distinct populations of precursor interneurons. Also, there was very little overlap between $Ascl1^+$ and $calretinin^+$ progenitors. $Ascl1^+$ cells however were $GABA^+$, so they may give rise to another interneuron subtype. $Ascl1^+$ cells were however sometimes also labeled with PDGRFα, but most of these cells were seen in the cortical plate, especially in the subplate[59]. Its percentage was much lower in the VZ/SVZ. Therefore in midgestation $Ascl1^+$ interneurons and $Ascl1^+$ OPC progenitors are present. There are also $Ascl1^+$ cells that express neither of these markers and therefore they are either not committed to cell fate yet or part of the interneuron and OPC lineage but at time of examining not expressing GABA or PDGRFα.

$Neuropeptide\ Y^+$, $somatostatin^+$ and $parvalbumin^+$ interneurons are sparse in midgestation[58] and are generated later in human neurodevelopment.

GLIAL CELLS: OLIGODENDROCYTES AND ASTROCYTES

Oligodendrocyte lineage cells have the highest turnover in the central nervous system and all ages of the cell are present throughout the brain at all times. OPC development starts in 2nd trimester and continues after birth[72,73]. $PDGFR\alpha^+$ cells are visualized at 10 gw in the forebrain for the first time, but the highest number of these cells is around 15 gw, when they are present mostly in the GE and VZ/SVZ. Cells with similar morphology as $PDGFR\alpha^+$ cells were often also labeled with NG2-chondroitin sulfate proteoglycans[72]. By midgestation 19-22 gw OPCs invade more dorsal areas as well as the cortical plate. During the majority of development OPCs are most dense in the SVZ. At around 20-22 gw $O4^+$ and $O1^+$ OPCs are present in the subplate layer, immediately below the cortical plate. As they mature they start to express MBP

and PLP. The first MBP$^+$ cells with mature morphology are seen at 18 gw. There is a ventral to dorsal progression of oligodendrogenesis. During development several classes of OPCs are discernable: there is a population that expresses Dlx2, Nkx2.1, present in both GE and VZ, a Dlx2$^-$ and Nkx2.1$^-$ class, and a class of OPCs expressing PDGRFα, NG2, Olig1, nestin, and also CD34 and CD68[72,73]. Next to this humans contain a subpopulation of NPCs that are Olig2$^+$ and Pax6$^+$ in cryosections of 15-20gw in GE and SVZ, indicating human-specific OPC populations.

Human astrocyte development is mostly unknown. In rodents astrocytes develop from transformation of RG, glial progenitors in the SVZ, glial progenitors in the MZ/layer I or from progenitors in the superficial layers of the cortex[74]. DeAzevedo et al. describes the transition of RG into astrocytes in human brain from 18 to 39 gw[75]. Transition is described by detachment of the ventricular process, followed by detachment of the pial process. However, also pial detachment before ventricular detachment is seen. In the late stages of astrocytes development stellate morphology is discerned. From 38-39 gw astrocytes are bilaminarly distributed. GFAP$^+$ and vimentin$^+$ astrocytes are seen in the upper CP and MZ and in the SP/IZ. After detachment of either of the processes, nuclei of the astrocytes migrate radially to their place in the cortex.

Most astrocytes nonetheless are generated after birth[36]. In adult humans four classes of astrocytes are found: protoplasmic astrocytes, interlaminar astrocytes, polarized astrocytes and varicose projection astrocytes[37,38]. It is unclear though how and when these develop.

MODELING HUMAN NEURAL CELLS WITH IPS CELLS

Regardless of the complexity of the human brain, the generation of neural cell types that resemble bona fide neural cells at the level of RNA, antigen expression and/or functionality have been generated using iPS as cell source.

Most protocols to classically differentiate neural cells from iPS are based on or modified from protocols to generate neural cells from mouse ES or hES cells. The majority of the protocols rely on mimicking the extracellular environment *in utero*[76]. In short, two pathways exist: guiding towards neuroepithelium with growth-factors and morphogens versus dual-SMAD inhibition[76]. In such a way neural progenitor cells (NPCs) are produced. They are then cultured for terminal differentiation into neurons or glial cells[77,78]. Protocols are also available to enrich for specified neurons such as cholinergic[79], dopaminergic[79], GABAergic[80] and serotonergic[81,82] populations. By combination of growth factors and mere time, cell populations could also be enriched for astrocytes[83,84], OPCs[85] and oligodendrocytes[86].

It became clear that the development *in vitro* was mimicking the order of development *in vivo*[87]. Many neuron-generating protocols show a neural rosette stage resembling neural tube formation[76,88]. This stage recapitulates progenitor zones similar to the VZ and SVZ including

its mixed population of progenitors. *In vitro* emergence of astrocytes and myelination takes place after terminal neuronal division also mimicking *in vivo* neurodevelopment. As a consequence, more so than a model for adult human brain neurons, stem cell-derived neurons *in vitro* represent best first trimester (up to 12gw) human fetal neurons[89,90], which are generated in at least 6 weeks *in vitro* from a neural progenitor stage[91]. Also, certain protocols recapitulate some structures of second trimester brain development[92]. As such, next to modeling specific cell types, human iPS technology allows modeling early human brain development[93].

Enhanced maturation is seen by using 3D culturing techniques[92,94,95]. Combinations of growing iPS in gels and scaffolds[96], or the self-organizing capacity of iPS in suspension are used to generate adherent 3D neural cultures, or free-floating brain organoids respectively[97]. The 3D environment allows next-level development of structures with enhanced and more mature capabilities[84] and model gene expression programs of fetal brain development[98]. 3D models have come as far as modeling hippocampal and cortical layers[99,100] as well as forebrain, midbrain and hypothalamic structures [92,101], where further development of the culture is commonly held back by lack of *in vitro* vascularization capacity[99]. However, recently Mansour et al.[102] implanted brain organoids in the mouse brain and showed enhanced development and vascularization, paving the way towards developmental progression of iPS-derived neural models and enhanced understanding of the brain and brain-related disease using iPS-based models.

SCOPE OF THIS THESIS

In this thesis we explore the use of IPS for modeling human brain development and disease.

In chapter 2 we describe a neural differentiation protocol that produces electrophysiological functional neural networks. This protocol allows for examination of iPS-derived neural networks for disease-related studies.

In chapter 3 we study the transcriptional regulation of human *BDNF.* Using our protocol described in chapter 2 we find novel *BDNF* transcripts in humans that are expressed upon activity of neural cells.

In chapter 4 we study the subcellular localization of mouse and human UBE3A in neurons, the lack of which in neurons causes the neurodevelopmental disorder Angelman Syndrome. We find differential localization of mouse and human UBE3A protein isoforms.

In chapter 5 we study the epigenetic modifications of the *FMRI1* gene. The absence of the *FMRI1* gene product, fragile X mental retardation protein (FMRP), causes the intellectual disability disorder Fragile X syndrome. We find that standard reprogramming procedures lead to epigenetic silencing of the fully mutated *FMR1* gene also in rare healthy individuals who carry a full mutation of *FMRI1* but show no hypermethylation of the gene's CGG repeats and promoter.

In chapter 6 we study long non-coding RNA (lncRNA) variants associated with Alzheimer's disease (AD). We find an associated variant that mediates regulation of AD-related genes in iPS-derived neural cells.

In chapter 7 I discuss the limitations of iPS technology that influence its capacity to model human brain diseases. I also discuss potential solutions.

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