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Studying the role of human Amyloid Precursor Protein (APP) during human cortical neurogenesis

Khadijeh Shabani Miankoushki

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Khadijeh Shabani Miankoushki. Studying the role of human Amyloid Precursor Protein (APP) during human cortical neurogenesis. *Neurons and Cognition [q-bio.NC]*. Sorbonne Université, 2021. English. NNT : 2021SORUS476 . tel-03771345

HAL Id: tel-03771345

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Sorbonne Université

Ecole Doctorale ED158

Studying the role of human Amyloid Precursor Protein (APP) during human cortical neurogenesis

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PhD Dissertation

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To my grandmother,

Who suffered from Alzheimer's disease. I hope that the results of this project will help in the treatment of Alzheimer's in the future.

با عشق و احترام تقدیم می شود به
مادر بزرگ عزیزم که از بیماری آلزایمر رنج برد.
امیدوارم که نتیجه این تحقیقات کمکی در راه درمان آلزایمر در آینده باشد.

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Acknowledgements

I am deeply grateful to you Bassem for the opportunity to be part of your lab. You are an incredible mentor with insightful comments and suggestions who always give space to your lab members to express themselves.

I also would like to thank:

All the collaborators in this project especially Scipio Bioscience members.

Hassan team, Paris and Berlin, for their supports and encouragement, especially Carlos PARRAS for all his help and support during this project and Julien Pigeon with whom I have shared the joys and the pains of this project the most.

All my friends in France and Iran who always have been by my side throughout these challenging times.

Philippe for his endless understanding, kindness and support.

The most special thanks go to my family; my parents, especially mom who supports me to follow my dreams. My lovely sister and brother who are the most supportive sibling's one could ask for.

Abbreviations and Acronyms

CNS	central nervous system
CSF	cerebrospinal fluid
PCW	post conception week
GM	gray matter
WM	white matter
VZ	ventricular zone
SVZ	subventricular zone
iSVZ	inner subventricular zone
oSVZ	outer subventricular zone
IZ	intermediate zone
PP	preplate
CP	cortical plate
MZ	marginal zone
CR	Cajal-Retzius
PN	projection neuron
IN	interneuron
DL	deep-layer
UL	up-layer
GE	ganglionic eminence
LGE	lateral ganglionic eminence
MGE	medial ganglionic eminence
CGE	caudal ganglionic eminence
PV	parvalbumin
SST	somatostatin
hESC	human embryonic stem cells
NPC	neural progenitor cell
NSC	neural stem cells
NEC	neuroepithelial cell
RGC	radial glial cell
aRG	apical radial glia

bRG	basal radial glia
IPC	intermediate progenitor cell
INM	interkinetic nuclear migration
MST	mitotic somal translocation
ECM	extracellular matrix
AP-1	activator protein1
APP	amyloid precursor protein
CRD	cysteine rich domain
AICD	APP intracellular domain
KPI	Kunitz-like protease inhibitor
TMD	transmembrane domain
CTF	c-terminal fragment
ADAM	A Disintegrin And Metalloproteinase domain-containing protein
BACE	β -site APP Cleaving Enzyme
NMJ	neuromuscular junction
CRD	cysteine rich domain
CAM	cell adhesion molecule
ECM	extracellular matrix
APLP1	APP-like protein 1
APLP2	APP-like protein 2
APL-1	APP-like 1
APPL	APP-Like
CAM	cell adhesion molecule
AD	Alzheimer disease
NFTs	neurofibrillary tangles
ACH	amyloid cascade hypothesis
PSH	Presenilin hypothesis
LOAD	late-onset Alzheimer disease
FAD	familial Alzheimer disease
Ach	acetylcholine
CAT	choline acetyltransferase
IEG	immediate early gene

HSP	heat shock protein
EE	early endosome
RE	recycling endosome
LE	late endosome
MVB	multi vesicular body
EV	empty vacuole
MAPK	mitogen-activated protein kinase
JNK	Jun kinase
MLK	mixed lineage kinase
CDK	cyclin-dependent kinase
TGN	trans Golgi network
EGR1	early growth response 1
Lnc RNA	Long non coding RNA
CP	Cycling progenitor
NP	Neurogenic progenitor

Thesis outline-objective

The neocortex is a highly elaborate part of the human brain which underwent significant expansion in primates, and specifically in humans, during evolution. This expansion is thought to be dependent on the capacity of human cortical progenitors to retain progenitor identity for a long period while generating neurons, before ultimately differentiating. In mammals, the cerebral cortex is typically structured into six radial layers of cells with a highly elaborate circuit of neurons, which is conserved among mammalian species and are key aspects of the cerebral cortex.

The Amyloid Precursor Protein (APP) is an evolutionary conserved protein, which is known for its participation in the pathology of Alzheimer disease. It is highly expressed in early embryonic days in human telencephalic neurospheres, along with differentiation, migration and maturation of cortical neurons. However, its function in human developing cortex is unexplored. It has been suggested that APP is essential for normal brain development and possibly adult brain plasticity however, the physiological functions of the full-length protein, especially in humans, have remained elusive. Several studies on brain injury models suggest that APP is a conserved stress response protein, which may have neuroprotective role. Therefore, the absence of APP may mimic cellular stress and affect the developing human cortex.

We hypothesized APP may play a role in human context development. This project outlines a strategy to unveil the physiological functions of human APP during cortical development.

To assess that, we used human induced pluripotent stem cells as a model. We produced APP knock-out iPSC in two different genetic backgrounds and studied cortical neurogenesis in 2D and 3D by focusing on neural progenitor cells.

I will introduce in the following chapter the relevant state-of-the-art knowledge on human cortex development, Amyloid Precursor Protein (APP), Alzheimer disease and stress response, which will follow by results of the project and discussion.



Chapter I-General Introduction

1. Overview of the cerebral cortex

1.1. Cerebral cortex

The human CNS contains on average 86 billion neurons in the brain and spinal cord (in adult males), along with an approximately equal number of glial cells (Azevedo *et al.*, 2009). The cerebral cortex (neocortex) is highly elaborate part of the brain with enormous diversity in cell type, morphology, connectivity and function (Harris, Tomassy and Arlotta, 2015). It is responsible for complex cognitive behavior including sensory perception and skilled motor planning, attention, language, emotion and consciousness (Fuster, 2010). During development, the neural tube is patterned into three major vesicles along the rostro-caudal dimension of the CNS: fore brain (prosencephalon), midbrain (mesencephalon), and hind brain (rhombencephalon) (Silbereis *et al.*, 2016). The prosencephalon develops into the telencephalon (cerebrum) and diencephalon. The telencephalon gives rise to the cerebral cortex, the allocortex, and the striatum, while the diencephalon develops into the thalamus and surrounding nuclei (Harris, Tomassy and Arlotta, 2015). In mammals, the cerebral cortex is typically structured into six radial layers of cells with a highly elaborate circuit of neurons (Mancinelli and Lodato, 2018). Construction of this cortical circuit includes two major steps: First, generation of a diverse cortical neuron from a restricted pool of progenitor cells within the ventricular and subventricular zones (VZ and SVZ). Second, migration of neurons from their place of birth to their final destination radially and furthermore tangentially, resulting in an inside-out lamination of the neocortex (Hanashima and Toma, 2015). These laminar and tangential organizations are conserved among mammalian species and are key aspects of the cerebral cortex (Rakic, 2009).

1.2. Classification of cortical neurons

1.2.1. Projection neurons (PNs)

The neocortex consists of a thin gray matter (GM) surrounding the underlying white matter (WM). The GM encompasses projection neurons and interneurons, supported by glial cells and blood vessels. The WM contains myelinated axons directed to or stemming from overlying GM (Florio and Huttner, 2014). Projection neurons (PNs) are glutamatergic neurons characterized by

a typical pyramidal morphology and extend axons to distant intracortical, subcortical and subcerebral targets. They are generated by cortical progenitor cells located in the dorsolateral wall of the telencephalon and constitute 80% of the cortical neurons. Interneurons (INs) are GABAergic neurons and make local connections and constitutes 20% of cortical neuron. They are produced outside of the cortex from progenitors in the ventral telencephalon and migrate long distances to their final locations in the cortex during development (Wonders and Anderson, 2006; Molyneaux *et al.*, 2007). In another classification, cortical neurons have been divided to deep-layer (DL) and up-layer (UL) neurons which are arranged in columns in the radial dimension of the neocortex (Mountcastle, 1997). Neuronal birthdate is highly correlated with final laminar fate, in which early born neurons occupy the deep layer and late born neuron reside in the upper layer (Hanashima and Toma, 2015; Nomura *et al.*, 2018). Deep-layer neurons, which include layers 5 and 6, consist mainly of corticofugal projection neurons and connect cortex to subcortical and subcerebral targets. Layer 6 neurons express the transcription factors TBR1, TLE4 and FOXP2 and project to different nuclei in thalamus while layer 5 neurons express Fezf2 and CTIP2 and project to midbrain, hindbrain and spinal cord (Molnár and Cheung, 2006; McKenna *et al.*, 2011). The Upper-layer consist of corticocortical neurons which project their axons to the ipsilateral and contralateral cortex, thereby establishing intracortical and inter-hemispheric connections (Fame, MacDonald and Macklis, 2011; Greig *et al.*, 2013). They are located mostly in layer 2 and 3 and express the transcription factors Cux1/2, Brn1/2, Satb2. Layer 4 neurons receive input from other brain regions notably the thalamus and act as a gateway for processing information from peripheral sensory organs (Figure 1) (Hanashima and Toma, 2015; Harris, Tomassy and Arlotta, 2015).

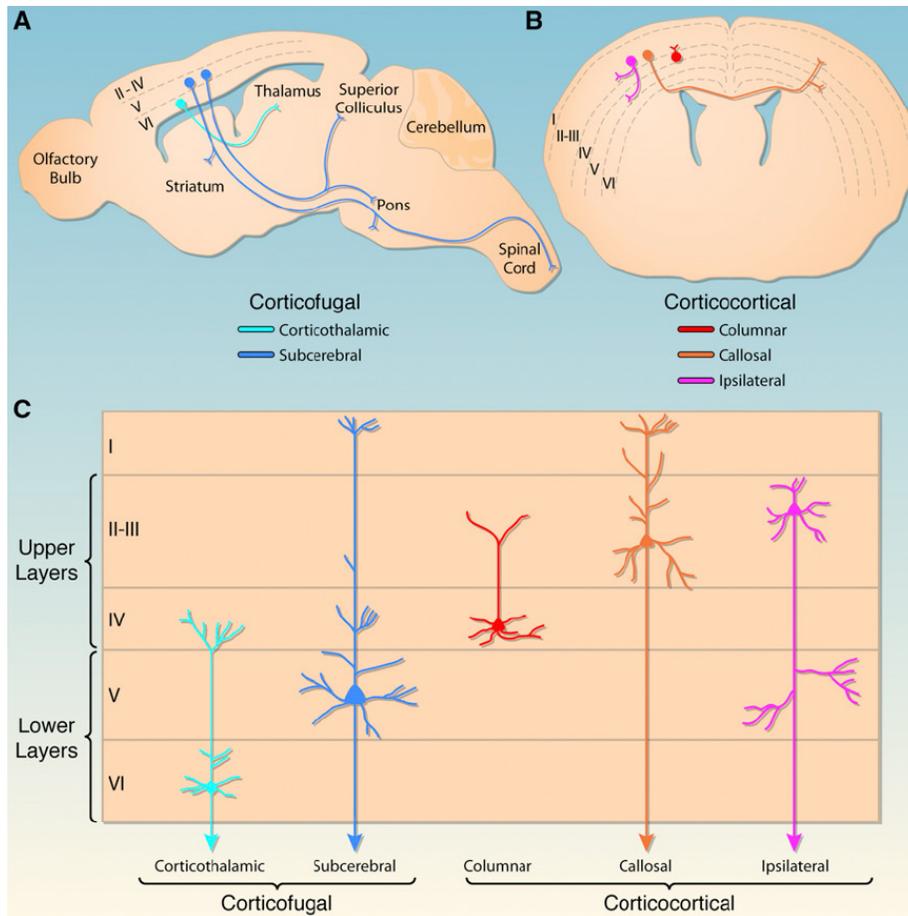


Figure 1. Subtypes of projection neurons in cerebral cortex.

A) Sagittal view of corticofugal projection neurons show that corticothalamic neurons are found mostly in layer VI and project to the thalamus. Subcerebral projection neurons are located in layer V and send primary projections to the spinal cord, pons and superior colliculus. B) Coronal view of corticocortical projection neurons show that ipsilateral and callosal projection neurons are located in layers II-III and project within the same hemisphere or to the contralateral hemisphere. Columnar projection neurons are found in layer IV and send short axons locally within a neocortical column. C) Detail of the relationships between laminar positions and projection patterns of projection neuron subtypes represented in (A) and (B).

Picture adapted from (Franco and Müller, 2013)

1.2.2. Interneurons (INs)

While PNs extend long-range axons to distant regions of the cortex and the central nervous system (CNS), interneurons which are GABAergic, usually modulate the activity of PNs within the local microcircuitry (Kepecs and Fishell, 2014). Interneurons are classified to three universally accepted groups by expression of either parvalbumin (PV), somatostatin (SST), or the ionotropic serotonin receptor 5HT3a (5HT3aR) (Rudy *et al.*, 2011). SST+ and PV+ interneurons are more abundant in the deep layers, while 5HT3aR+ interneurons are found mainly in the upper layers (Lee *et al.*, 2010; Lodato *et al.*, 2011). Within these three broad classes, PV+ interneurons represent about 40% of cortical interneurons, SST+ cells make up about 30% of cortical interneurons and 5HT3aR+ interneurons comprise the remaining 30% of cortical interneurons (Kelsom and Lu, 2013). Unlike PNs, which originate from dorsal telencephalon, Interneurons are derived from two regions in ventral telencephalon named the ganglionic eminence (GE). GE is divided into medial (MGE), lateral (LGE) and caudal regions (CGE). PV+ and SST+ interneurons arise primarily from Nkx2.1+ progenitors in the medial ganglionic eminence (MGE). In contrast, 5HT3aR+ interneurons derived from progenitors expressing COUP-TFII in the caudal ganglionic eminence (CGE) (Xu *et al.*, 2004; Corbin and Butt, 2011).

1.3. Cortical development in human

The human brain has expanded over fifteen-fold since our divergence from old world monkeys and three-fold since our divergence from a common ancestor with chimpanzee (Herculano-Houzel, 2012). Neurogenesis in the human CNS begins shortly after the fusion of neural folds. The first neurons to appear are motor neurons in the spinal cord and brainstem at 4 pcw (Silbereis *et al.*, 2016). Human cortical neurogenesis begins at around 5 gestational weeks and is mostly completed by 28 weeks (Rabinowicz *et al.*, 1996; Meyer *et al.*, 2000). In early development, radial glia in the dorsal forebrain produce glutamatergic neurons which migrate toward cortical plate using radial glial process and their journey is coordinated by a complex network of signaling pathways and molecular factors (Rakic, 1988; Marín and Rubenstein, 2003). Cajal-Retzius (CR) and subplate (SP) cells are the first neurons produced in the cerebral cortex, and their neurogenesis occurs prior to the generation of excitatory neurons (Angevine and Sidman, 1961;

Price *et al.*, 1997; Hevner *et al.*, 2003). However, a second wave of Cajal-Retzius cells may appear around mid-gestation in humans (Meyer and González-Gómez, 2018). Both of these waves are generated not in the cortex but in other regions of the brain and migrate tangentially to settle in the cortex (Griveau *et al.*, 2010; Pedraza *et al.*, 2014). CR and SP cells form a transient structure called the preplate (PP) above the VZ, which is divided to cortical plate (CP) and marginal zone (MZ) later (Hanashima and Toma, 2015). Cajal-Retzius cells and subplate neurons mostly vanish during early development in mouse (Chowdhury *et al.*, 2010; Hoerder-Suabedissen and Molnár, 2013). In human, Cajal-Retzius cells persist in the marginal zone (MZ) and subplate neurons give rise to the interstitial white matter neurons (Marín-Padilla, 2015). Excitatory neurons are sequentially generated and migrate to occupy cortical plate (CP) resulting in lamination of the neocortex (Kumamoto and Hanashima, 2014). Synapse formation begins between weeks 9 and 10 of human fetal development and continues after birth (de Graaf-Peters and Hadders-Algra, 2006). Similar to other mammals, neurons undergo a phase of over connectivity and reduction in neuronal connectivity due to synaptic pruning (Kirwan *et al.*, 2015).

1.4. Cortical development in human versus mouse

There are several important differences between the human and the mouse cortex (Figure 2). First, the human brain has undergone the largest expansion in volume during mammalian evolution whereby the cerebral cortex makes up over three-quarters of brain size (Mountcastle, 1998). Second, whereas the surface of the mouse neocortex is smooth (lissencephalic), the surface of the human neocortex is highly convoluted (gyrencephalic). Third, there are 16 billion neurons in human versus 14 million neurons in mice. Finally, the timing of cortical neurogenesis is much more protracted in human characterized by an extended period of initial amplification of neuroepithelial cells (NECs) (6-8 days in mice versus 30 days in human) followed by a much prolonged period of neurogenesis (6-7 days in mice 3-4 months in human) (Florio and Huttner, 2014; Otani *et al.*, 2016). Although mouse models have enabled pioneering studies of cortex development, their ancestors diverged from human ones almost 90 million years ago. Therefore, some features of human cortex development may be difficult to model in mouse (Mostajo-Radji *et al.*, 2020). At the genomic level, it is difficult to reconstruct many human-specific genetic

changes in mouse because these changes often occur in regions with poor homology. Likewise, at the cellular level, some cell types that are important for human brain development, such as basal radial glia (bRG), are rare or absent in the mouse brain (Hansen *et al.*, 2010; Wang *et al.*, 2011). Finally, at the tissue level, the human brain is over 1000 times larger than the mouse brain and needs long range connections which are absent in mouse brain (Mostajo-Radji *et al.*, 2020).

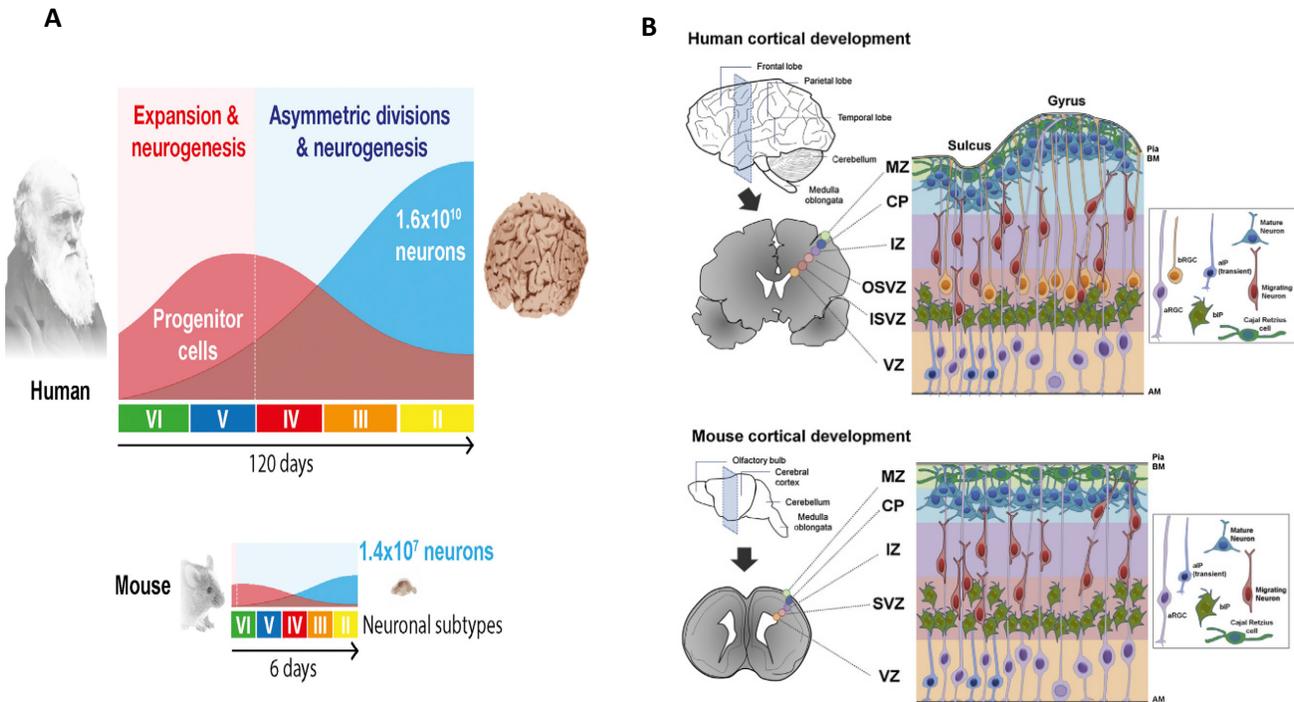


Figure 2. Cortical neurogenesis in human versus mouse.

A) Human brain is different from mouse brain at four levels: 1) Size (1000 fold larger than mouse brain); 2) Number of neurons (16 billion versus 14 million in mice); 3) Pace of development (120 days versus 6 days in mouse); 4) the surface of the human neocortex is highly convoluted, while the surface of the mouse neocortex is smooth. B) Coronal view of human developing cortex versus mouse which different color circles represent different developing cortical region. The basal radial glial cells (bRGCs) are located in the outer subventricular zone (OSVZ) and mainly present in gyrencephalic species.

Pictures Adapted from (Otani *et al.*, 2016; Romero, Bahi-Buisson and Francis, 2018)

1.5. Neural progenitor cells in the cortex

During early development, neuroepithelial cells (NECs) are the first progenitors that are located in the dorsolateral wall of the rostral neural tube and undergo dramatic expansion. As neurogenesis proceeds, neural progenitor cells in the dorsal telencephalon populate two regions: the ventricular zone (VZ), which is the layer immediately adjacent to the ventricle, and an additional proliferative layer known as the subventricular zone (SVZ) above the VZ. There are at least two basic types of neurogenic progenitors within these two zones: radial glial cells (RGCs) and intermediate progenitor cells (IPCs). These progenitors give rise to projection neurons of the different neocortical layers in a tightly controlled temporal order (Rakic, 2003; Götz and Huttner, 2005; Molyneaux *et al.*, 2007). The intermediate zone (IZ), located above the SVZ and radially migrating glutamatergic neurons and tangentially migrating interneurons (IN) passing through it to reach to cortical plate (Cárdenas *et al.*, 2018).

1.5.1. Neuroepithelial cells

Neuroepithelial cells (NECs) are neural stem cells derived from the anterior neuroectoderm during cortical development. They undergo symmetric expansive divisions before neural tube closure to expand the pool of progenitors that give rise to the forebrain (Franco and Müller, 2013). NECs are characterized by four basic features in mammals: First, they are highly polarized cells exhibiting apico-basal polarity. Second, NECs only undergo symmetric self-amplificative divisions to generate two daughter NECs, hence massively increasing their number. Third, they are attached to each other by adherens and tight junctions at the level of the apical surface, where they contact with cerebrospinal fluid, which provides a proliferative niche for neural progenitor cells. Finally, NECs undergo interkinetic nuclear migration (INM), i.e. their nucleus moves between the apical and the basal areas in coordination with the cell cycle. During G1, their nuclei move basally and they exit S-phase at the basal surface. Then the nuclei undergo basal-to-apical migration and mitosis will occur at the apical (ventricular) surface. It is important to mention that adult neural stem cells originate from a proportion of NECs (Lee and Norden, 2013; Furutachi *et al.*, 2015; Arai and Taverna, 2017). Since NECs pool size defines the numbers of their derived neurogenic progenitor cells and the final number of cortical neurons, NECs have a

fundamental impact on the size and folding of the mature cerebral cortex (Fernández, Llinares-Benadero and Borrell, 2016).

1.5.2. Radial Glial Cells

1.5.2.1. apical Radial Glia

After neural tube closure at gestational week (GW4), neuroepithelial cells (NECs) transform into apical radial glia (aRG), a process that occurs at E9-10 and GW5-6 in mouse and human, respectively. At these stages, the ventricular zone (VZ) is the only proliferative region (Zecevic, Chen and Filipovic, 2005). aRG -also known as ventricular radial glia (vRG) or inner radial glia (iRG)- are primary progenitors and share some features but not all with the NECs. Like NECs, I) they are highly polarized with an apical-basal orientation; II) aRG undergo interkinetic nuclear migration (INM) too and divide at the apical surface of the developing cortex. Unlike NECs, I) they start losing tight junctions and begin expressing glial markers such as brain lipid-binding protein (BLBP), astrocyte-specific glutamate transporter (GLAST), Vimentin (VIM), and PAX6. II) Although they undergo self-amplifying divisions, they gradually start dividing asymmetrically to generate one aRG plus a different cell. This different cell can be either a neuron or secondary progenitor which moves basally to form the subventricular zone (Nowakowski and Pollen, 2016; Romero, Bahi-Buisson and Francis, 2018). Most neurons of the brain are derived, either directly or indirectly from RGCs. RGCs have long processes that extend from the ventricular wall to the basal surface which prepare the scaffold for migration of newly born neurons through the intermediate zone to the cortical plate (Rakic, 2003). This process also allows attachment to the ventricular cavity containing key elements of signaling pathways necessary for proliferation, including fibroblast growth factor (FGF), insulin-like growth factor (IGF), retinoic acid (RA), sonic hedgehog (Shh), transforming growth-factor beta/bone morphogenetic proteins (TGF β /BMPs) and Wnts (Lehtinen *et al.*, 2011; Taverna, Götz and Huttner, 2014). The transition from NECs to RGCs is directed by several signaling molecules such as Fgf10 and retinoic acid (RA). Genetic deletion of Fgf10 cause delayed onset of RG markers, GLAST and BLBP which results in the tangential expansion of prefrontal areas in the Fgf10 mutants (Kang *et al.*, 2009; Sahara and O'Leary, 2009). Similarly, retinoic acid (RA) expressed in the meninges is necessary for the transition from

symmetric to asymmetric divisions. Lack of meninges-derived RA signaling due to *Foxc1* mutation, results in a notable decrease in the number of neurons and hence symmetric cell divisions and prolonged NEC stage (Siegenthaler *et al.*, 2009).

1.5.2.2. basal Radial Glial Cells

The human neocortex comprises a second population of radial glia cells that emerge around GW14 called basal radial glia (bRG). They were first described in the developing cortex of gyrencephalic species and are observed rarely or at much lower abundance in mouse (Nowakowski and Pollen, 2016). Comparative studies in mouse, ferret, macaque, and human brain show that the expansion of bRG cells associates with brain size. In mammals with a large brain, the subventricular zone (SVZ) is subdivided into inner and outer zones where aRG remain in inner SVZ (iSVZ) and bRG mostly concentrated in outer SVZ (oSVZ). While the oSVZ is negligible in rodents, its volume in macaque and human is much more expanded and is linked to evolutionary expansion of the human cortex, as well as formation of folds and fissures. At the cellular level, the bRG -also known as outer radial glia (oRG)- are distinguished from aRG by position, morphology, and cell behavior during cell division (Figure 3). In contrast to aRG, bRG cells possess unipolar morphology, lack apical junctions, reside in different niches, and undergo mitotic somal translocation (MST), in which the soma rapidly translocate towards the cortical plate immediately prior to cytokinesis. At the molecular level, oRG cells are transcriptionally enriched by genes such as *TNC*, *PTPRZ1*, *FAM107A*, *HOPX*, and *LIFR* that are involved in extracellular matrix formation, migration, and stemness. Similar to aRG, they are mitotic cells that express several RGC-specific markers and undergo asymmetric divisions to self-renew and produce IPCs, neurons, and astrocytes (Smart *et al.*, 2002; Hansen *et al.*, 2010; Franco and Müller, 2013; Pollen *et al.*, 2015).

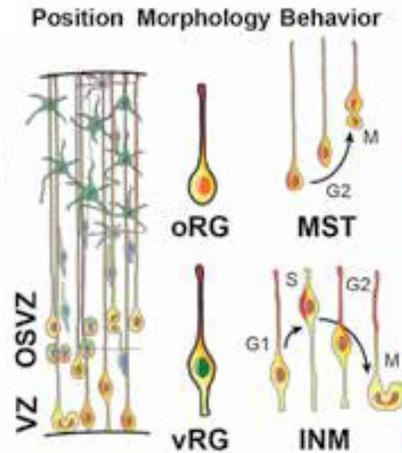


Figure 3. vRG and oRG differences in term of morphology, position and behavior.

vRG cells are bipolar, reside in VZ and undergo interkinetic nuclear migration (INM), i.e. their nucleus is moving between the apical and the basal arises during cell cycle. In contrast to vRG, oRG cells has unipolar morphology, reside in SVZ, and undergo mitotic somal translocation (MST), in which the soma rapidly translocate towards the cortical plate immediately prior to cytokinesis. Picture adapted from (Nowakowski and Pollen, 2016)

1.5.2.3. Intermediate Progenitor Cells

Intermediate progenitor cells (IPCs) are non-polar neuron-producing progenitors found in the SVZ and in the basal VZ before the formation of the SVZ. The majority of cortical projection neurons in mice derive from IPCs and only 10% them come directly from RGCs. IPCs are distinct from NECs and RGCs in several ways. First, they are non-polar progenitors, which lose contact with the ventricular surface and migrate to more basal positions creating a distinct proliferative region, the SVZ. Second, they express the IPC-specific transcription factor *Tbr2* and they lose expression of RGC-specific transcription factor *Pax6*. Third, IPCs function as neuron-restricted transient amplifying cells but can undergo a limited number of symmetric divisions to generate more IPCs before making neurons (Molyneaux *et al.*, 2007; Arai and Taverna, 2017).

1.6. Neural progenitor cells and the canonical WNT signaling pathway

The canonical WNT pathway contains different proteins; In the absence of WNT ligand, a destruction complex containing AXIN, adenomatous polyposis coli (APC), and Glycogen Synthase Kinase 3 (GSK-3) phosphorylates β -catenin, leading to its ubiquitylation and subsequent proteasomal degradation. In the presence of WNT ligand, WNT binds to Frizzled and LRP co-receptors, Axin is recruited to LRP and GSK-3 is blocked by DVL. So the destruction complex dissociates, followed by inhibition of β -catenin phosphorylation; unphosphorylated β -catenin accumulates, translocates to the nucleus and induces Wnt target gene expression (Figure 4) (Bengoa-Vergniory and Kypta, 2015; Bhavanasi and Klein, 2016).

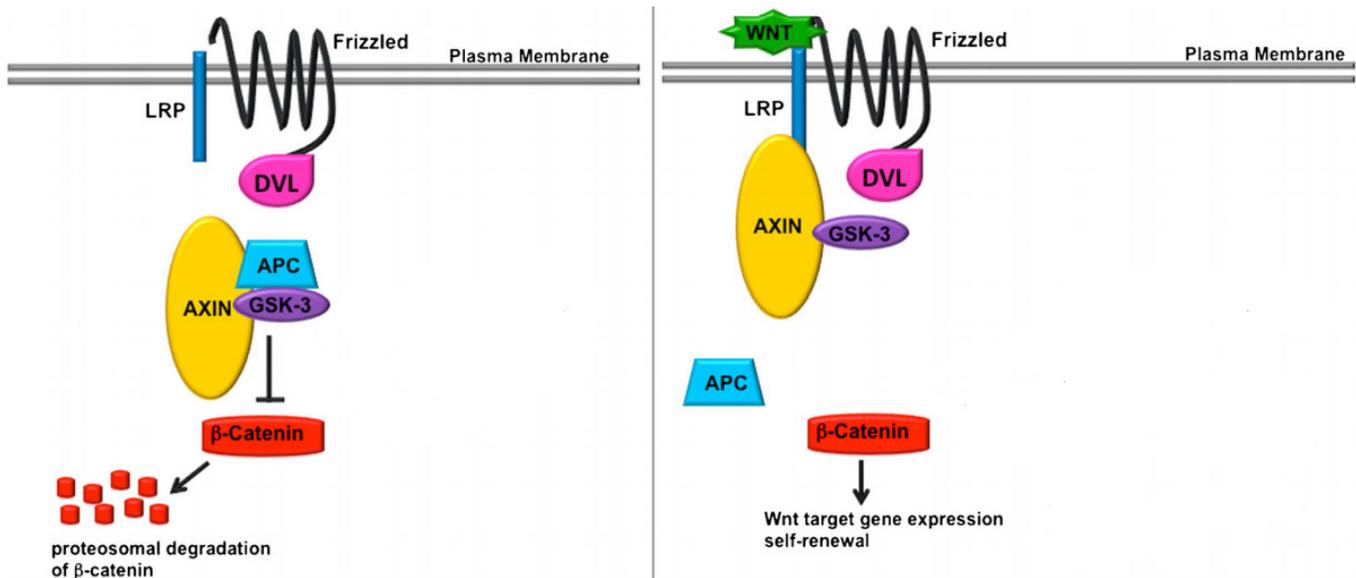


Figure 4. Canonical WNT pathway.

A) In the absence of Wnt, a destruction complex consisting of Axin, APC, GSK-3 catalyzes sequential phosphorylation of β -catenin, leading to its ubiquitylation and subsequent proteasomal degradation. B) Upon Wnt binding to Frizzled and LRP co-receptors, Axin is recruited to LRP and APC dissociates from the complex. GSK-3 phosphorylation of β -catenin is inhibited; unphosphorylated β -catenin translocates to the nucleus, leading to Wnt target gene expression. Picture adapted from (Bhavanasi and Klein, 2016)

In rodents, β -catenin has been established as a central regulator of RGC proliferation versus differentiation by affecting transcriptional activity or as a component of adherent junctions. Transcriptionally, it is downstream of canonical WNT signaling pathway which is activated by WNT ligands derived from cerebrospinal fluid (CSF) (Machon *et al.*, 2003; Junghans *et al.*, 2005; Zhou *et al.*, 2006). Constitutive activation of Wnt/ β -catenin signaling cause excessive proliferation in RGCs, resulting in expansion of the RGC pool at the expense of neurogenesis (Chenn and Walsh, 2002). In contrast, blocking β -catenin forces premature differentiation of RGCs into neurons (Woodhead *et al.*, 2006). Consistent with this idea, WNT- β -catenin signaling is decreased in IPCs compared to RGCs and deletion of *CTNNB1* (gene encoding β -catenin) in RGCs causes increased production of IPCs (Mutch *et al.*, 2010). β -catenin is also a component of cadherin-based adherent junctions between RGCs which maintaining RGCs contact with the CSF in apical surface and serving as docking sites for Par proteins (Manabe *et al.*, 2002).

2. Overview of the Amyloid Precursor Protein (APP)

2.1. APP family and evolution

Human APP is a member of the APP family of proteins with three members: APP, APP-like protein 1 (APLP1) and APP-like protein 2 (APLP2) (van der Kant and Goldstein, 2015). This family is evolutionarily conserved and can be found in vertebrates and invertebrates like *C.elegans* (APP-like 1, APL-1) and *D. melanogaster* (APP-like, APPL). APLP1 is the closest human homolog to the invertebrate APP-like proteins (APL-1 and APPL) due to sequence homology (Joshi *et al.*, 2009). Prokaryotes, plants, and yeasts do not possess members of the APP family of proteins (van der Kant and Goldstein, 2015). The complicated trajectory of the evolution of the APP protein shows highly selective evolutionary forces on this family. They appeared in fly (*Drosophila melanogaster*) and roundworms (*Caenorhabditis elegans*) 800 million years, underwent duplication and contraction during evolution and appeared 100 million years ago in mammals (Shariati and De Strooper, 2013) (Figure 5). APP family genes are outcomes of gene duplication whereby duplicated genes can acquire three roles: non-functionalization, neo (sub) functionalization or increased gene dosage (Innan and Kondrashov, 2010). The non-functionalization is the outcome of the accumulation of lethal mutations which lead to pseudogenization (J. and Ohno, 1971). In neo-functionalization, duplicate gene acquire new functions distinct from the ancestral ones (Force *et al.*, 1999). Sub-functionalization is a version of neo-functionalization in which the function of the ancestral protein is subdivided into two new proteins with more specialized functions. Finally, duplication can provide redundancy by producing genes without any functional innovation but increasing the gene dosage for dosage sensitive genes (Conrad and Antonarakis, 2007). In mammals, while triple knock out for APP family member (APP/APLP1/APLP2) is lethal, knock out mice for any one is viable and fertile with minor defects. Double knockouts for APP/APLP2 or APLP1/APLP2 die early after birth while double knockout for APP/APLP1 is viable (Heber *et al.*, 2000; van der Kant and Goldstein, 2015). These findings could indicate that other APP family members may compensate for APP loss (Von Koch *et al.*, 1997; Müller and Zheng, 2012). However, it is formally possible that APP paralogues have unrelated non-redundant functions (Shariati and De Strooper, 2013). Because I) APLP1 and APLP2 levels are not upregulated in APP knockout mice (Zheng *et al.*, 1995); II) APP, APLP1, APLP2

have different tissue distributions; III) their interactome overlap only modestly (Bai *et al.*, 2008). So, the complex evolutionary tree of APP proteins cannot be simplified into the “redundancy” or “divergence” model. On the one hand, some functions of the family like neuron specific functions at the synapse are clearly conserved and on the other hand, innovation happened in this gene family causing divergence of their expression and interaction networks. Therefore, more studies are needed to understand the specific functions of each member which are not complemented by the other APP members (Shariati and De Strooper, 2013).

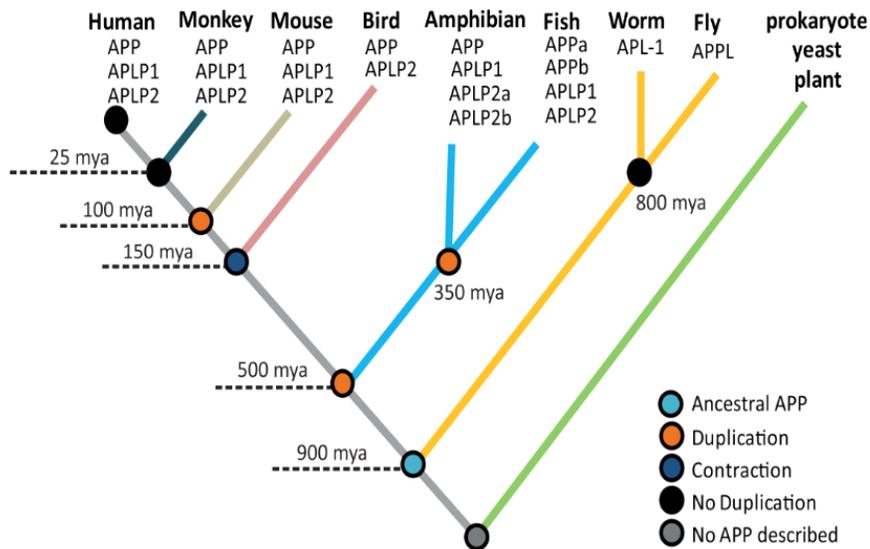


Figure 5. Phylogenetic tree shows important events in the evolution of APP gene family.

APP does not exist in prokaryote, yeast and plant. They appeared in Fly 90 million years ago and after some duplication and contraction through evolution, they appeared in mammals 100 million years ago. The duplication and contraction nodes are color-coded. Mya: million years ago. Picture adapted from (Shariati and De Strooper, 2013)

2.2. APP gene structure

Amyloid precursor protein (APP) has been studied intensively in the last 30 years since its discovery in 1987 (Coronel, Palmer, *et al.*, 2019). The mammalian APP gene is located on chromosome 21 and contains 18 exons, which undergoes alternative-splicing events and produce three main isoforms: APP770, APP751, and APP695. APP splice variants 770 and 751 are expressed in many tissues such as the thymus, heart, muscle, lung, kidney, adipose tissue, liver, spleen, skin, and intestine (Puig and Combs, 2013) while APP695 is expressed predominantly in the brain (Tanaka *et al.*, 1988). There are also some other variants like APP714 and APP639 expressed in fetal tissue and adult liver (Golde *et al.*, 1990; Tang *et al.*, 2003) and the leukocyte-derived APP (L-APP) splice variants (APP677, APP696, APP733, APP752). L-APP express highly in leukocytes and can be upregulated in microglia and activated astrocytes (BEYREUTHER *et al.*, 1993).

2.3. APP protein structure

APP is a type I transmembrane protein with a large extracellular domain and a short cytoplasmic domain. Extracellular domain contain dimerization domains (E1 and E2) while the intracellular domain is a short motif called APP intracellular domain (AICD). E1 and E2 are linked to each other by an acidic domain (AcD) which is rich in glutamic and aspartic acid (Figure 6). There are two domains which are not present in all the APP isoforms; while APP751 and APP770 contain a Kunitz-like protease inhibitor domain (KPI domain; encoded by exon 7) and a short OX-2 homology domain (encoded by exon 8), APP 695, the first cloned APP splice variant and the major brain isoform, excludes exons 7 and 8. Importantly, the A β sequence which is found only in APP and not in the APLPs, is located partially in juxtamembrane and partially in transmembrane domain (TMD) (Müller, Deller and Korte, 2017).

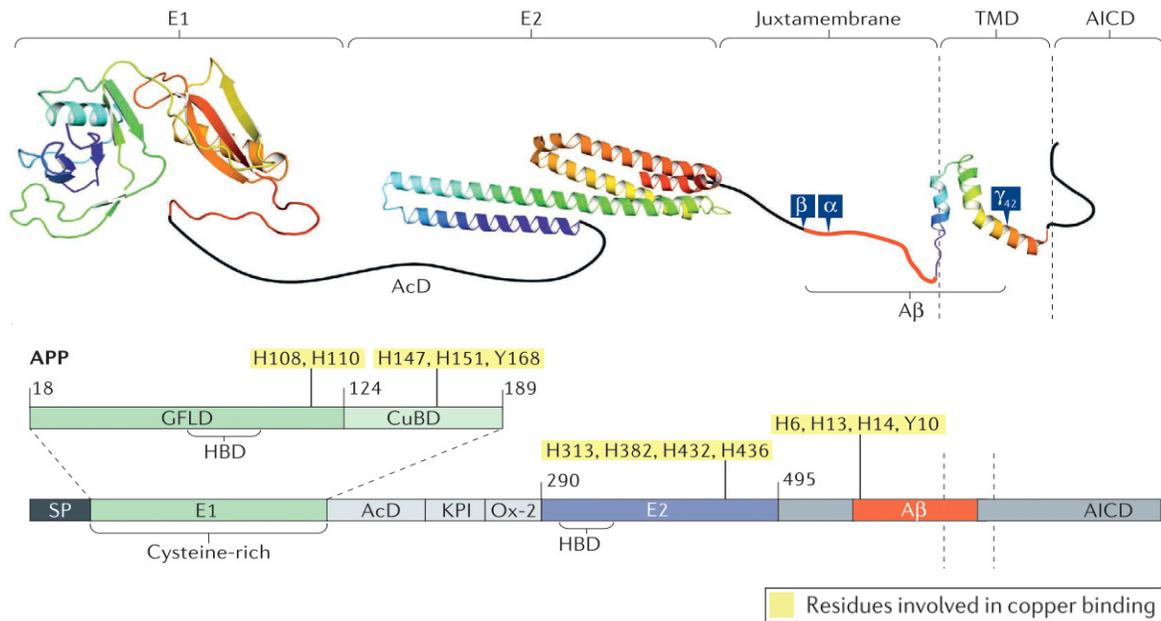


Figure 6. APP protein structure.
 All APP family proteins share conserved extracellular E1 and E2 domains, Acidic domain (AcD), and APP intracellular domain (AICD). KPI and OX-2 are not present in all the APP isoforms. Amyloid- β ($A\beta$) domain which is known in relation to Alzheimer's disease (AD) is unique to APP.
 Picture adapted from (Müller, Deller and Korte, 2017)

2.4. APP processing

APP is synthesized in the endoplasmic reticulum and transported to the cell membrane through the secretory pathway. Post-translational modifications such as phosphorylation, glycosylation, and sulfations occur in this pathway which give rise to the mature form of the protein (Adela Bernabeu-Zornoza *et al.*, 2019). After reaching to cell surface, APP can be processed by canonical and non-canonical processing yielding fragments that may each have specific and even opposing functions (Müller, Deller and Korte, 2017).

2.4.1. Canonical APP processing

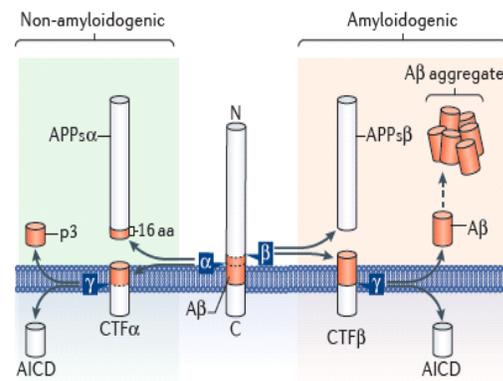
Canonical processing is classified into the amyloidogenic and non-amyloidogenic pathway depending on secretases' cleavage (Figure 7A). In the non-amyloidogenic pathway, which is physiologically predominant and neuroprotective, APP is cleaved by α -secretase within $A\beta$

sequence to produce sAPP α fragment and α -CTF (c-terminal fragment). This is followed by γ -secretase cleavage of α -CTF, resulting in release of the P3 peptide and AICD. The amyloidogenic pathway is initiated by α -secretase cleavage at the amino terminus of A β in APP resulting in sAPP β and β -CTF, followed by β -secretase cleavage which liberates A β and AICD (Nicolas and Hassan, 2014; Müller, Deller and Korte, 2017). Cleavage of β -CTF by γ -secretase generates several A β peptides: A β 37, A β 38, A β 39, A β 40, A β 42, of which 90% are A β 40 in healthy brain (Portelius *et al.*, 2012). In the Alzheimer's disease (AD) brain, A β peptides are prone to assemble and form oligomers, protofibrils, and fibrils, which are insoluble, stable and resistant to proteolytic cleavage. A β 42 has higher tendency to aggregate due to increased hydrophobicity in C terminus and the ratio of A β 42/ A β 40 is increased in Alzheimer patients (Coronel *et al.*, 2018; Long and Holtzman, 2019). ADAM10 (A Disintegrin And Metalloproteinase domain-containing protein 10) and BACE1 (β -site APP Cleaving Enzyme) are the major physiological α and β secretase in the brain (Vassar *et al.*, 2009; Saftig and Lichtenthaler, 2015).

2.4.2. Non-Canonical APP processing

In addition to canonical pathways, APP can be processed through several non-canonical pathways. APP can be processed by δ , β and γ -secretase (δ -pathway), meprin- β and γ -secretase (meprin pathway) and η , α and β -secretase (η -pathway) yielding to other N-terminal APP fragments (Figure 7B). The APP fragments from these pathways were noticeably reduced after genetic knockout of the enzymes, showing that these pathways are physiological and not degradation artefacts. Some of these APP fragments have been involved in AD pathogenesis but very little is known about their physiological relevance, except A η - α which is known to attenuate neuronal activity. The other non-canonical pathway is caspase cleavage which APP C terminus is cleaved by caspase and γ -secretase, sequentially to yield C31, which has been implicated in neuronal apoptosis and small peptide Jcasp, which may inhibit presynaptic transmitter release (Nhan, Chiang and Koo, 2015; Andrew *et al.*, 2016; Müller, Deller and Korte, 2017).

A Canonical processing



Secretases of canonical processing

- α -secretase: mainly ADAM10
- β -secretase: BACE1 and BACE2
- γ -secretase complex:
 - PS1 and PS2 (catalytic core)
 - NCT
 - PEN2
 - APH1 and APH2

B Non-canonical processing

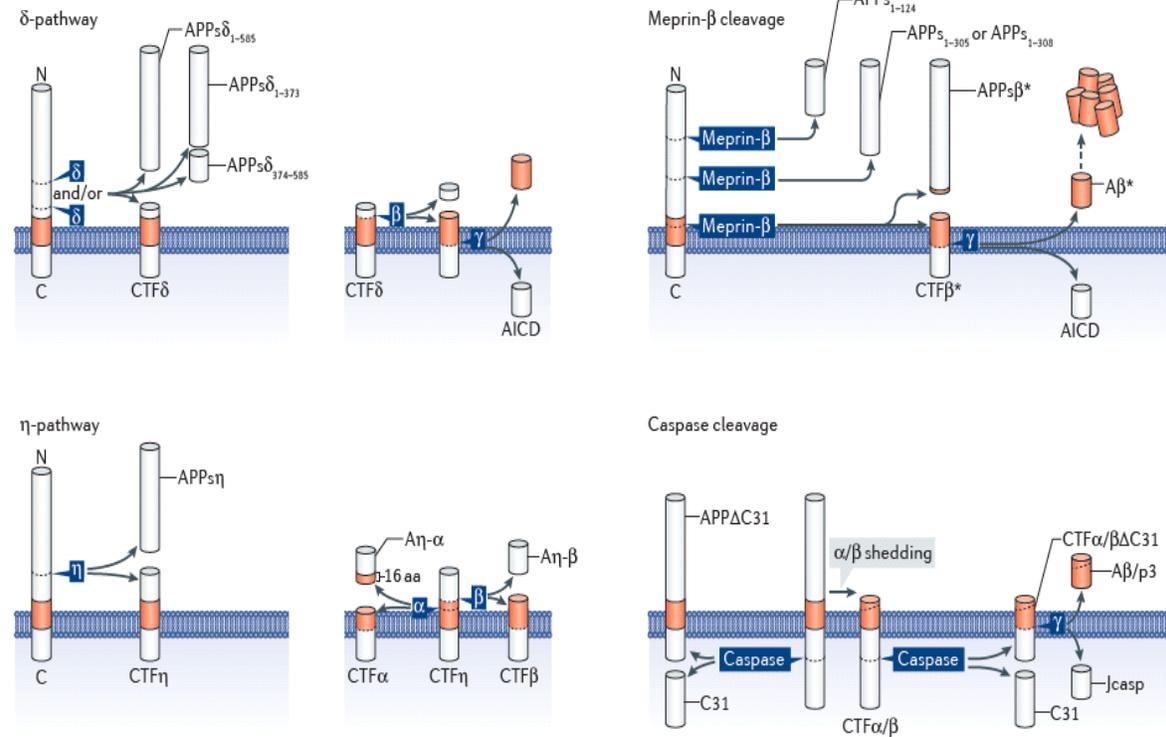


Figure 7. APP canonical and non-canonical processing.

A) Canonical processing include non-amyloidogenic pathway which APP cleave by α and γ -secretase and amyloidogenic pathway which APP cleaved by β and γ -secretase. B) Non-canonical APP processing contain δ -pathway, meprin- β cleavage, η -pathway and caspase cleavage.

Picture adapted from (Müller, Deller and Korte, 2017)

2.5. APP expression in different model organisms

APP expression can be detected very early in embryogenesis at E9 in rat and E9.5 in mouse (Sarasa *et al.*, 2000; Ott and Bullock, 2001). In the adult rodent brain, APP is also abundantly expressed in neural progenitors found in the VZ, SVZ (Young-Pearse *et al.*, 2010) suggesting an important role for APP in cortex development. Important clues for the role of APP in development comes from organisms that have only a single APP family member. In *C. elegans*, APL-1 knockout is lethal due to a molting defect (Hornsten *et al.*, 2007; Wiese, Antebi and Zheng, 2010). APPL knockout *Drosophila* is viable but have minor defects including conditional learning defects (Luo, Tully and White, 1992), defects in maintenance of synaptic boutons at the neuromuscular junction (NMJ) (Torroja *et al.*, 1999), more vulnerability to brain injury (Leyssen *et al.*, 2005) and defect in the endo-lysosomal pathway (Kessissoglou *et al.*, 2020). APP knockout mice have reduced body weight, reduced locomotor activity, disturbed forelimb strength and gliosis. These mice also have altered long term potentiation responses (Zheng *et al.*, 1995; G.R. *et al.*, 1999; Seabrook *et al.*, 1999), reduced brain weigh and axonal growth/white matter defects (Magara *et al.*, 1999) and axonal transport defects (Kamal *et al.*, 2001; Smith *et al.*, 2007, 2010; Goldstein, 2012).

2.6. APP functions

APP is a multimodal protein that regulates various biological processes including transcriptional regulation, intracellular transport, cell signaling processes and synaptic functions. It has been suggested that APP interacts with more than 200 extra cellular and intracellular binding partners (Müller, Deller and Korte, 2017). APP is suggested to be a cell surface receptor due to significant features resembling those of cell surface receptors such as a large extracellular domain and cysteine rich domain (CRD) which is important for protein interaction (Kang *et al.*, 1987). It is also suggested to be a cell adhesion molecule (CAM) because it shows important CAM features in both the extracellular and intracellular domains (Sosa *et al.*, 2017). The extracellular domain is capable of cis or trans dimerization and binding to multiple proteins of the extracellular matrix (ECM) such as collagen, heparin, heparan sulfate proteoglycans and Laminin (Soba *et al.*, 2005; Deyts, Thinakaran and Parent, 2016). It also binds to Reelin, an extracellular protein involved in

neural migration during cortical development (Hoe *et al.*, 2009) and F-Spondin, a protein which participates in cell-cell interaction and axonal outgrowth (Ho and Südhof, 2004). Similar to other adhesion proteins such as cadherins, integrin b1 and L1, the APP cytoplasmic domain interacts with cytoskeleton-associated scaffold proteins (Sosa *et al.*, 2017). Examples of important intracellular interactors are brichos domain-containing 2 (BRI2) and BRI3, which inhibit APP processing by α -secretase, β -secretase and γ -secretase (Matsuda *et al.*, 2005; Matsuda, Matsuda and D'Adamio, 2009) and adaptor proteins such as Fe65 and X11 (MINT) (Tamayev, Zhou and D'Adamio, 2009). There is evidence indicating the involvement of APP in neural development both in vitro (Bolós *et al.*, 2014) and in vivo (Wang *et al.*, 2014). It has been suggested that APP promotes neuronal differentiation (Demars *et al.*, 2011), neurite outgrowth and maturation (Milward *et al.*, 1992; Soldano *et al.*, 2013). Deyts et al showed that overexpression of AICD domain of APP induces neurite outgrowth in vitro through a G α s protein–adenylyl cyclase–cAMP cascade (Deyts *et al.*, 2012). APP has also been described as a regulator of cell surface N-methyl-D-aspartate (NMDA) receptor homeostasis due to co-association of APP with the two major NMDA receptors expressed in adult brain (Cousins *et al.*, 2009; Cousins, Innocent and Stephenson, 2013). APP also has effects on neuroplasticity (Marik *et al.*, 2016), neuronal migration and synaptogenesis (Chen and Dou, 2012; Tyan *et al.*, 2012). It is suggested that APP functions as a trophic factor during the proliferation and differentiation of stem cells (Small *et al.*, 2014; Coronel, Palmer, *et al.*, 2019). APP is upregulated in the adult brain in response to injury and ischemia which suggest it play a role in neural regeneration (Koistinaho and Koistinaho, 2005).

2.7. APP and cortical development

APP is highly expressed in human developing cortex in progenitors, differentiating and migrating neurons (Polioudakis *et al.*, 2019) (Figure 8). In vitro models of human cortical neurons showed that APP mRNA and protein expression remained stable during cortical neuron differentiation over 100 days, whereas APP processing changed. As an example, sAPP α was secreted early during differentiation from neuronal progenitors, while sAPP β was first secreted after emergence of early born neuron. Short A β peptides such as A β 1-15/16 were highly produced at the progenitor

stage, while longer peptides including A β 1-40/42 peaked when post-mitotic neurons appeared. This indicates that APP processing is regulated throughout differentiation of cortical neurons and that amyloidogenic processing of APP is more associated with mature cortical neurons (Bergström *et al.*, 2016). In vivo model of rodent brain showed that full-length APP is required for proper migration of cortical neurons into the cortical plate during the development of the mammalian brain. While knockdown of APP inhibited cortical plate entry, overexpression of APP caused accelerated migration of cortical neurons. Triple knock-out of APP family protein causes neurons to migrate past the cortical plate boundary and present focal dysplasia regions placed within the marginal zone (Figure 9). Full length APP can fully rescue migration defects, revealing that both the extracellular and intracellular domains of APP are required for efficient rescue (Young-Pearse *et al.*, 2007; Smith *et al.*, 2010). Young-perase et al, showed that APP induces cytoskeleton remodeling and cell migration by activating Disabled-1 (Dab1), a well-established adaptor protein in cortical cell migration followed by activation of another downstream protein disrupted-in-schizophrenia 1 (DISC1). They indicated that the intracellular domain of APP interacts with the N-terminal domain of DISC1 and regulate translocation of DISC1 to the centrosome where it plays an important role in neuronal migration during cortical development (Young-Pearse *et al.*, 2010).

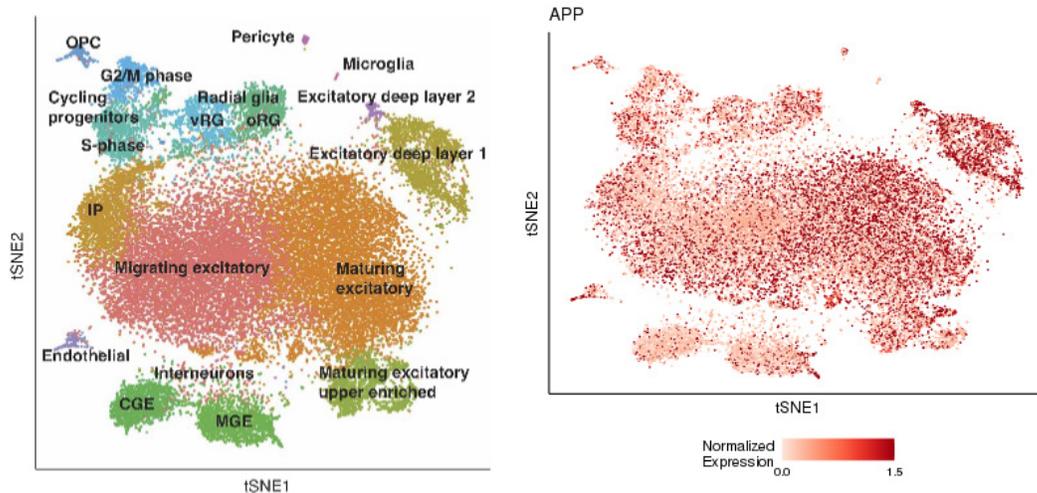


Figure 8. APP expression in developing human cortex.

Single cell RNA seq data of human developing cortex shows that APP is expressing in all cell type with an enrichment in excitatory neurons.

(Picture adapted from <http://solo.bmap.ucla.edu/shiny/webapp/>; Polioudakis *et al.*, 2019)

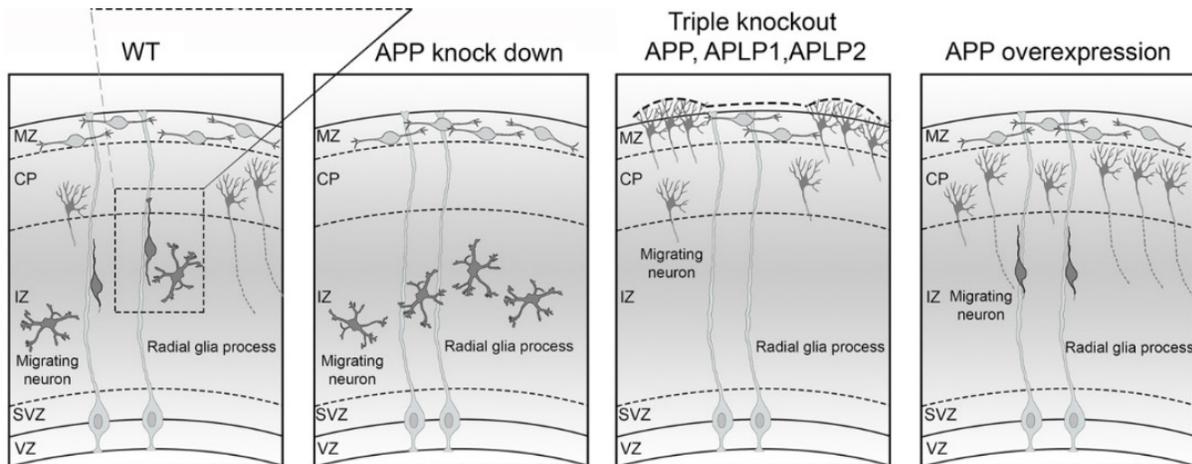


Figure 9. APP defects on cortical neuron migration.

Schematic diagram shows mis-expression of APP cause aberrant neuronal migration in developing cerebral cortex. When APP is knocked down, neurons fail to enter in the cortical plate, being arrested in the intermediate zone, whereas over-expression of APP caused an acceleration of the neuronal migration into the cortical plate. The triple knock-out neurons for all APP family proteins present focal dysplasia regions within the marginal zone.

Picture adapted from (Sosa *et al.*, 2017)

2.8. APP and neural progenitor cells

Neural progenitor/stem cells (NPCs/NSCs) are multipotent cells capable of self-renewal and differentiation into all cell types of the CNS, including neurons and macroglia. These cells can be obtained from fetal, neonatal and adult brains, as well as from the differentiation of pluripotent stem cells (Coronel, Palmer, *et al.*, 2019). NPCs constitute an endogenous reservoir for neurons in healthy brains that could be potentially used for regenerative therapies in neurodegenerative diseases such as AD (Papadimitriou *et al.*, 2018). APP can have different effects on NPCs biology depending on whether full length APP or its processed products are studied and whether the study is *in vitro* or *in vivo*. In the adult rodent brain, full length APP is expressed abundantly and it is involved in proliferation of neural progenitor cells (Wang *et al.*, 2016). *In vivo* models show that sAPP α increase proliferation of mouse NPC in SVZ and SGZ (Caillé *et al.*, 2004; Demars *et al.*, 2011, 2013). In Human, APP is abundantly expressed in ESCs (Porayette *et al.*, 2007), iPSC derived NPCs (Ochalek *et al.*, 2017), dividing and differentiating human NSCs and telencephalic neurospheres (Lee *et al.*, 2013; Small *et al.*, 2014). sAPP α increases the differentiation of human neural stem cells (hNSCs) *in vitro* in a dose dependent manner. While low dose of it increase neural differentiation, high dose of sAPP α treatment or wild-type APP gene transfection directs hNSCs into astrocytes rather than neurons. In addition, hNSCs transplanted into APP-knock out mouse brain also showed more glial differentiation than neural differentiation (Kwak *et al.*, 2006). Coronel *et al.* suggest that APP has a dual role in the differentiation of human NPCs, favoring gliogenesis and inhibiting the generation of neurons (Coronel, Lachgar, *et al.*, 2019). Contrary to sAPP α , the effect of sAPP β on NPCs biology is less known and it is even considered as a neurotoxic factor (Chasseigneaux and Allinquant, 2012). While some authors suggest that sAPP β fails to show a proliferative effect *in vitro* and *in vivo*, others propose that sAPP β strengthens age-associated deficits in the number of proliferating cells in the SVZ and SGZ of the adult mouse brain (Demars *et al.*, 2011). APP intracellular domain (AICD) decrease proliferation and neurogenesis by negatively regulating EGFR (Zhang *et al.*, 2007), dysregulation of SHH pathway (Trazzi *et al.*, 2011), dysregulation of GSK3 β (Trazzi *et al.*, 2014) and promoting expression of miR-633, which is a well-known suppressor of genes (FBXL15 and CDK6) implicated in human neurogenesis (Shu *et al.*, 2015). A β has different effects on NPCs biology depending on

the isoform (A β 40 and A β 42) and aggregation state (soluble fibrillary and oligomeric form). While freshly prepared A β 40 enhances neurogenesis in human and mouse NPC, A β 42 favors gliogenesis in the same cells (Chen and Dong, 2009; Fonseca *et al.*, 2013; A. Bernabeu-Zornoza *et al.*, 2019). A β 42-not A β 40- induce neurogenesis in rat hippocampal NPCs as oligomers but not as fibrils (López-Toledano and Shelanski, 2004).

3. Overview of Alzheimer's disease

3.1. Alzheimer's disease

Alzheimer's disease (AD) was originally described by Alois Alzheimer in 1906 and characterized by progressive cognitive deficiency due to degeneration of neurons (Barnes and Yaffe, 2011). The worldwide prevalence of dementia is estimated to be 46.8 million and predicted to increase to 74.7 million by 2030 and 131.4 million by 2050 (Whyte *et al.*, 2017). It is the most common cause of neurodegenerative dementia with ~50 million patients worldwide and it is characterized by two hallmarks: intra-neuronal hyper phosphorylated tau protein deposits known as neurofibrillary tangles (NFTs) and amyloid plaques, which are mainly composed of amyloid β (A β) peptide (Sharma *et al.*, 2019). The majority of AD cases are late-onset AD (LOAD) in which the disease occurs sporadically after age 65 (Long and Holtzman, 2019). LOAD risk factor genes play key functions in neuroinflammation, vascular dysfunction, calcium homeostasis, cholesterol metabolism, innate immune system, and endo-lysosomal pathway (Behl, 2017). Most of the genetic risk determinants in sporadic AD (APOE, TREM2, BIN1, CD33, INPP5D, CTSB, CTSD, and PICALM) are non-neural genes expressed in glial cells (Sala Frigerio *et al.*, 2019). Early-onset AD (EOAD) which also known as familial AD (fAD), constitutes 1-5% of cases and occurs before the age of 65 (Long and Holtzman, 2019). It is caused by fully penetrant mutations in the Amyloid Precursor Protein (APP), Presenilin1 (PSEN1) or more rarely Presenilin2 (PSEN2), ADAM metallopeptidase domain 10 (ADAM10), ADAM metallopeptidase domain 17 (ADAM17) (Hunter and Brayne, 2018).

3.2. Molecular mechanism of Alzheimer disease

Various hypotheses associated with AD pathway have been proposed, which some of them are listed below. However, none could fully explain the mechanism of the disease. It is unlikely to say that only one hypothesis is the reason of such a complex disease. Rather, it is likely that a combination of different pathways, together lead to appearance and progression of Alzheimer's disease.

3.2.1. Cholinergic hypothesis

Acetylcholine (ACh) is a neurotransmitter available in the brain with a significant role in neuromodulation of learning, memory, and cognitive functions. The reduction of choline acetyltransferase (CAT) -an enzyme involved in ACh synthesis- observed in the post-mortem AD brain, led to the idea of a substantial role of ACh in the AD (Perry *et al.*, 1977; Perry, 1980). Moreover, cholinergic abnormalities such as choline transport, ACh release, nicotinic and muscarinic receptor expression can contribute significantly to behavioral dysfunctions in AD patients (Terry and Buccafusco, 2003). These findings revealed that cholinergic therapy should be the primary approach in the treatment of AD. However, cholinesterase –an enzyme involved in degradation of ACh- inhibitors only provides moderate improvement of cognitive impairment and is not successful in stopping disease progression (Tayeb *et al.*, 2012).

3.2.2. Excitotoxic hypothesis

Glutamate is the excitatory neurotransmitter in the hippocampus and cortex whose excessive activation leads to neural excitotoxicity, thereby causing neurodegeneration and cell death (Olney, 1997). Under normal conditions, glutamate binds to the N-methyl-D-aspartate receptor (NMDAR), and depolarization proceeds, followed by magnesium mediated closing of the cationic channel to prevent the entry of calcium at resting state. In AD, there is excessive activation of NMDA receptors in neurons that causes the release of bound magnesium ions and allows the additional entry of calcium ions into neurons (Sharma *et al.*, 2019). The excessive activation can also occur in the presence of A β , as Zott et al. show that soluble A β dimers and oligomers block glutamate reuptake by neurons and astrocytes followed by hyperactivation and eventually neurodegeneration (Zott et al., 2019). Although experimental work indicates that the glutamate pathway may play role in the pathogenesis since early stages of the disease, antagonists targeting NMDAR showed only temporary and limited effects (Wang and Reddy, 2017).

3.2.3. Amyloid cascade hypothesis

APP and its cleavage product amyloid β (A β) have been studied extensively in relation to AD. The amyloid cascade hypothesis (ACH) was proposed in 1992 by Hardy and Higgins (Hardy and Higgins, 1992), suggesting deposition of A β in brain is the initiating phase in the pathology of AD. A β 40

and A β 42 –products of amyloidogenic processing of APP- exists in several forms such as monomers, dimers, oligomers, and fibrillary polymers. A β 42 has very high tendency to aggregate and form amyloid plaques and it is the dominant form in the brain of AD patient. Progressive accumulation of A β through increased A β production, A β 42/A β 40 ratio, A β deposition, and decreased A β clearance cause senile plaque formation followed by series of complex inflammatory responses, microglia activation, cytokine release, and astrogliosis (O'Brien and Wong, 2011). The amyloid beta hypothesis is supported by: I) Genetic forms of AD such as Autosomal-dominant Alzheimer's disease (ADAD), Down syndrome and APP locus duplications, produce an increase in the A β 42/40 ratio and total A β production (Tcw and Goate, 2017); II) Protective APP mutation A673T that reduces risk of developing AD, causes decreased A β production (Jonsson *et al.*, 2012; Martiskainen *et al.*, 2017); III) Apo lipoprotein E (APOE)- the strongest genetic risk factor for LOAD- increases the risk by influencing A β seeding and clearance (Verghese *et al.*, 2013). However, while Amyloid beta accumulation is important in the pathology of AD, it is not sufficient to drive the disease and other downstream factors may play key roles. Furthermore, minimal correlation was reported between phases of amyloid deposition and degree of cognitive decline (Nelson *et al.*, 2012) and there is no association between age of onset and amyloid beta level. Moreover, none of the therapies targeting A β have been successful in limiting the progression of cognitive decline in symptomatic AD (Long and Holtzman, 2019).

3.2.4. Presenilin hypothesis (PSH)

Besides APP, there are other genetic factors such as Presenilin (PSEN1/PSEN2) which are associated with Alzheimer's disease. PSEN1 encodes the catalytic subunit of γ -secretase enzyme –involved in APP cleavage- and its mutation is responsible for ~95% of FAD (Hunter and Brayne, 2018). Some studies suggest that mutations in PSEN1 could give rise to A β production and deposition. However, Sun *et al.* analyzed 138 pathologic mutation in PSEN1 gene and reported about 90% of these mutations lead to reduced production of A β 42 and A β 40 but majority of them change the ration of A β 42/A β 40 (Sun *et al.*, 2017). The results from the trial studies of the γ -secretase inhibitor Semagacestat showed a worsening of dementia. However, how PSEN1

mutation cause dementia and whether it is through amyloid beta production is not fully understood yet (Hunter and Brayne, 2018).

3.2.5. Tau Hypothesis

The Tau protein (encoded by *MAPT* gene) plays a significant role in the stability of microtubules and it exists in six isoforms due to alternative splicing. In the healthy brain, Tau protein mainly exists in the phosphorylated form in the axonal membrane, while it gets hyper-phosphorylated and loses the affinity to bind to microtubules in the AD brain. These hyper-phosphorylated forms bind to each other, tying themselves in insoluble intracellular structures called neurofibrillary tangles (NFTs) that disrupt neuronal plasticity and cause neurodegeneration (Almansoub et al., 2019). It has been suggested that hyper-phosphorylated, aggregated forms of Tau may be a principal driver of neurodegeneration in AD because unlike A β , the stage of tau pathology correlates more strongly with the progression of cognitive impairment (Long and Holtzman, 2019). Several studies showed that tau hyper-phosphorylation is due to over-activation of kinases and inactivation of phosphatases. Mechanistically, different stimuli induce the cascade of events in microglial cells and astrocytes, which will lead to the release of NF-kB and overproduction of pro-inflammatory mediators such as TNF- α and interleukins (ILs). They cause the inflammatory reactions, neuritic injury, activation of the different kinases such as MAP kinase (MAPK), cyclin-dependent kinase-5 (CDK- 5) and glycogen synthase kinase-3 β (GSK-3 β) and Janus kinase (JNK), etc which ultimately lead to the tau phosphorylation (Sharma *et al.*, 2019).

3.2.6. ApoE hypothesis

ApoE is a glycoprotein consisting of 299 amino acid residues mainly produced by astrocytes. It functions in cholesterol transport through ApoE receptors, which are members of the low-density lipoprotein (LDL) gene family. ApoE has three allele: ApoE2 (ϵ 2), ApoE3 (ϵ 3) and ApoE4 (ϵ 4) which only vary by one or two amino acid residues at 112 and 158 positions (Hatters, Peters-Libeu and Weisgraber, 2006). The ϵ 4 allele is the most common genetic risk factor linked with the late onset of the AD, whereas the ϵ 2 allele decreases the risk of the AD (Michaelson, 2014). ApoE ϵ 4 has high tendency to bind to A β and it is involved in AD pathogenesis by increasing A β aggregation,

reducing A β clearance, inducing neuroinflammation, and reduced rate of glucose metabolism in the brain of AD patients (Sharma *et al.*, 2019). Moreover, TREM2 (triggering receptor expressed on myeloid cells- 2) which increase 2-4 fold risk of developing AD is a receptor for ApoE ϵ 4. TREM2 is highly expressed in microglia –the resident immune cell type in the brain – and there is a strong decrease in microgliosis surrounding A β plaques in TREM2-deficient mice. TREM2 as an AD risk factor points to new directions, namely AD-linked changes in the metabolism of lipids and glucose which interestingly Alois Alzheimer already described the accumulation of lipids in the dementia brain. TREM2 itself binds anionic lipids and lipoproteins, suggesting that the lipid-sensing function of microglia in the brain might be disease-relevant as well (Behl, 2017).

4. Overview of stress response

4.1. Stress

Stress is defined as a state in which homeostasis of a living organism is endangered. All organisms have developed systems to detect and react to stress in order to minimize stress related damage. Many environmental variations impose stresses that can damage or kill the organism in the absence of an appropriate response. Mammals have evolved biological processes such as complex buffering mechanisms, complex intercellular interactions and programmed cell death to minimize environmental variation. These biological processes associated with stress responses play important roles in normal development and homeostasis. Defects in systems associated with the stress responses contribute to human diseases such as cancer, diabetes, Alzheimer disease, Parkinson disease, Osteogenesis Imperfecta, cardiovascular disease and others (Helfand *et al.*, 2003). From the molecular perspective, cells respond to stress by expressing a wide range of molecules which early immediate genes (IEGs) and heat shock proteins (HSPs) are two well-known examples. In the following, I will describe IEGs and particularly point out JUN and FOS, two important members of this family.

4.2. Immediate-early genes

Immediate-early genes (IEGs) are a class of genes rapidly induced by a large number of stimuli, which stress is one of them. IEGs' induction occurs within minutes and they regulate the expression of the so-called delayed response genes. IEGs encompass many functionally different products such as secreted proteins (e.g., cytokines and chemoattractants), cytoplasmic enzymes (the phosphatase 3CH134/MKP-1/Erp and the cyclooxygenase COX-2), ligand-dependent transcription factors (NGFIB/ Nur77/N10), and inducible transcription factors (**Jun**, **Fos**, Krox, Myb, Myc, NGFI-C, SRF) (Pérez-Cadahía, Drobic and Davie, 2011).

4.3. Activator Protein 1

Activator Protein 1 (AP-1) is one of the first mammalian transcription factors to be identified. It is not a single protein, but a large family of dimeric protein complexes. The well-known canonical

AP-1 members are Jun proteins (c-Jun, JunB, and JunD) and Fos proteins (c-Fos, Fra-1, Fra-2, and FosB), which share two highly conserved domains: the leuzine zipper (dimerization region) and a basic DNA-binding region (Shaulian and Karin, 2002). JUN (c-Jun) is the principal component of the AP-1 transcription factor whose deletion is embryonically lethal in mouse (Hilberg *et al.*, 1993). It can homodimerize and heterodimerize with other Jun or Fos members to form transcriptionally active complexes. In addition, Jun proteins can also heterodimerize with other transcription factors, such as members of the activator transcription factor (ATF) family and other basic zipper containing transcription factors such as CBP, MyoD, NFat or c-rel (Raivich and Behrens, 2006).

AP-1 activity is induced by a variety of physical and chemical stresses, growth factors, inflammatory cytokines, neurotransmitters, polypeptide hormones, cell–matrix interactions, bacterial and viral infections (Shaulian and Karin, 2002). The up-regulation of Jun and Fos protein is common in the developing, adult as well as in injured nervous system and their activity dramatically increases in cerebral ischaemia and stroke, seizure, axotomy and other forms of trauma as well as in the post-traumatic repair (Raivich and Behrens, 2006). The induction of AP-1 by pro-inflammatory cytokines and stress is generally mediated by c-Jun N-terminal kinases JNK1, JNK2 and JNK3 that are known as stress-activated protein kinases (SAPKs). The JNK-signalling pathway is a central stress signaling pathway involved in neuronal plasticity, neuroregeneration, neurodegeneration and brain aging and it is critical upstream regulators of the mitochondrial pathway of apoptosis. JNKs translocate to the nucleus after activation, phosphorylate c-Jun and thereby enhance its transcriptional activity (Shaulian and Karin, 2002; Raivich and Behrens, 2006). Many AP-1 target genes are related to neuronal regeneration and repair after injury. These genes are involved in process extra cellular matrix proteins (tenascin, laminin, fibronectin), structural proteins (TUBULIN, MAP-2, TAU, GAP-43, GFAP), matrix metalloproteinases, growth factors (NGF, BFGF), synaptic proteins (SYNAPSIN-1) and genes related to Alzheimer's disease (APP, APOE, PRESENILINS) (Pennypacker *et al.*, 2000). AP-1 transcriptional activity can be regulated in four level by affecting : I) Jun and Fos mRNA expression and turnover; II) Jun and Fos protein turnover; III) post-translational modifications of Jun and Fos

proteins; IV) interactions with other transcription factors that can either enhance or inhibit AP-1 activity (Shaulian and Karin, 2002).

4.4. The endo-lysosomal pathway

The endo-lysosomal system plays a critical role in maintaining homeostasis by protein degradation and recycling within cells. Disruption of this pathway causes cellular stress, and stress can induce endolysosomal disruption. The endo-lysosomal system is a cellular trafficking mechanism that relies on the transport of vesicles in the cell. It is made of different types of vesicles including the early endosome (EE), recycling endosome (RE), late endosome (LE), and lysosome (Lys) as a final destination where proteolytic degradation takes place (Huotari and Helenius, 2011). While cytoplasmic pH is about 7.0, the endosomal and lysosomal lumen pH is retained in a range of 6.5 to 4.5 which is optimal for the activity of hydrolases and other enzymes (Diering and Numata, 2014). Protein sorting along the endocytic pathway is regulated by membrane associated small GTPases called Rab family of proteins. They act as molecular switches that alternate between two states: the GTP-bound activated form and the GDP-bound inactivated form (Villarroel-Campos *et al.*, 2014). Different Rab proteins are often considered as markers of different endosomal compartments due to their corresponding host organelles. Endocytosis often starts by internalization of clathrin-coated vesicles derived from the plasma membrane. The EE is the first sorting station for endocytosed proteins and it is associated with Rab5. Recycling cargos can return to plasma membrane thorough RE and this process is regulated by Rab4 and Rab11 (Hsu and Prekeris, 2010). Degradative cargos are progressively sorted into intraluminal vesicles in the EE, creating multivesicular body (MVB) which is an important stage in the transition of EE into LE. During this transition, Rab5 is replaced by Rab7, which this process is known as “Rab conversion”. LE often labeled by Rab7 and undergo fusions with each other or with lysosomes for cargo degradation (Winckler *et al.*, 2018). Within the lysosome lumen, there are more than 50 hydrolases concentrated which fusion of LE with lysosomes generally initiates the activation of hydrolase precursors or proenzymes. LE can also join auto phagosome and proceed cargo destruction in autophagy pathway (Hu *et al.*, 2015).

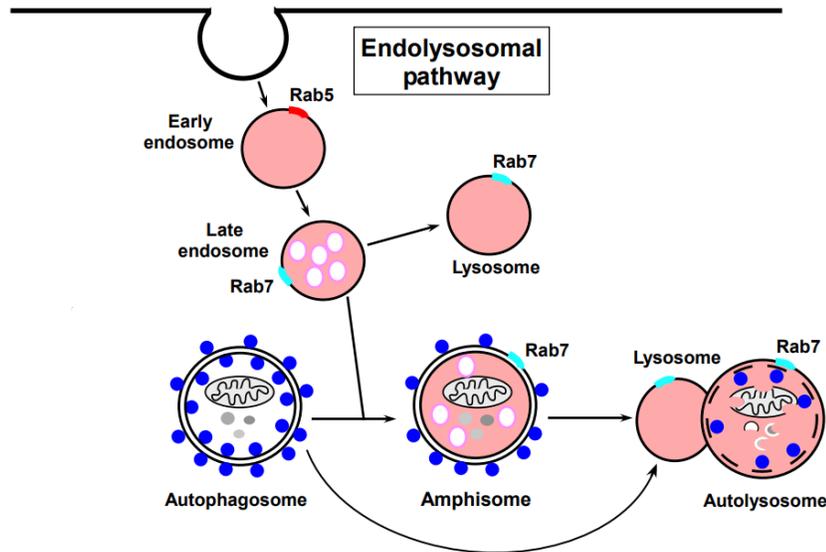


Figure 10. Endo-lysosomal pathway.

Schematic drawing showing the endo-lysosomal pathway contain early endosome, late endosome followed by fusion to either lysosome or autophagosome.

Picture adapted from (Hansen and Johansen, 2011)

4.5. APP and the endolysosomal pathway

The endo-lysosomal network is critical for APP processing where proteolytic generation of APP-CTFs and A β proceeds within this network (Figure 11). However, if APP is not cleaved in the endo-lysosomal network, it is trafficked to the Golgi apparatus or recycled back to the plasma membrane, where it can be processed by α -secretase via the non-amyloidogenic pathway (Whyte *et al.*, 2017). Abnormalities in endolysosomal components and dysregulation of their trafficking play a key role in neurodegenerative disorders such as Alzheimer disease (AD) and Parkinson's disease (PD) (Nixon, 2017). Extensive endosomal pathology such as increases in endosomal size, accumulation of lysosomal dense bodies and autophagic vacuoles has been reported consistently in human AD brain, but not in normal aged brains (Cataldo *et al.*, 1997, 2000, 2004; Nixon *et al.*, 2005). Remarkably, a number of late-onset Alzheimer disease (LOAD) risk genes including Bin1 and CD2AP are encoding regulators of endocytic trafficking (Guimas Almeida *et al.*, 2018). Moreover, A β has been detected in some of the compartments within

degradative pathways including endosomes, autophagic vesicles, multivesicular bodies/late endosomes, and lysosomes (Whyte *et al.*, 2017). APP mutations also disrupt the function of endosome, lysosome and autophagy in iPSC-derived human cortical neurons (Hung and Livesey, 2018).

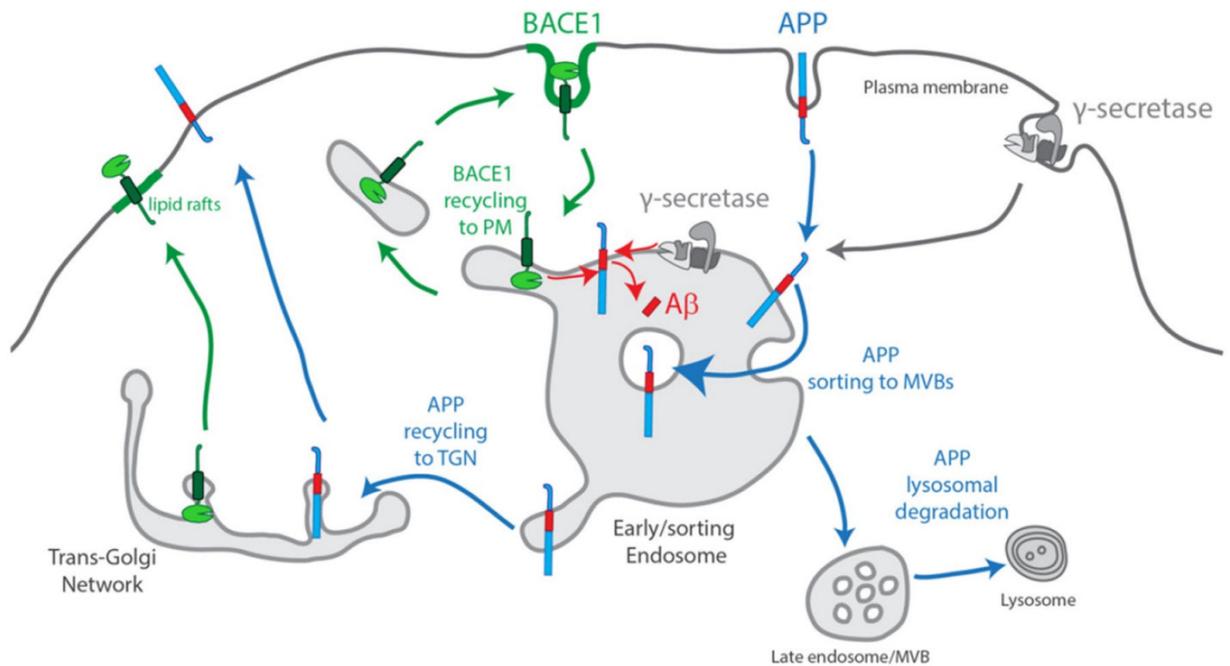


Figure 11. APP and BACE processing and trafficking through endo-lysosomal pathway.

Scheme of APP processing in endo-lysosomal pathway. APP and BACE1 exit the Trans-Golgi Network (TGN) to the plasma membrane in separate post-Golgi secretory vesicles. At the plasma membrane, BACE1 prefers lipid rafts, and is endocytosed independently of APP. Less clear is γ -secretase complex assembly and endocytic trafficking. Upon endocytosis, APP, BACE1 and γ -secretase reach early/sorting endosomes. BACE1 recycles fast out of sorting endosomes to the plasma membrane, while APP is sorted into inner luminal vesicles during MVB biogenesis. $A\beta$ production occurs upon acidification of sorting endosomes which favors BACE1 activity and APP processing at the endosomal limiting membrane. APP degradation occurs upon fusion with the lysosome.

Picture adapted from (Guimas Almeida *et al.*, 2018)

4.6. APP, AP-1 and stress

APP is known as stress response gene due to its elevated expression in brain injury and ischemia, suggesting a role for this protein in the repair of the injured brain (Pennypacker *et al.*, 2000). *In vivo*, APP upregulation has been observed following brain injury in mammals and *Drosophila*. Overexpression of APP in transgenic mice decreases chronic forms of excitotoxicity and injection of sAPP α or sAPP α domains into the brain of rats with brain injury improved motor or cognitive outcome (Kögel, Deller and Behl, 2012). However, the *in vivo* data obtained from APP knockout mouse models does not show consistency. Heber *et al.* did not observe significant differences in the cell death sensitivity of cortical neurons obtained from APP-KO animals compare to wild type neurons (Heber *et al.*, 2000). Steinbach *et al.* reported higher susceptibility to kainate-induced epilepsy in APP-KO mice, but couldn't detect any difference in cortical and cerebellar neurons obtained from these animals *in vitro* by glutamate- and NMDA-induced toxicity (Steinbach *et al.*, 1998). In contrast to *In vivo* data, there is extensive experimental evidence for neuroprotective function of APP and sAPP α in neuronal culture *in vitro*. APP showed a neuroprotective effect by repressing JNK/c-Jun signaling and subsequent apoptosis in response to stress. Cells over expressing APP or treated with exogenous sAPP α showed reduction of JNK signaling pathway activity and cell death (Kögel *et al.*, 2005; Copanaki *et al.*, 2010; Eckert *et al.*, 2011). In contrast, overexpression of the Swedish mutant of APP did not prevent stress-mediated JNK activation and cell death (Kögel *et al.*, 2005). It is not completely clear how APP mediates neuroprotection but based on literature sAPP α is the strongest candidate. sAPP α -dependent neuroprotection may be mediated by activation of anti-apoptotic signaling pathways such as PI3K/Akt, ERK and NF- κ B and subsequently: I) activation of antioxidative defense genes involved in stress responses and neuronal survival such as manganese-superoxide dismutase (MnSOD), peroxiredoxin-2 and catalase, the anti-amyloidogenic gene transthyretin, insulin-like growth factor 2 (IGF2) and insulin-like growth factor-binding protein 2 (IGF-BP2); II) inhibition of mixed lineage kinase (MLK) family which are upstream of JNK and protective effect of APP/ sAPP α could be mimicked by an MLK inhibitor; III) antagonizing stress induced pro-death pathway GSK3 β ; IV) blocking apoptosis inducer proteins such as Bad and Bcl-xL (Figure 12). Moreover, neuroprotective effects of sAPP α may be associated with its effect on the function of ion channels. sAPP α activates potassium

channels and suppresses NMDA currents to limit Ca^{2+} overloading and excitotoxic damage in neurons (LaFerla, 2002; Stein *et al.*, 2004; Kögel *et al.*, 2005).

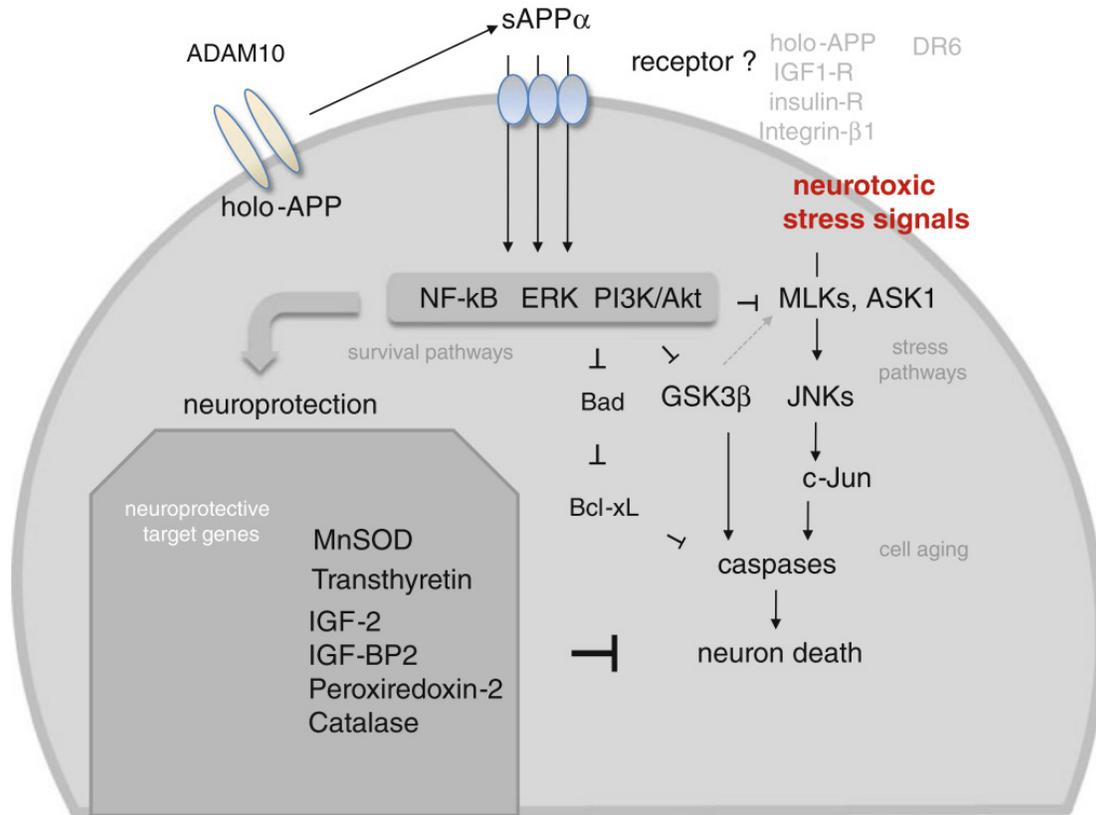


Figure 12. Model of APP-dependent neuroprotection.

APP is cleaved by α -secretase along the secretory pathway to generate sAPP α which can act in paracrine and autocrine way. By binding to a hitherto unidentified cellular receptor, sAPP α can trigger several neuroprotective signaling pathways (NF- κ B, PI3K/Akt, ERK), enhance the expression of downstream target genes and antagonize stress-induced pro-death pathways (JNK/c-Jun, GSK3 β).

Picture adapted from (Kögel, Deller and Behl, 2012)

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Chapter II-Results

The human Amyloid Precursor Protein regulates the balance between self-renewal and differentiation in human cortical neural progenitors

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Abstract

The human neocortex is a highly elaborate brain structure responsible for complex cognitive behavior. The approximately 16 billion neurons of the human neocortex are derived from a relatively limited number of cortical neural progenitor cells (NPCs). During embryogenesis, human cortical NPCs initially generate neurons at a particularly slow rate while preserving their progenitor state for a relatively long time, in part contributing to increased human cortical size. How this balance between the progenitor state and neurogenic state is regulated, and whether it contributes to species-specific brain patterning, is poorly understood. Here we study the impact of Amyloid Precursor Protein (APP), whose mutations cause familial Alzheimer's disease (fAD), in human cortex development. We find that loss of APP drives human cortical NPCs towards a neurogenic state and reduced proliferation, without changing the temporal order of neuronal fate acquisition or the rate of neuronal maturation. Mechanistically, loss of APP cell-autonomously triggers conversion of cortical NPCs to a neurogenic state through activation of immediate early genes and repression of the Wnt pathway. We propose that APP is a homeostatic regulator of the fine balance between proliferation and differentiation, which may contribute to human-specific patterns of neurogenesis.

Introduction

The highly conserved class I transmembrane Amyloid precursor protein (APP) is broadly expressed throughout nervous system development and has been extensively studied because its proteolytic processing is linked to Alzheimer's disease AD^{1,2,3}, yet its physiological function, especially in humans, is unclear. APP and its homologues in various species are involved in many biological processes such as axonal outgrowth after injury⁴, endo-lysosomal pathway^{5,6,7}, stress response after hypoxia/ischemia^{8,9}, cell signaling processes and brain development and plasticity^{10,11}. In the worm, *C. elegans* loss of the APP homologue *Apl* is lethal due to molting deficits, while causing mild and low penetrance defects in various aspects of neuronal differentiation, function, and survival in mouse and *Drosophila*¹². In humans, point mutations of even a single copy of *APP* can cause familial AD¹³. Furthermore, genetic loss of function data obtained from the human population by the GENOMAD project show that the *APP* gene has a loss of function observed/expected (LOEUF) score of 0.42, meaning that complete loss of function of APP causes >50% developmental mortality^{14,15} indicating that human development may be sensitive to the complete loss of *APP* and that APP may play an unknown essential function in humans. APP is highly expressed in human telencephalic neurospheres, and during the differentiation and migration of cortical neurons^{16,17,18} leading to suggestions that it may be involved in NPC proliferation, differentiation and/or maturation^{19,20}. In the adult rodent brain, APP is also abundantly expressed in NPCs found in the VZ-SVZ^{21,22}, but its loss does not cause obvious deficits in neurogenesis. To assess APP's role in human cortical development, we generated *APP*-knockout iPSCs in two different genetic backgrounds and queried the potential effect on cortical neurogenesis.

Results

Loss of APP increases cortical neurogenesis

We generated *APP-KO* iPSC by CRISPR/Cas9 (fig. S1, A to K) and chose three *APP-KO* clones and one *APP* wild type (control) clone which received Cas9 but was not mutated, for further analysis. Following induction of cortical differentiation, the morphology of NPCs appeared normal in all

clones, and they expressed canonical molecular markers of corticogenesis (fig. S2). Seven days after initiation of neuronal differentiation, we observed many more cells with neuronal-like morphology in *APP-KO* NPC than in control (fig. S3, A and B). We stained the cells for SOX2 (NPCs), Doublecortin (DCX; immature neurons), β -TubulinIII (TUJ1; neurons) to determine cell type. All three *APP-KO* cultures showed increased TUJ1 and DCX expression 7 days post differentiation (Fig. 1, A to H and fig. S3, C to J). To determine the neurogenic state of control and *APP-KO* NPCs, we transduced a small subset of them (70 NPCs in the control line control and an average of 63.6 NPCs in the 3 *KO* lines, Supplementary Table 1) with a GFP-expressing lentiviral vector^{23,24} (Fig. 1I) and quantified the number of GFP+ NPCs (SOX2) and neurons (TUJ1) at days 0, 7 and 30 after differentiation (Fig. 1, J to S and fig. S3, I-R). We observed significant decrease in NPCs (Fig. 1T) accompanied by significantly more production of neurons over time (Fig. 1U) in all three *APP-KO* compared to control after 7 days. The neuron-to-progenitor ratio was strikingly higher in all 3 *APP-KO* lines compared to controls at day 7, but was not significantly different by day 30. Focusing on this initial burst of neurogenesis in *APP-KO* NPCs, we found that 1 control NPC produced on average 8 NPCs and 3.6 neurons after 7 days of differentiation. In contrast, within the same time frame each *APP-KO* NPC produced on average 1.25 NPCs and 22.9 neurons (Supplementary Table 1), indicating a major switch of cortical NPCs from a self-amplifying state to neurogenic state.

7 days post differentiation

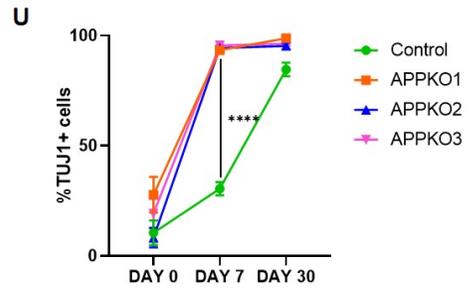
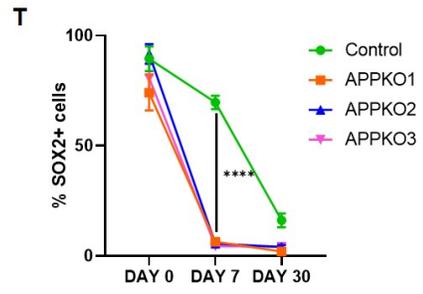
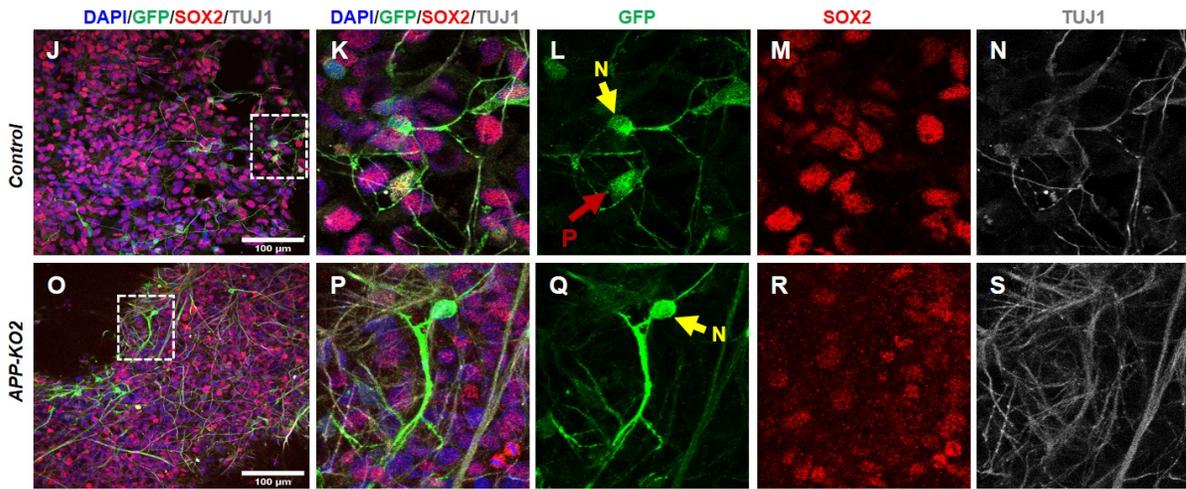
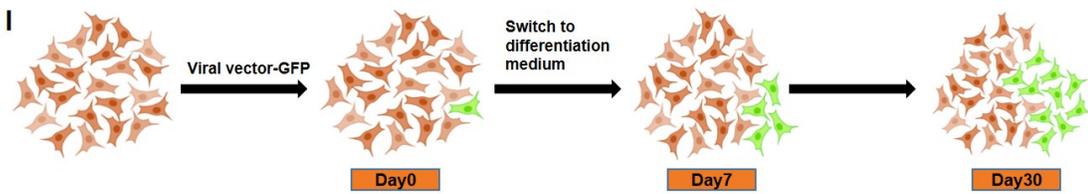
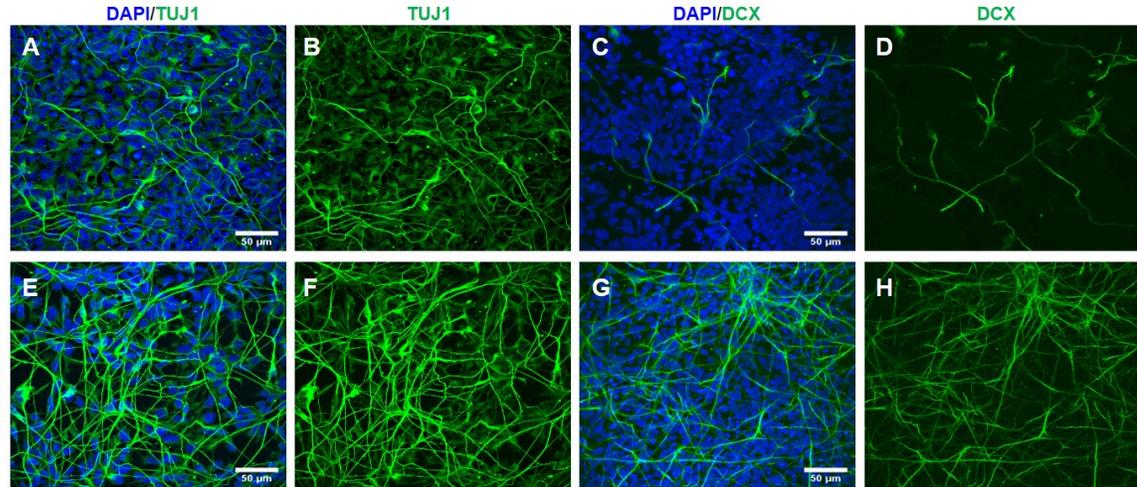


Fig. 1. Increased differentiation of *APP-KO2* NPCs within 7 days of neuronal differentiation.

A-H, Many more cells express the neuronal markers TUJ1 and DCX in *APP-KO2* compared to control (Scale bar 50 μm). **I**, Schematic illustration of sparse labelling using a lentiviral vector approach to follow neurogenesis. **J-S**, Expression of SOX2 and TUJ1 within GFP+ cells in control and *APP-KO2* 7 days post differentiation (Scale bar 100 μm , N=neuron, P=Progenitor). **T**, Decrease in SOX2 and **U**, Increase in TUJ1+ expression in *APP-KOs* neurons compared to control within 7 days (2way ANOVA, $p < 0.0001$), whereas levels are almost equal at 30 days post-differentiation.

APP is broadly expressed in the developing nervous system. In order to examine whether this effect is general or brain-region specific, we generated motor neurons²⁵ from control and *APP-KO2* iPSCs. We confirmed the expression of APP protein in motor neurons precursor cells by western blot (fig. S4A) and examined them at 1 and 4 days post-differentiation. No significant differences were detected in phase contrast morphology (fig. S4, B to E) or the number of ISLET1+ cells (MN marker) in *APP-KO2* and control (fig. S4, F-R) at either time point. These data suggest that loss of APP preferentially affects cortical progenitors and causes either an increased neurogenic state of cortical NPCs or accelerated differentiation of their daughter neurons.

Loss of APP does not alter the order or timing of cortical neuron differentiation

One of the key features of cortical development is the sequential generation of different neuronal subtypes that will occupy different layers of the neocortex. This feature is preserved in NPC cultures, whereby deep layer (early born) neuronal fate markers appear before upper layer (late born) cell fate markers^{26,27}. To assess whether loss of APP affects this order, differentiation was launched and cells were tracked for 7, 15, 30, 60- and 120-days post-differentiation. We first observed clear expression of early born neuronal marker CTIP2 only at day 15 in both genotypes, despite the fact that *APP-KO* NPCs had generated ~3-fold more neurons than control NPCs (Fig. 2, A1-F4). Within 30 days, the number of CTIP2+ and STAB2+ cells increased in both control and *APP-KO2* (Fig. 2, G1-H4). The same results were observed for *APP-KO1* and *APP-KO3* at 7, 15 and 30 days post-differentiation (data not shown). We next focused on markers of neuronal maturation namely the emergence of synapse markers 60 and 120 days post-differentiation. No

difference was observed between control and *APP-KO* in the appearance of MUNC13 (presynaptic), PSD95 (postsynaptic) and HOMER1 (synapse marker of glutamatergic neurons). MUNC13 began to be detectable 60 days' post-differentiation in both control and *APP-KO* while no PSD95 was detected in both (fig. S5, A to L). MUNC13, PSD95 and HOMER1 were clearly detectable 120 days post-differentiation and showed the same pattern in control and *APP-KOs* (fig. S6, A to H). These data suggest that loss of APP does not significantly accelerate maturation of new born neurons, and hint that the deficit is in the NPCs themselves.

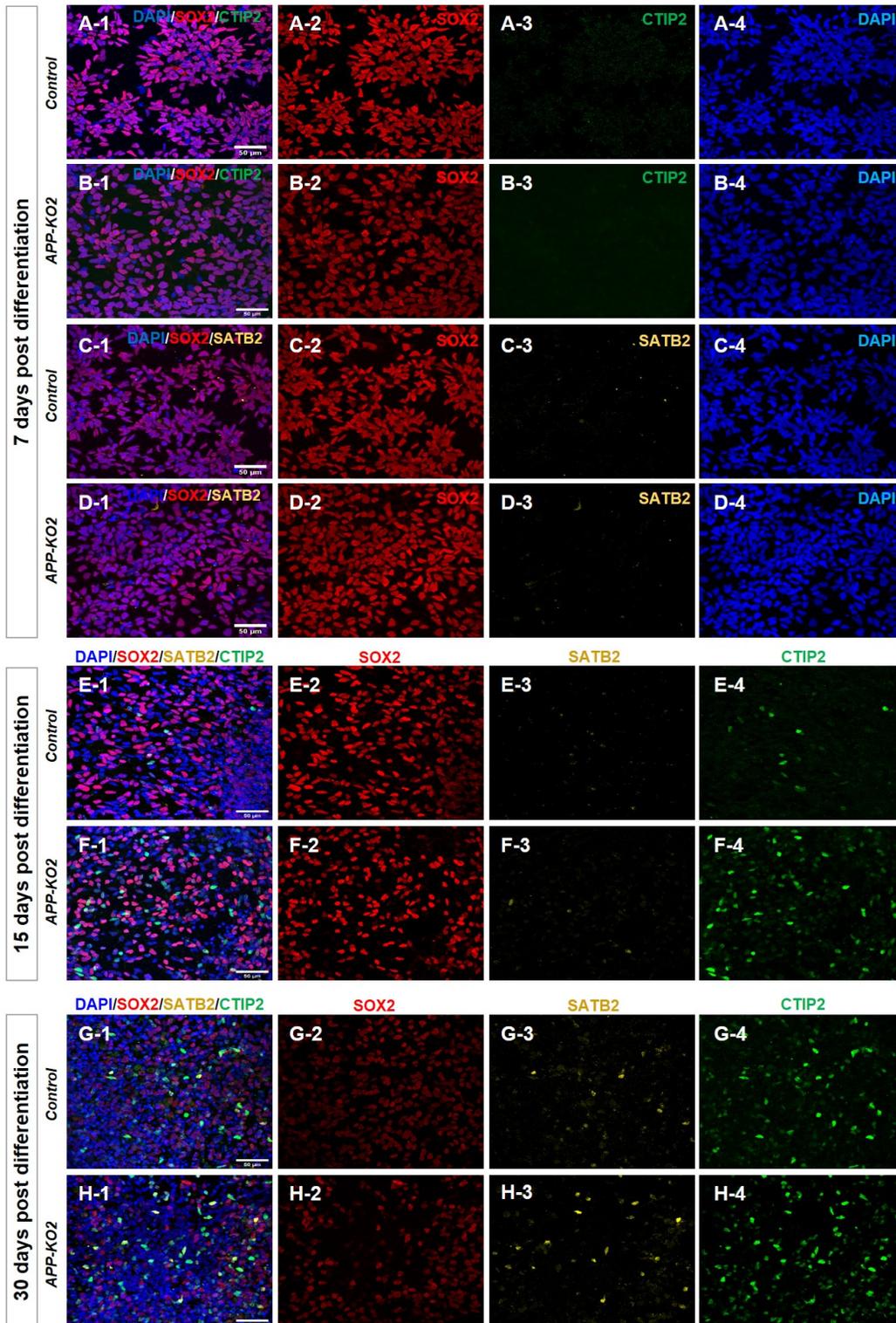


Fig.

2.

Cortical neuron fate markers appear with same temporal order in APP-KO and controls.

A1-D4, No expression of CTIP2 (early born neuron) and SATB2 (late born neuron) was observed 7 days post-differentiation. **E1-F4**, CTIP2 and SATB2 appeared at the same time 15 days post-differentiation in

control and *APP-KO2* (scale bar 50 μ m). **G1-H4**, Expression of CTIP2 and SATB2 30 days post-differentiation in control and *APP-KO2* (scale bar 50 μ m).

APP knockout NPCs are in an elevated neurogenic state

Temporal analysis of *APP* expression (<http://www.humous.org>) in scRNAseq data of the developing human cortex²⁸ shows higher expression levels in early NPCs, when self-amplifying divisions dominate, and a decrease over time as NPCs switch to neurogenic divisions. To unravel the earliest detectable defect in the absence of APP, NPCs were maintained under progenitor conditions without induction of differentiation. We confirmed APP expression in the control NPCs under proliferating conditions by western blot (fig. S7, A), and stained for SOX2/ TUJ1 and SOX2/ DCX (Fig. 3, A to L and fig. S7, B to M). We observed a significant decrease in the ratio of SOX2+ cells (Fig. 3M and fig. S7N) and significant increase in the ratio of DCX+ cells (Fig. 3N and fig. S7O) in *APP-KOs* relative to control, even in the absence of induction of differentiation. This was accompanied by a significant reduction in proliferative NPCs in *APP-KOs* compared to controls (~20% fewer Ki67+ cells; Fig. 3, O to Q and fig. S7, P to R). These data suggest that APP is required to maintain human cortical NPCs in a self-renewing progenitor state by inhibiting premature neurogenesis. To rule out genetic background effects, we generated an *APP-KO* clone from a different independent iPSC line (fig. S1, L to N) and observed the same results in those NPCs (fig. S7, S to Z). We performed a rescue experiment by transfecting *APP-KO2* NPCs with PiggyBac vectors containing either GFP alone (*pPB-CAG-IRES-EGFP*) or GFP and APP (*pPB-CAG-hAPP-IRES-EGFP*) and staining for SOX2 and DCX. Quantification of GFP+/SOX2+ and GFP+/DCX+ cells showed that *APP-KO* NPCs were restored to a progenitor state and the number of their neuronal progeny was reduced back to control levels upon rescue with APP (Fig. 3, R1 to V), but not with GFP alone. We noted that restoring APP only rescued NPCs expressing it, but not neighboring cells (arrows in Fig. 3T2), suggesting a cell-autonomous requirement. Next, we produced cortical organoids from control and *APP-KO* clones from the two independent iPSC lines. We stained the organoids for SOX2 and DCX at day 15, when the majority of cells are in the NPC state, and observed larger DCX+ clusters with higher levels of DCX expression in *APP-KO* organoids of both iPSC lines (fig. S7, Z'1 to Z'8 iPSC line1 and Z''1 to Z''8 iPSC line 2, Supplementary movies 1-4).

Finally, to test whether this effect on cortical neurogenesis was specific to human NPCs, we tested for signs of premature neuronal differentiation in the *APP-KO* mouse cortex at a stage when most cells should still be progenitors. Previous work on cultured mouse NPCs reported contradictory observations with respect to the effect of loss of APP on neuronal differentiation^{29,30} and a third study reported no evidence for a role of APP or its paralogues in mouse neurogenesis *in vitro* or *in vivo*³¹. We examined the developing mouse cortex at E10.5 and observed no Tuj1+ cells in the progenitor layer and no difference in Pax6 (NPC) and Tuj1 expression between *App+/+* and *App-/-* brains (fig. S8, A-J). Together, these data show that APP is cell autonomously required to repress neurogenic divisions of human cortical NPCs.

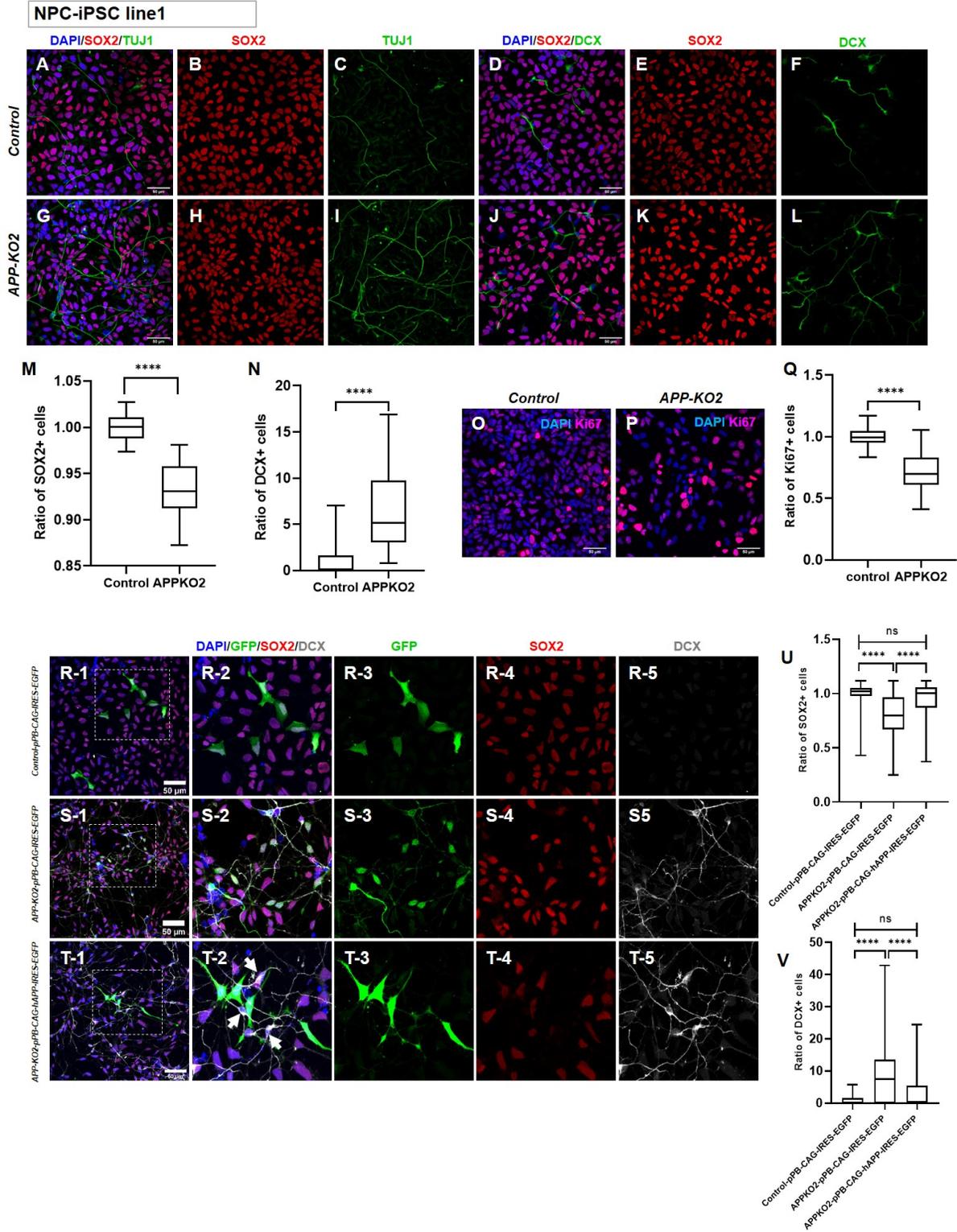


Fig. 3. Loss of APP drives increased differentiation of neural progenitors.

A-L, NPCs in proliferating medium from control and APP-KO2 stained for SOX2/TUJ1 and SOX2/DCX. **M**, Significant decrease in ratio of SOX2+ cells and **N**, Significant increase in ratio of DCX+ in APP-KO2 (n=3, unpaired t-test, p<0.0001, scale bar 50µm). **O-Q**, Significant decrease in ratio of Ki67+ cells in APP-KO2 (n=3, unpaired t-test, p<0.0001, scale bar 50µm). **R1-V**, APP cell autonomously maintains NPCs in a progenitor stage. **R1-R5**, Control NPCs transfected with pPB-CAG-IRES-EGFP **S1-S5**, APP-KO2 NPCs transfected with pPB-CAG-IRES-EGFP **T1-T5**, APP-KO2 NPCs transfected with pPB-CAG-hAPP-IRES-EGFP and stained for GFP/SOX2/DCX. APP rescued the phenotype of transfected cells but not neighboring cells (arrows in T2). **U**, Ratio of SOX2+ and **V**, DCX+ cells in all 3 conditions (n=2, Ordinary one-way ANOVA, p<0.0001, scale bar 50µm).

Loss of APP promotes neurogenic divisions in human cortical NPCs

To gain insight into how APP regulates the fate of human cortical NPCs, we performed single cell RNA sequencing using a novel, non-microfluidics-based method, on control and *APP-KO* NPCs (Supplementary Table 2, BioProject accession number PRJNA678443). Cluster analysis identified five clusters in control NPCs and seven clusters in *APP-KO* NPC (*APP-KO* 2) that presented very close relationship between genotypes and lineage progression stages as shown by uniform manifold approximation and projection (UMAP) 2D-representation (Fig. 4A and fig. S9, A to C). We used the main marker genes of each cluster to generate heat maps, and based on these markers, the five clusters of control NPCs were annotated as radial glial cells (RGC), cycling progenitors 1 and 2 (CP1 and CP2), intermediate progenitors (IP) and neurons (N) (fig. S9, D to F). These same five categories were also found in *APP-KO* together with two additional clusters. One cluster, which we refer to as “neurogenic progenitors” (NPs), is composed of cells expressing both radial glial markers (e.g. *FABP7* and *VIM*) as well as neuronal differentiation markers (e.g. *PNT*, *SPARC*, and *TUBB2A*; Supplementary Table 2). In addition, we found a very small cluster of cells (3%; “other”) enriched in pericyte markers, while the neuronal cluster represented twice as many cells (5% vs 2%) in the *APP-KO* sample. Interestingly, NPs represent 13% of *APP-KO* cells and together with the neurons appear at the expense of cycling progenitors, which show a reduction of 20% in *APP-KO* cells compared to control (Fig. 4B), matching the reduction we observed with Ki67 immunofluorescence (Fig. 3Q). Furthermore, pseudotime analysis shows a clear temporal

shift towards a differentiated state in all *APP-KO* clusters compared to similar control clusters (Fig. 4, C and D). To further test these observations, we performed SOX2/NEUROG2/DCX triple staining (Fig. 4, E to L) on control and *APP-KO* NPCs. NEUROG2 expression marks commitment to differentiation of mammalian NPCs^{32,33}. We observed a 15-fold increase in the number of NEUROG2+ NPCs (SOX2+/NEUROG2+; Fig. 4, I and K) in *APP-KO* compared to control (Fig. 4M). This almost exactly corresponds to the ratio of neurons-to-progenitors produced by *APP-KO* NPCs, confirming that APP transforms cortical NPCs from self-amplifying progenitors to neurogenic progenitors.

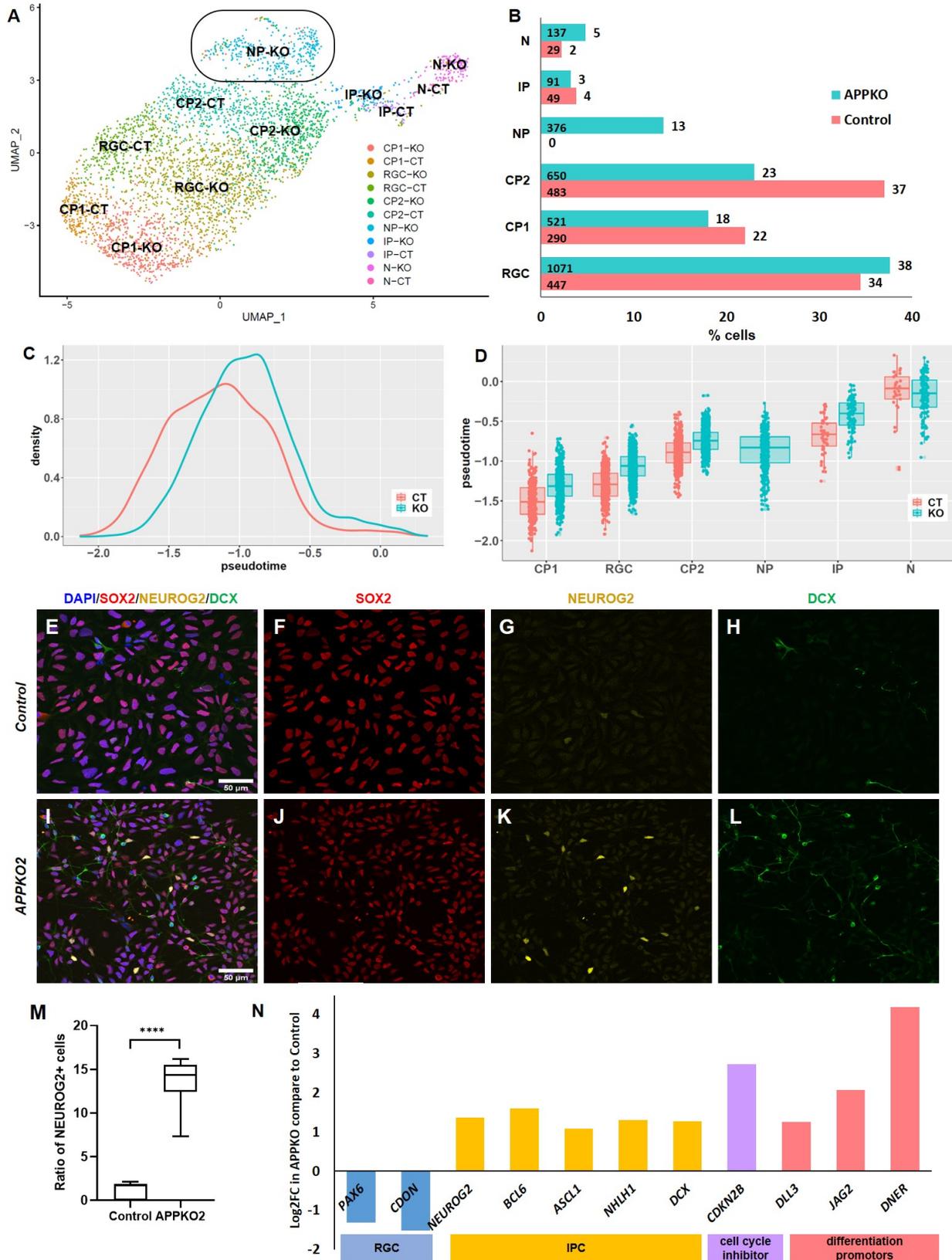


Fig. 4. Neurogenic progenitors explain the increased differentiation of APP-KO NPCs.

A, Clusters of control and *APP-KO2* NPCs (CT=control, KO=APPKO2). **B**, Emergence of a new “neurogenic progenitors” cluster in *APP-KO2*, number and percentage of cells in each cluster in control and *APP-KO2* (RGC=radial glial cell, CP1=cycling progenitor1, CP2=cycling progenitor2, IP=intermediate progenitor, NP=neurogenic progenitor, N=neuron). **C-D**, Pseudotime analysis show a shift toward neural fate in *APP-KO* clusters. **E-L**, Staining of control and *APP-KO2* NPCs for NEUROG2/SOX2/DCX. **M**, Ratio of NEUROG2+ cells is 15-fold higher in *APP-KO2* (unpaired t-test, $p < 0.0001$) with the majority being SOX2+/NEUROG2+ (I-K). **N**, Bulk RNAseq shows downregulation of RGC markers (*PAX6* and *CDON*) and upregulation of neurogenic genes (*NEUROG2*, *BCL6*, *ASCL1*, *NHLH1*, and *DCX*), cell cycle inhibitor (*CDKN2B*) and differentiation promoters Notch ligands *DLL3*, *JAG2*, *DNER*.

Loss of APP in cortical NPCs triggers the immediate early gene response to drive neurogenesis

To gain insight into the mechanisms of the gain of neurogenic state in the absence of APP, we performed bulk RNA sequencing in *APP-KO* and control NPCs. We observed 763 differentially expressed genes (558 up; 205 down including *APP* as expected) between control and *APP-KO2* NPC (Supplementary Table 3 and fig. S10, A and B). Consistent with the *APP-KO* phenotype, bulk RNAseq showed downregulation of primary progenitor’s markers *PAX6* and *CDON*, and upregulation of neurogenic genes *NEUROG2*, *BCL6*, *ASCL1*, *NHLH1*, and *DCX*. Moreover, *CDKN2B* a well-known cell cycle inhibitor and NPC differentiation promoters *DLL3*, *JAG2*, and *DNER* were also upregulated (Fig. 4N). One pathway whose activity is linked to APP function in a variety of organisms and neuronal contexts is the JUN N-terminal Kinase (JNK) pathway³⁴. It is a central stress signaling pathway whose effector proteins JUN and FOS are immediate early genes that combine to form the AP1 transcription factor. Some studies showed an important role of APP in the control of JNK/c-Jun signaling, target gene expression and cell death activation in response to stress. Kogel et al., showed APP-dependent transcriptional repression of c-Jun and reduced basal c-Jun N-terminal kinase (JNK) activity in PC12 cells³⁵. Stress-induced activation of the JNK signaling pathway and subsequent apoptosis were likewise reduced either by over expression of APP or treatment with exogenous sAPP α ³⁵⁻³⁷. In contrast, overexpression of the Swedish mutant of APP did not inhibit stress-triggered JNK activation and cell death³⁵. The protective effect of

APP/sAPP α could be mimicked by an MLK inhibitor that inhibits upstream JNK-activating kinases of the mixed lineage kinase (MLK) family³⁵ or by direct inhibitors of the JNK pathway³⁷. Moreover, APP is involved in transcriptional regulation of well-known members of stress response genes belonging to a large family of immediate early genes. Hendrickx et al., showed that APP epigenetically down regulates expression of some stress response genes including *c-Fos*. In the absence of App, *c-Fos* expression together with other stress response genes were also upregulated in prefrontal cortex of *App*^{-/-} mice compare to *App*^{+/+} mice^{38,39}. So, we hypothesized that loss of APP mimics stress and induce immediate early genes (IEGs) JUN and FOS to form the AP1 transcription factor AP1 and drive neurogenesis. Indeed, we found increased expression of *JUN* and *FOS* in *APP-KO* NPCs (1.3 and 2.7 Log₂FC, respectively; Fig. 5A), suggesting that APP also regulate their expression in human context. JUN is activated upon phosphorylation by JNK^{40,41,42}, and we observed a 1.5-fold increase in phospho-JUN in *APP-KO* NPCs compared to control (Fig. 5, B and C). Interestingly, while recent work in the immune system has shown that AP1 regulates the expression of BCL6⁴³ in germinal center B cells, in the developing mouse cortex BCL6, which we found to be upregulated in *APP-KO* NPCs, drives neurogenesis of cortical progenitors⁴⁴. Investigating the genes characterizing the *APP-KO* specific NP cluster showed enrichment for binding of JUN and JUND by chromatin immunoprecipitation (ChIP-seq) associated with several of these genes (ChEA 2016; Fig. 5, D and E). We also found consensus AP1 binding sites in the regulatory regions of genes upregulated in *APP-KO* NPCs, including *DCX* (Supplementary Table 4). To test whether AP1 activation might specifically underlie the increased neurogenesis phenotype, we treated *APP-KO2* NPC with a specific AP1 inhibitor (SR11302)^{45,46} and stained them for SOX2, NEUROG2 and DCX (Fig. 5, F to Q). While the ratio of SOX2⁺ cells remained unchanged after treatment (Fig. 5R), the number of NEUROG2⁺ and DCX⁺ cells was strongly rescued (Fig. 5, S and T). These results suggest that loss of APP cause premature differentiation through AP1 transcription factor and this pathway is not affecting proliferation of NPCs.

Fig. 5. Loss of APP increases differentiation of NPCs through affecting JNK pathway.

A, Upregulation of *JUN* and *FOS* in *APP-KO2* NPCs in bulk RNA sequencing results. **B-C**, Western blot shows significant increase in phospho-JUN in *APP-KO2* NPCs compared to control (n=3 biologically independent repeats, unpaired t-test, $P < 0.0001$). **D-E**, JUN family members bind to marker genes of neurogenic progenitors (NPs) in the ChEA 2016 CHIP-seq database. **F-Q**, NPCs treated with AP inhibitor (SR11302) and stained for SOX2/DCX/NEUROG2 showed **R**, no rescue of ratio of SOX2+ in *APP-KO2* treated with SR11302 compared to untreated *APP-KO2* NPCs (n=3, Ordinary one-way ANOVA, $p < 0.0001$ for control-DMSO vs *APP-KO2*-DMSO and *APP-KO2*-DMSO vs *APP-KO2*-SR11302). **S**, Significant decrease in NEUROG2+ cells in *APP-KO2* treated with SR11302 compared to untreated *APP-KO2* NPCs (n=3, Ordinary one-way ANOVA, $p < 0.0001$ for control-DMSO vs *APP-KO2*-DMSO and *APP-KO2*-DMSO vs *APP-KO2*-SR11302). **T**, Significant decrease in DCX+ cells in *APP-KO2* treated with SR11302 compared to untreated *APP-KO2* NPCs (n=3, Ordinary one-way ANOVA, $p < 0.0001$ for control-DMSO vs *APP-KO2*-DMSO and *APP-KO2*-DMSO vs *APP-KO2*-SR11302). Scale bar 50 μ m for all the images.

Premature differentiation is a stress response behavior

APP is known as stress response gene due to its elevated expression in brain injury and ischemia⁴⁷ and there are several studies showing that increased stress drives premature neurogenesis in human cortical progenitors^{48,49}. We hypothesized that loss of APP mimics stress-induced AP1 activation and premature neurogenesis. We asked whether *APP-KO* NPCs show features of stress such as cell death or defects in the endo-lysosomal pathway. We investigate cell death by immunostaining for cleaved caspase 3 and FACS for Annexin V. We observed significant increase in cell death in *APP-KO2* NPC compare to control in both experiments (fig. S11, A-D). Several studies suggest that loss of APP causes endolysosomal stress in fly⁷ and human cortical neurons⁵. Therefore, we first performed Western blot analysis to assess any possible alterations in proteins regulating the endo-lysosomal pathway, and we observed a significant and specific increase in LAMP1 (Lysosomal marker) while RAB5 (early endosome marker) and RBA7 (late endosome marker) remained unchanged (fig. S11, E-F). Moreover, we observed a 6 log₂FC increase in expression of the brain specific lysosomal marker, LAMP5, and two other components in endolysosomal pathway RAB30 and OPTN in bulk RNA seq data (fig. S11, G). Next, we performed

Electron Microscopy imaging on NPCs (fig. S11, H-I). We observed a significant increase in the ratio of multivesicular body (MVB; a transient structure between early and late endosome) size to cell size, number of MVB and empty vacuole (EV) in *APP-KO* compare to control (fig. S11, J-L, black arrow show MVB and white arrows point to EV). These results point to the fact that neural precursor cells are in a state of endo-lysosomal stress in the absence of APP.

Loss of APP causes less proliferation thorough canonical WNT pathway

We showed that loss of APP directs NPCs to the neurogenic state thorough activation of the immediate early genes (JUN and FOS). Blocking of AP1 can rescue the differentiation phenotype however, proliferation of NPC remain unchanged. Therefore, an additional mechanism must be involved in the proliferation of NPCs. Gene ontology (GO) and pathways analysis suggested that canonical WNT signaling may be altered in *APP-KO* NPCs (fig. S10,C to F), with upregulation of WNT inhibitors *DKK1*, *DKK3*, *APCDD1*, *ALPK2* (Fig. 6A). Since WNT activity is required in a variety of contexts for stem cell maintenance⁵⁰, we tested whether changes in WNT signaling could modify the *APP-KO* phenotype. We compared *APP-KO* NPCs treated with WNT3a for 48 hours to untreated control and *APP-KO* NPCs and stained them for SOX2 and DCX (Fig. 6 B to J). We observed a strong rescue of the loss of SOX2+ without reduction in DCX+ cells (Fig.6, K and L), suggesting that APP regulates the balance between NPC proliferation and differentiation through partially parallel mechanisms.

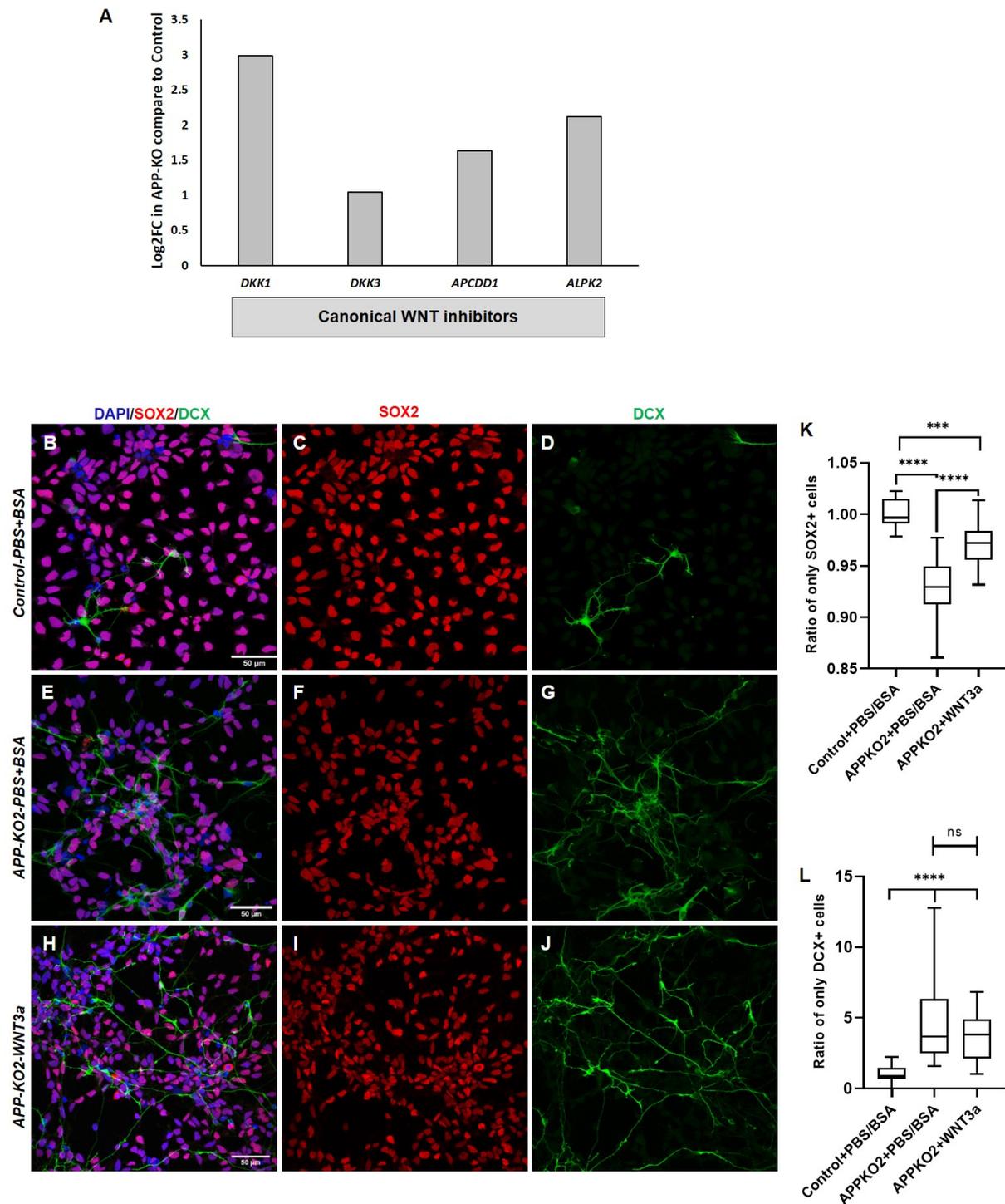


Fig. 6. Loss of APP decrease proliferation of NPCs through canonical WNT pathway.

A, Upregulation of canonical WNT inhibitors *DKK1*, *DKK3*, *APCDD1*, and *ALPK2* in *APP-KO2* NPC compared to control. **B-J**, *APP-KO2* NPCs treated with canonical WNT ligand (WNT3a) and stained for SOX2 and DCX.

K, Ratio of SOX2+ was significantly rescued in *APP-KO2* NPCs treated with WNT3a (n=3, Ordinary one-way ANOVA, $p < 0.0001$ for control-PBS+BSA vs *APP-KO2*-PBS+BSA and *APP-KO2*-PBS+BSA vs *APP-KO2*-WNT3a, $p = 0.0002$ for control vs *APP-KO2*-WNT3a). **L**, Ratio of DCX+ cells was not rescued after treatment with WNT3a (n=3, Ordinary one-way ANOVA, $p < 0.0001$ for control-PBS+BSA vs *APP-KO2*-PBS+BSA and control vs *APP-KO2*-WNT3a).

Discussion

APP is expressed during human cortex development but its function is unclear. Here, we present evidence supporting a specific requirement for APP during human cortical development. Loss of APP caused a striking increase in the generation of neurons in a very short time window from human cortical NPCs. Three observations suggest that human brain development may be particularly sensitive to loss of APP. First, there is strongly reduced tolerance to complete loss of APP in human genetic data^{14,15}, and second both *in vivo* and *in vitro* studies on mouse NPCs lacking APP have shown no consistent evidence for altered neurogenesis^{29–31}. Third, loss of APP affects the expression of genes located within human accelerated regions (HARs) which are conserved genomic loci with elevated divergence in humans. They are enriched for neural regulatory elements and they seem to play an important role in exceptionally developed human brain⁵¹. Doan et al., 2016, reported HARs regions related to cognitive disorders such as autism spectrum disorders (ASD)⁵². By comparing to their data set, we observed an upregulation ranging from 1.1 to 6.1 log₂FC in some of the HARs-related genes in *APP-KO* NPC such as ADAMTS14, AHRR, BNC2, CCDC102B, CDH11, CHST7, COL6A3, CPEB2, CYP1B1, DLC1, DOK5, EPHB3, FAM196B, FAM198B, FST, ITGA11, KCNIP2, KCTD12, LRRTM2, MASP1, MEIS2, MPPED2, NFIA, NFIB, NFIX, NTM, PDGFRB, POU2F2, SAMD4A, SAMD5, SOCS2, TGFB2, ZIC3. These results show that presence of APP is necessary for maintaining the specific features of human brain/cortex acquired during evolution. We also did not observe an effect on human motor neurogenesis, suggesting the human cortical neurogenesis is particularly sensitive to loss of APP. We speculate that this greater requirement for APP in the human cortex is not due to changes in the highly-conserved APP protein itself but to the context of human cortical neurogenesis, especially the evolutionary pressure to maintain human cortical progenitors in a proliferative undifferentiated state for a

relatively long time during embryogenesis. Several studies suggest that increased stress drives premature neurogenesis in human cortical progenitors^{48,49}. APP is a conserved stress response protein, as are JUN and FOS. Therefore, the absence of APP may mimic cellular stress, activate AP1-mediated responses and drive premature neurogenesis. Whether this enhanced requirement for APP in humans may also offer a clue into fAD is worth further investigation. We note that we observed an upregulation of the *MAPT* gene, encoding the other key AD protein Tau, in *APP* mutant progenitors (Supplementary Table 3). It is tempting to speculate that fAD mutations in APP have subtle effects on early human cortical development that cause reduced cortical robustness and resistance to a lifetime of neuronal and glial stress eventually contributing to premature neurodegeneration.

Acknowledgements

This work was supported by the Investissements d'Avenir program (ANR-10-IAIHU-06), Paris Brain Institute-ICM core funding and the Roger De Spoelberch Foundation Prize (to B.A.H.). Tengyuan Liu is funded by the Chinese Scholarship Council (CSC). We thank Natalia Baumann and Dr. Denis Jabaudon for sharing the pseudotime analysis code, Dr. Bart De Strooper for sharing the anti-APP antibody, Drs. Stephane Nedelec, Joris De Wit, Denis Jabaudon, members of the Hasan lab as well as Antoine Graindorge, and Stuart Edelstein from Scipio biosciences for helpful discussions. Scipio bioscience was supported by the Investissements d'Avenir program and the Région Île-de-France. This work benefited from equipment and services from the ICM's genotyping and sequencing core facility (iGenSeq). Mouse breeding work was conducted at the PHENO-ICMice facility. The Core is supported by 2 Investissements d'Avenir grants (ANR-10-IAIHU-06 and ANR-11-INBS-0011-NeurATRIS) and the "Fondation pour la Recherche Médicale". iPSC work was carried out at the CELIS core facility with support from Program Investissements d'Avenir (ANR-10-IAIHU-06) Light microscopy was carried out at the ICM.Quant facility. We thank all core technical staff involved and in particular Stephanie Bigou from CELIS for her advice on iPSCs and CRISPR/Cas9 work and Claire Lovo from ICM.Quant for help with image analysis.

Author contribution

Kh. Sh., C.P., and B.H. conceived the project, interpreted the data, and wrote the manuscript with input from all authors. Kh.Sh. designed and performed experiments and data analysis for most of the project. J.P. performed experiment on NPC derived from second iPSC line and 3D culture derived from both iPSC lines. M.B.T.Z performed the viral approach experiment. T.L performed mouse embryo staining. J.K. performed sample preparation for single cell RNA-seq A.S. and C.P performed single cell data analysis. N.D. generated the vectors for rescue experiment. E.L. and D.B. performed motor neuron culture and staining. R.L. participated to optimize iPSCs differentiation into cortical neurons protocol

Statement of competing interest

Azadeh Saffarian and Jun Komatsu are employees of Scipio biosciences.

Material and Methods

iPSC culture and maintenance

iPSC cell lines WTS002 (iPSC line1) and WTS008 (iPSC line2) were purchased from EBISC (European bank for induced pluripotent cells). They were maintained on Geltrex LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (ThermoFisher Scientific) in Essential 8™ Flex Media Kit (ThermoFisher Scientific) with 0.1% penicillin/streptomycin. Cultures were fed every other day and passaged every 5–7 days by ReLeSR™ (STEMCELL Technologies).

Creating *APP-KO* clones by CRISPR/Cas9

iPSC line1

Four guide RNAs were designed by Crispor (<http://crispor.tefor.net/>) to target the APP first exon and cloned in the vector pCAG-CAS9-GFP (Extended data Fig. 1a, b). To evaluate the cleavage efficiencies of the guide RNAs, HEK293 cells were transfected by expression vector p-CAG-Cas9-GFP containing four guide RNAs (Extended data Fig. 1c). DNA extraction and PCR were performed 48 hours post transfection for the cleaved region. Sequenced PCR products were analyzed in TIDE (Tracking of Indels by Decomposition) to obtain the cleavage efficiency. The best guide RNA with 8.5% total efficiency and lowest off-target was chosen for further experiments (Extended data Fig. 1d). The iPSC line was transfected by the expression vector containing the best guide RNA using Lipofectamin stem reagent (ThermoFisher Scientific). GFP positive cells isolated by FACS 48 hours post-transfection and seeded at low density. A total of 118 clones were picked after 5 days, expanded, and 60 screened for possible mutation (Extended data Fig. 1e-h), and 16 out of the 60 clones had homozygous mutations for APP. These mutations are classified as: 1, 2, 3, 20, 22, 33bp deletions and 1bp and 2bp insertions (Supplementary Table 5). The absence of expression of the APP protein was confirmed by western blot in 14 clones (Extended data Fig. 1i). The efficiency of producing APP knock-out clones was 23% (Figure 1j). Three APP knock out clones were chosen for further experiments and low APP mRNA levels were confirmed by quantitative-PCR (Extended data Fig. 1k). The control, which was chosen for further experiments, expressed guide RNA and CAS9 but the APP gene remained un-mutated. The pluripotency of *APP-KO* cells and the control were confirmed by OCT4 staining (Extended data Fig. 2 d-g).

iPSC line2

The guide RNA 1 and the ribonucleoprotein approach were used to generate *APP-KO*. 1×10^6 iPSCs were nucleofected with the RNP complex from IDT (225pmol of each RNA and 120pmol of Cas9 protein). The iPSCs were plated 48 hrs later at very low density (10 cells/cm²) on Laminin-521 with Cloner supplement (Stem Cell Technology) for clonal selection. One week later, iPSC clones were picked under a stereomicroscope and cultured on Laminin-521 in 96 well plates (Duscher). The resulting iPSC clones were duplicated after confluency and used for cryoconservation and DNA extraction. One base-pair deletion was observed in screening results and the absence of expression of APP protein was confirmed by western blot. The APP knock out clone and control were used for further experiments and the low level of APP mRNA was confirmed by quantitative-PCR (Extended data Fig. 1l, m, n).

Cortical differentiation of human iPSC

Three *APP-KO* and one *APP-WT* control clones underwent a cortical differentiation protocol to produce cortical neurons in 2D. The protocol is divided into 2 main stages: neural induction and neural differentiation. At day 0, iPSCs were detached by Accutase (ThermoFisher Scientific) and transferred to T75 ultra-low attachment flasks (VWR) in Essential 8 medium with 0.1% penicillin/streptomycin and Stemgent hES Cell Cloning & Recovery Supplement (Ozyme, 01-0014-500) to form Embryoid body (EB). At day 1, the medium was switched to EB medium contain DMEM/F12 Glutamax, 20% knockout serum replacement, 1% nonessential amino acids, 1% penicillin/streptomycin, 0.55mM 2-mercaptoethanol and supplemented with Dorsomorphin (1μM, Sigma-Aldrich P5499), SB431542 (10μM, Abcam ab120163) for 8 days. At day 8, Embryoid bodies (EBs) were collected and seeded on Geltrex and maintained for 6-8 days in Neurobasal without vitamin A medium and B27 without vitamin A supplemented with Human EGF(10ng/ml) and human FGF2 (10ng/ml). Cells were fed every day from day 0 to day 16. Rosettes were manually picked and dissociated with Accutase, then seeded on poly-ornithine and laminin-coated dishes for expansion. They were maintained with passage for two additional weeks to achieve a large pool of neural precursor cells (NPCs) (Extended data Fig. 2 a-c). Characterization for NPCs was performed by staining against Nestin, SOX2, SOX1, and PAX6 (Extended data Fig. 2

h1-k6). NPCs were seeded on 24-well plates with coverslips and the day after seeding, the medium was switched to the B-27 Plus Neuronal Culture System (ThermoFisher Scientific) supplemented with Ascorbic acid. Cortical neurons were stained for early-born neural markers and late-born neural markers at different time point. They were kept for 3 to 4 months in culture and stained for synaptic markers at 60 days and 120 days post differentiation.

Motor neuron differentiation and staining

Control and mutant iPSC clones were differentiated into motor neurons as described²⁵. In summary, after dissociation of EBs containing motor neuron progenitors at D10, single cells were plated on poly ornithine-laminin coated coverslips at 2×10^5 cells/coverslip for 1, 4 and 7 more days. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes RT. For staining, cells were treated with 5 % goat serum with 0.1% Triton X100 (Thermofisher Scientific) for 1hour at RT, and then incubated overnight at 4°C with a mouse anti- β III-Tubulin (TUJ1, Sigma, 1/500) and a rabbit anti-ISLET1 (Abcam, 1/100) antibodies in 5% goat serum. Secondary antibodies (goat anti-mouse IgG2a-Alexa 555 (1/2000) and goat anti-rabbit IgG Alexa 488 (1/2000) from ThermoFischer Scientific) were incubated one hour at RT with Hoechst33342 to stain nuclei.

Neural differentiation follow-up by viral vector

Neural differentiation follow-up was performed by the viral vector approach described in previous studies^{23,24}. *APP-KO* and control neural precursor cells were seeded on poly ornithine-laminin coated coverslips and transduced by virus Lenti-Synapsin-GFP. The medium was changed to fresh Neurobasal without vitamin A and B27 without vitamin A supplemented with EGF and hFGF2 to wash out lentiviruses one day after initial infection. When the GFP+ cells appeared, it was considered as day 0 and the medium was switched to differentiation medium (B-27 Plus Neuronal Culture System) and kept for 7 and 30 days. Lentiviral expressing cells were detected by anti-GFP antibody and cell type was determined by anti-SOX2 and anti-DCX antibodies.

Immunocytochemistry

Cells were fixed in PFA 4% and incubated for 30 min in blocking buffer (phosphate-buffered saline and Triton X-100 0.3% and 5% horse serum). Primary antibodies (Supplementary Table 6) were diluted in antibody solution (phosphate-buffered saline and Triton X-100 0.3% and 5% horse serum) and applied overnight at 4°C. After three washes in phosphate-buffered saline, secondary antibodies conjugated to Alexa fluorophores (Molecular Probes, Eugene, OR, USA) were diluted at 1:1000 in blocking buffer and applied for 1 hr at room temperature. Cells were washed in phosphate-buffered saline followed by nuclear staining by DAPI (Sigma) for 10 min. Cells were washed three more times and mounted by VECTASHIELD® Mounting Medium-Vector Laboratories. Confocal image acquisition was performed using Olympus FV1200 and SP8 Leica DLS.

Generation of cortical organoids and immunohistochemistry

Cerebral organoids were generated from human iPSCs using a previously reported protocol⁵³ with some modifications. The hiPSCs were incubated with Accutase (Life Technologies, A11110501) at 37 °C for 7 min and dissociated into single cells. In order to obtain uniformly sized spheroids, approximately 3×10^6 single cells were added per well in the AggreWell 800 plate (STEMCELL Technologies, 34815) with Essential 8 flex medium supplemented with Stemgent hES Cell Cloning & Recovery Supplement (1X, Ozyme, STE01-0014-500) and incubated at 37°C with 5% CO₂. After 24 hr, spheroids from each microwell were collected by firmly pipetting medium (with a cut end of a P1000 tip) in the well up and down and transferring it into Corning® non-treated culture dishes (Merck, CLS430591-500EA) in TeSR™-E6 (StemCell Technologies, #05946) supplemented with two inhibitors of the SMAD signalling pathway, dorsomorphin (2.5 μM, Sigma-Aldrich, P5499) and SB-431542 (10 μM, Abcam, ab120163). From day 2 to day 5, TeSR™-E6 supplemented with dorsomorphin and SB-431542 was changed every day. On the sixth day in suspension, neural spheroids were transferred to neural medium containing Neurobasal minus vitamin A (Life Technologies, 10888), B-27 supplement without vitamin A (Life Technologies, 12587), GlutaMax (1%, Life Technologies, 35050), Penicillin-Streptomycin (1%, Life technologies, 15140122) and 2-mercaptoethanol (5mM, Life technologies, 31350010). The neural medium was

supplemented with 10 ng/mL epidermal growth factor (PreproTech, AF-100-15) and 10 ng/mL basic fibroblast growth factor (R&D Systems, 234-FSE-025) for 19 d (until day 24), with medium changed daily during the first 10 days and every other day for the subsequent 9 days. At day 15, spheroids were fixed with 4% paraformaldehyde in PBS overnight at 4°C. Following 2 washings with PBS + 2% Triton-X100 for 2 hr, spheroids were treated overnight at RT with PBS + 2% Triton-X100 + 2% Tween20 + 20% DMSO. Blocking/permeabilization was performed with PBS + 10% Horse serum, 3% BSA, and 2% Triton-X100 for 24 hr at RT. Primary antibodies, (Sox2 1/500, Milipore AB5603) and (DCX 1/2000, Milipore AB2253), were incubated in the same solution supplemented with 0.05% Azide for at least 3 days at 4°C. After multiple washing with PBS + 0.5% Tween-20 until the next day, spheroids were then incubated for at least 3 days with secondary antibodies. After 2 days of washing with PBS + 0.5% Tween-20, samples were cleared overnight in RapiClear 1.49 (SunJin lab) before mounting on cavity slides. Samples were imaged using confocal microscope Nikon A1R HD25 with a 10X objective (MRD71120).

Embryo collection, immunohistochemistry, and antibodies

E10.5 embryos were collected from APP-wild type or APP-knockout pregnant mice, whole embryos were fixed in 2% paraformaldehyde (PFA) in PBS at 4 °C for 2-3 hours, then dehydrated in 30% sucrose in 1X PBS overnight (o/n). After all the samples sank into the bottom of the tube, they were embedded in OCT compound (TissueTek) and frozen at -20 °C. Sagittal sections were performed by cryostat (Leica) at 20 µm and then slices stored at -80 °C. For the immunostaining, sections were fixed with 4% PFA for 10 minutes at RT, then blocked with 10% normal donkey or goat serum in 1 X PBS with 0.1% Triton (PBT) for 1 hour at RT followed by 3 washes in 1 X PBT. Thereafter, these sections were incubated with primary antibodies diluted in 0.1% 1 X PBT containing 1% normal donkey or goat serum o/n at 4 °C or 3-4 hours at RT. After 3 washes with 1X PBT, samples were incubated with appropriate secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 (1:500, Invitrogen) in 0.1% 1 X PBT containing 1% normal donkey or goat serum for 1-2 hours at RT. After washing with 1X PBT for 3 times, then counterstained with DAPI (1:2000, Sigma), the slides were mounted by using Vectashield (Vector) after rinsing. Primary antibodies used in this study were mouse anti-TUJ1 (1:300, Biolegend:

801202), rabbit anti-Pax6 (1:300, Biolegend: PRB-278P). After staining, images were obtained by using confocal microscope Olympus FV-1200.

qPCR

The cells were lysed directly in the wells by addition of 300 μ l Buffer RLT supplemented with 15 mM beta-mercaptoethanol (Thermo Fisher Scientific) after a wash with Dulbecco's Phosphate-Buffered Saline (DPBS, Life Technologies). Total RNAs were isolated using the RNeasy Mini extraction kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. RNA levels and quality were quantified using a Nanodrop spectrophotometer. cDNA synthesis was performed by Thermo Scientific Verso cDNA Synthesis Kit and Quantitative PCR assay was performed by Sybergreen Gene Expression Assays in triplicate wells of 96-well plates. Primers are listed in supplementary Table 7 and $2^{-(Cp\ GOI - Cp\ internal\ gene)}$ was used for analysis. Housekeeping gene (GAPDH) was selected to control variation in cDNA amounts.

Bulk RNA sequencing and analysis

The cells were lysed directly in the wells by addition of 300 μ l Buffer RLT supplemented with 15 mM beta-mercaptoethanol (Thermo Fisher Scientific) after a wash with Dulbecco's Phosphate-Buffered Saline (DPBS, Life Technologies). Total RNA was isolated using the RNeasy Mini extraction kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. RNA levels and quality were quantified using a Nanodrop spectrophotometer. RNA sample purity/integrity was assessed using an Agilent 2200 TapeStation. mRNA library preparation was completed following manufacturer's recommendations (KAPA mRNA hyperprep ROCHE). Final samples pooled library prep were sequenced on Nextseq 500 ILLUMINA with MidOutPut cartridge (2x130Million 75 base reads) with 1 run, corresponding to 2x20Million reads per sample after demultiplexing. The quality of raw data was evaluated with FastQC. Poor quality sequences were trimmed or removed with fastp software to retain only good quality paired reads without adapters. Star v2.5.3a⁵⁴ was used to align reads on the hg19 reference genome using standard options. Quantification of gene and isoform abundances was carried out with rsem 1.2.28⁵⁵, prior to normalisation on library size with the edgeR⁵⁶ bioconductor package. Finally, differential analysis was conducted with the glm framework likelihood ratio test from edgeR. Multiple

hypothesis adjusted p-values were calculated with the Benjamini-Hochberg⁵⁷ procedure to control FDR.

Single cell RNA sequencing and analysis

For each control and knockout cell lines, single cell samples were prepared for 3' mRNA sequence determinations using the Scipio bioscience protocol (to be published) with barcoding beads (NxSeq Single-cell RNA-seq Beads, LGC Biosearch Technologies). For beads that captured mRNA molecules, reverse transcription, PCR amplification of cDNA, and sequencing library preparation were performed according to published procedures⁵⁸. A total of 35,106,546 reads, 56,108,512 reads, and 54,702,467 reads were generated by NovaSeq from two replicates of *APP-KO2* (KO2-1 and KO2-2) and one CT (control) sample, corresponding to 2000, 2000, and 1500 cells. The reads passed QC process using FASTQC v0.11.8. Sample analyses were performed using UMI-tools v1.0.0. Reads were aligned to GRCh38.94 using STAR v2.7 with default parameters. FeatureCounts v1.6.4⁵⁹ (Ensembl GRCh38.94.GTF) was used to count the number of aligned reads per feature, followed by umi_tools dedup to collapse those reads belonging to a barcode and mapping the same position of a gene. To build the count table umi_tools count was applied. Seurat V3.1.4⁶⁰ was used: i) to filter cells having more than 200 genes and less than 20% mitochondrial genes, with 1326, 1594, and 1299 cells passing the QC filters for the KO2-1, KO2-2, and CT samples, respectively.; ii) to pool both KO samples (2920 total); iii) to cluster KO and CT cells using Seurat clustering with 10 PCA dimensions and resolution 0.5; iv) to identify cluster markers using FindAllMarkers with default parameters. Visualizations were performed with Seurat and ggplot2 packages. DEGs in KO vs CT were analyzed for enrichment in gene ontology, biological and disease associations using Enrichr tools to further explore ChIP-seq datasets linking transcription regulators with DEGs⁶¹.

Rescue experiment

Neural precursor cells of Control and *APP-KO2* were transfected with *pPB-CAG-IRES-EGFP* and *APP-KO2* NPC were transfected by *pPB-CAG-hAPP- IRES -EGFP* and kept for 4 days. Cells were

fixed and stained for GFP/SOX2/DCX. The GFP+ cells were quantified, and cell type was determined by SOX2 and DCX as progenitor and differentiated cell, respectively.

Neural precursor treatment

WNT3a (R&D Systems) and SR11302 (TOCRIS) were used at final concentrations of 150 ng/ml and 10 μ M, respectively. Neural precursor cells of control and *APP-KO2* were treated for 48 hours and then stained for SOX2 and DCX.

Flow cytometry

Cell death was assessed using FITC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend, 640922). Cells were washed twice with cold PBS and then suspended in Annexin V Binding Buffer at a concentration of 0.25-1.0 x 10⁶ cells/ml. 100 μ l of cell suspension was transferred in a 5 ml test tube. 5 μ l of FITC Annexin V and 7-AAD Viability Staining Solution were added, respectively. After gentle vortex, the cells were incubated for 15 min at room temperature (25°C) in the dark. Finally, 400 μ l of Annexin V Binding Buffer was added to each tube. Flow cytometry analysis was performed at 488 and 650 nm to detect Annexin V and 7-AAD.

Western blot

Cells were placed on ice, lysed directly in wells by adding RIPA buffer (Sigma) supplemented with Complete protease inhibitor cocktail (Roche) and agitated for 20 min. Thereafter, the samples were collected and centrifuged at >14000 g, 30min at 4 °C and the supernatant was transferred to a fresh tube. Protein determination was performed using the Pierce™ Detergent Compatible Bradford Assay Kit (ThermoFisher Scientific). Sample buffer (BOLT LDS, Life technologies) was added to equal amounts of protein, and samples were loaded onto a BOLT 4–12% Bis-tris gel and transferred using MES buffer (all from Life technologies). Proteins were blotted onto a 0.2 μ M nitrocellulose membrane (GE Healthcare) using semi-dry technique. Membranes were blocked in 5% Nonfat-Dried Milk bovine (Sigma) and incubated over night at 4 °C with primary antibody. After washing, membranes were incubated with HRP-conjugated secondary antibodies (supplemented table) for 1 hour at room temperature. Protein detection was performed by

Pierce ECL Western Blotting substrate (ThermoFisher Scientific) and bands were visualized using ChemiDoc™ Touch Imaging System (BioRad laboratories). Band intensities were calculated using Image Lab software.

Statistical analysis

Statistical analysis was performed by GraphPad Prism. Data in figure panels reflect 3 independent experiments performed on different days. An estimate of variation within each group of data is indicated using standard error of the mean (SEM). We performed unpaired t test for assessing the significance of differences in the analyses containing two conditions, one-way ANOVA correction in the analyses containing more than three conditions and two-way ANOVA in the group analysis.

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Fig. S1. Generation of iPSC derived APP knockout clones.

A-K, Individual steps in generating *APP* knock out from iPSC line1. **A**, Targeting exon1 of *APP* gene by **B**, Plasmid vector containing guide RNA, CAS9 and GFP. **C**, HEK293 cell 24 hours after transfection. **D**, Cleavage efficiency of Guide RNA1 that was used for transfection of iPSC. **E-H**, iPSC transfection by Guide RNA1, post FACS morphology and sequencing results of clones. **I**, Western blot results, which confirm no expression of APP protein in 14 out of 60 clones. Control and three *APP-KO* clones were chosen for further experiments. **J**, Distribution of clones with different *APP* genotypes (23%=*APP-KO*, 23%=*APPWT* and 53%=heterozygote and 1%=other mutation). **K**, qPCR confirms low level of *APP* mRNA expression in *APP-KO* clones. **L-N**, Generation of *APP-KO* clones from iPSC line 2 and confirming no expression of APP protein.

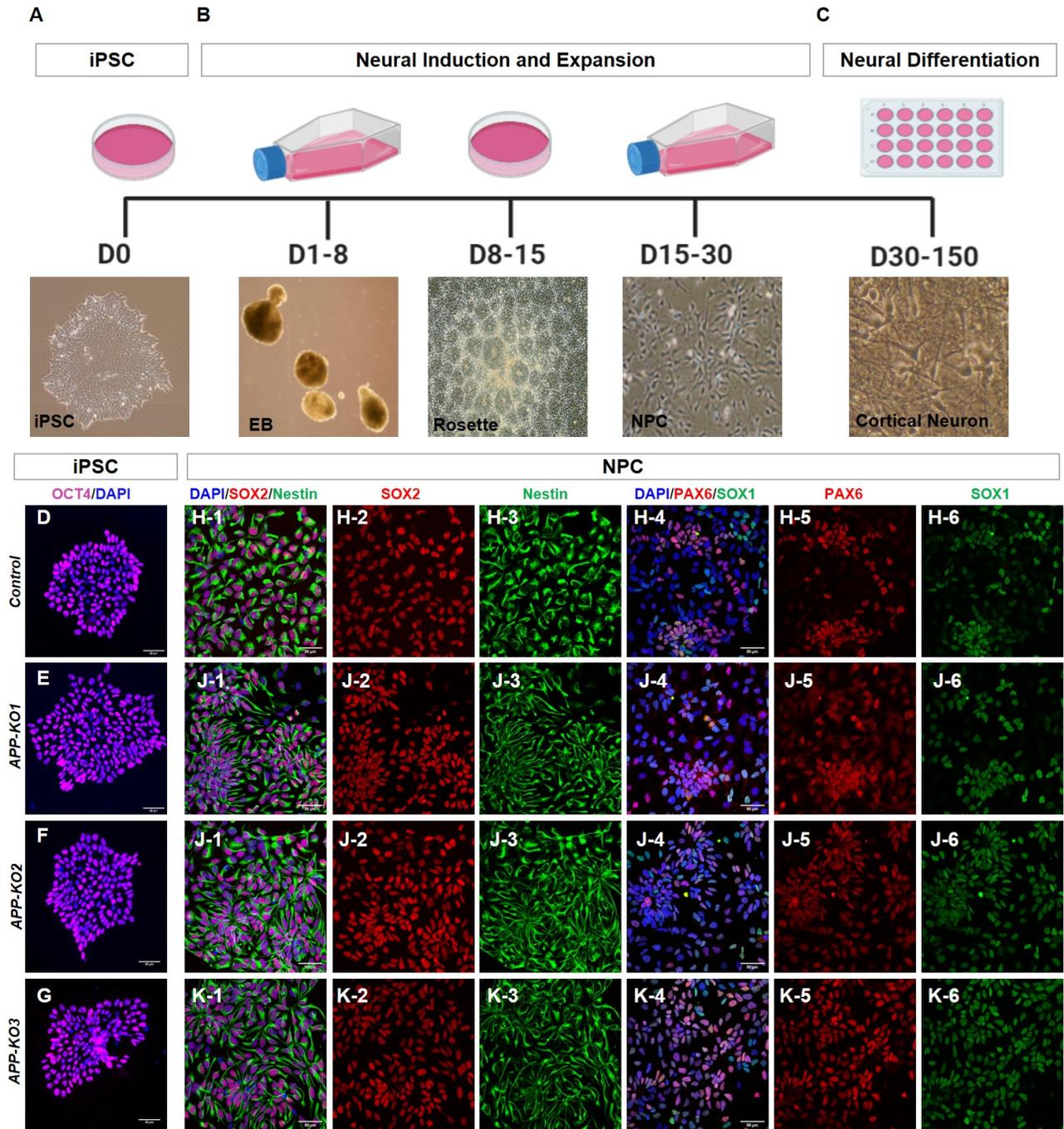


Fig. S2. Cortical differentiation protocol and characterization of neural precursor cells.

A-C, Schematic showing different steps in generating cortical neurons. **D-G**, Confirming pluripotency of control and *APP-KOs* iPSCs with pluripotency marker OCT4. **H1-K6**, Characterization of control and *APP-KOs* derived neural precursor cells with markers: Nestin, SOX2, PAX6, SOX1 (scale bar 50µm).

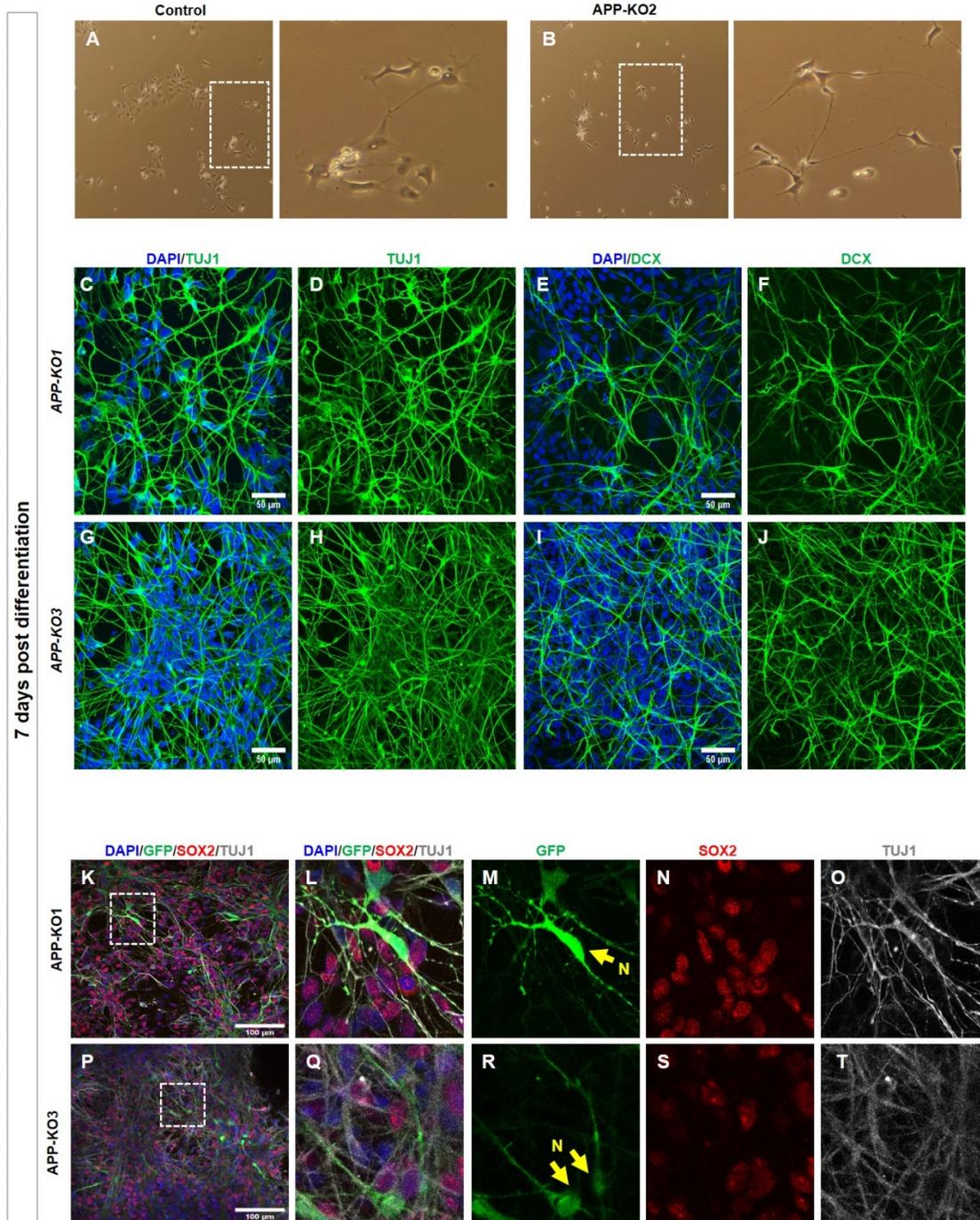


Fig. S3. Increased differentiation of APP-KO1 and APP-KO3 neural precursor cells within 7 days post-differentiation.

A-B, Morphological changes 7 days post-differentiation neurons for control and *APP-KO2*. **C-J,** Appearance of neural markers TUJ1 and DCX occurring earlier in *APP-KO1* and *APP-KO3* compared to

control 7 days post-differentiation (Scale bar 50 μ m). **K-T**, Expression of SOX2 and TUJ1 within GFP+ cells in *APP-KO1* and *APP-KO3* (Scale bar 100 μ m, N=neuron).

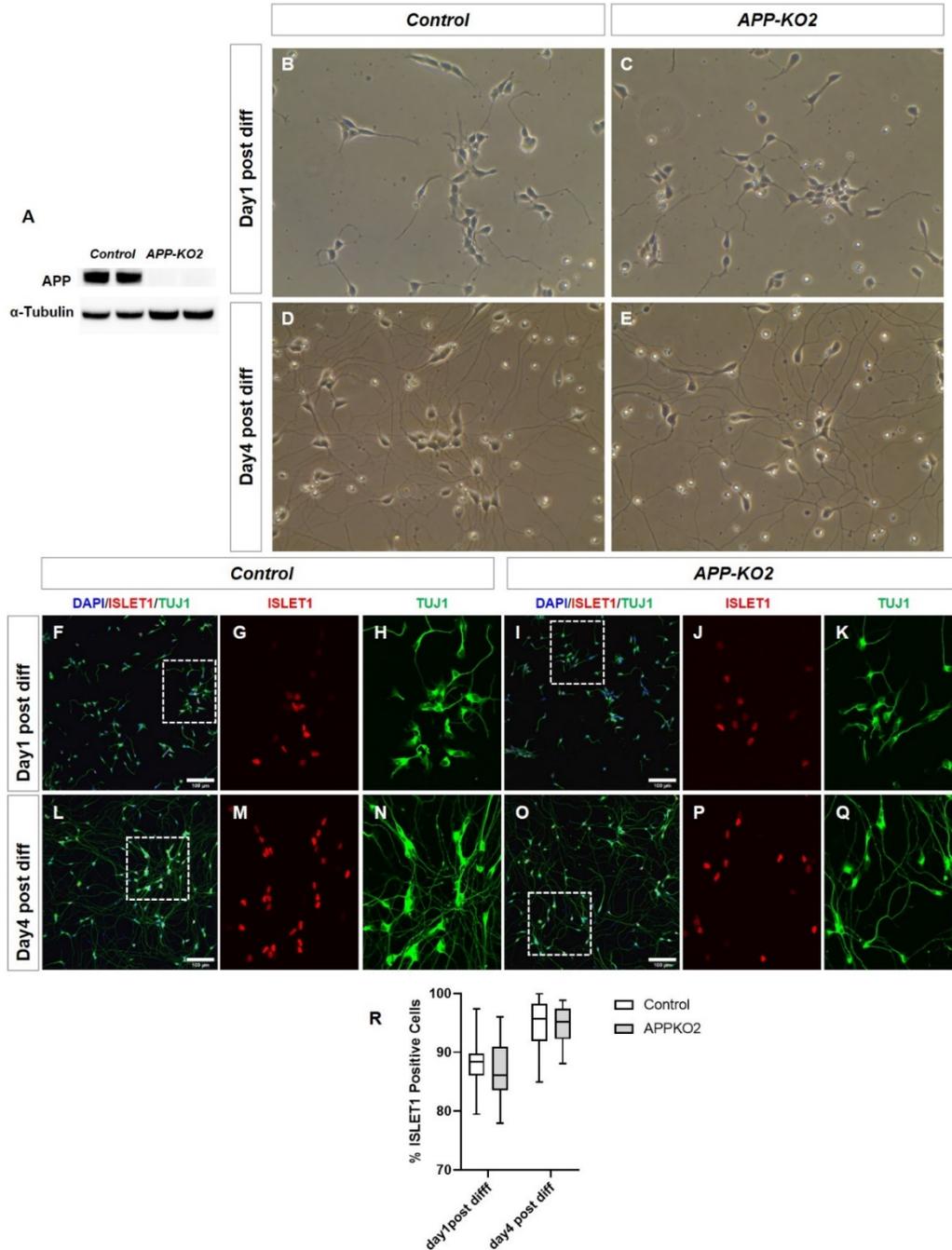


Fig. S4. No differences in ISLET1 motor neurons derived from control and APP-KO2.

A, APP expression was confirmed in control motor neuron precursor cells by western blot. **B-E**, Morphological changes in 1 day and 4 days post-differentiation motor neurons. **F-Q**, 1 day and 4 days post-differentiation motor neuron stained for ISLET1 and TUJ1 (scale bar 100 μ m). **R**, No significant

differences were observed in the percentage of ISLET1+ cells at 2 different time points, 1 day and 4 days post-differentiation (n=3 biological independent repeat, 2way ANOVA).

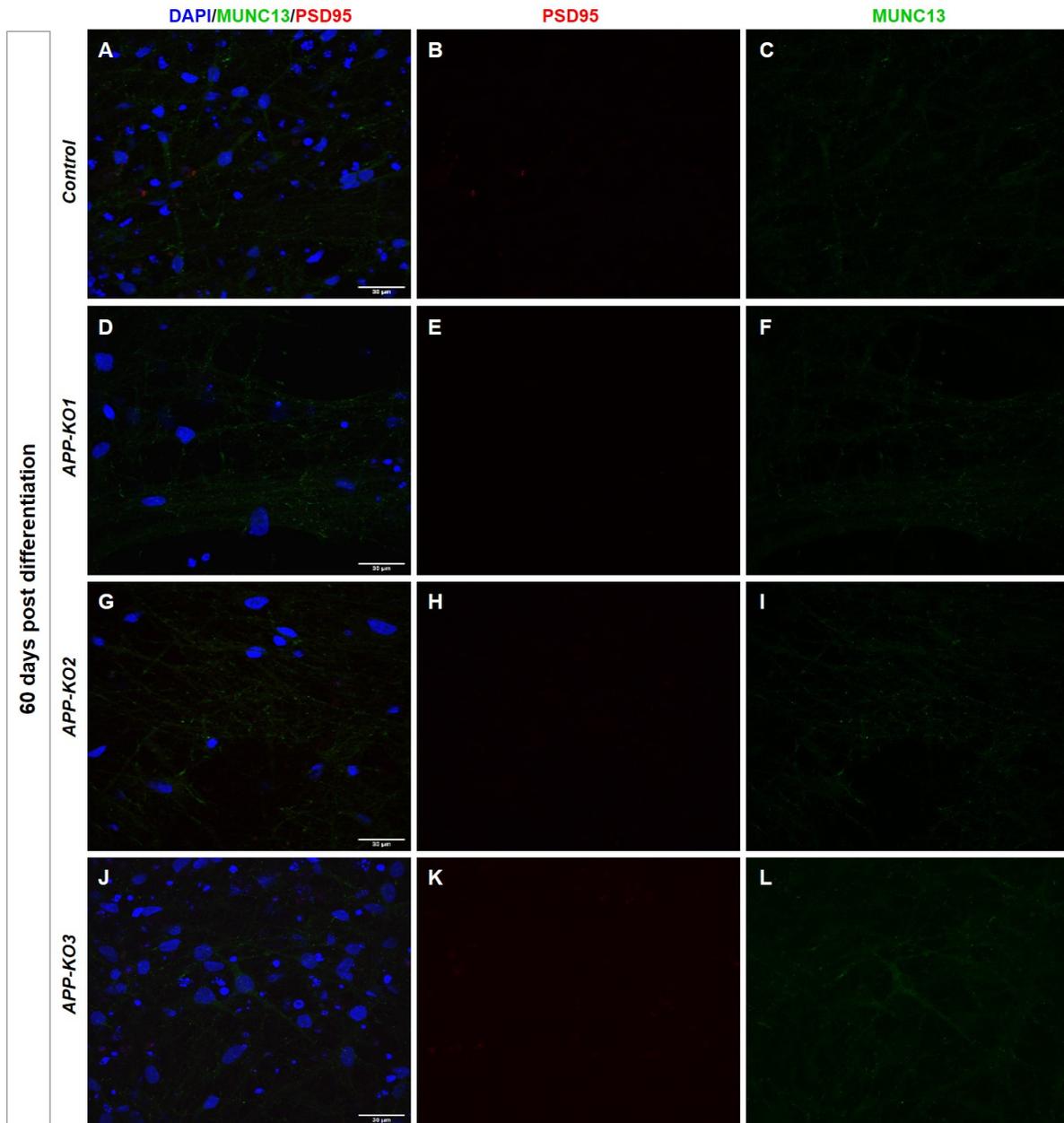


Fig. S5. Presynaptic markers begin to be expressed 60 days post differentiation in both APP-KO and controls.

A-L, Weak MUNC13 (presynaptic marker) in control and all 3 *APP-KOs*, while no expression of PSD95 (postsynaptic marker) expression was detected in either condition at this stage (scale bar 30 μ m).

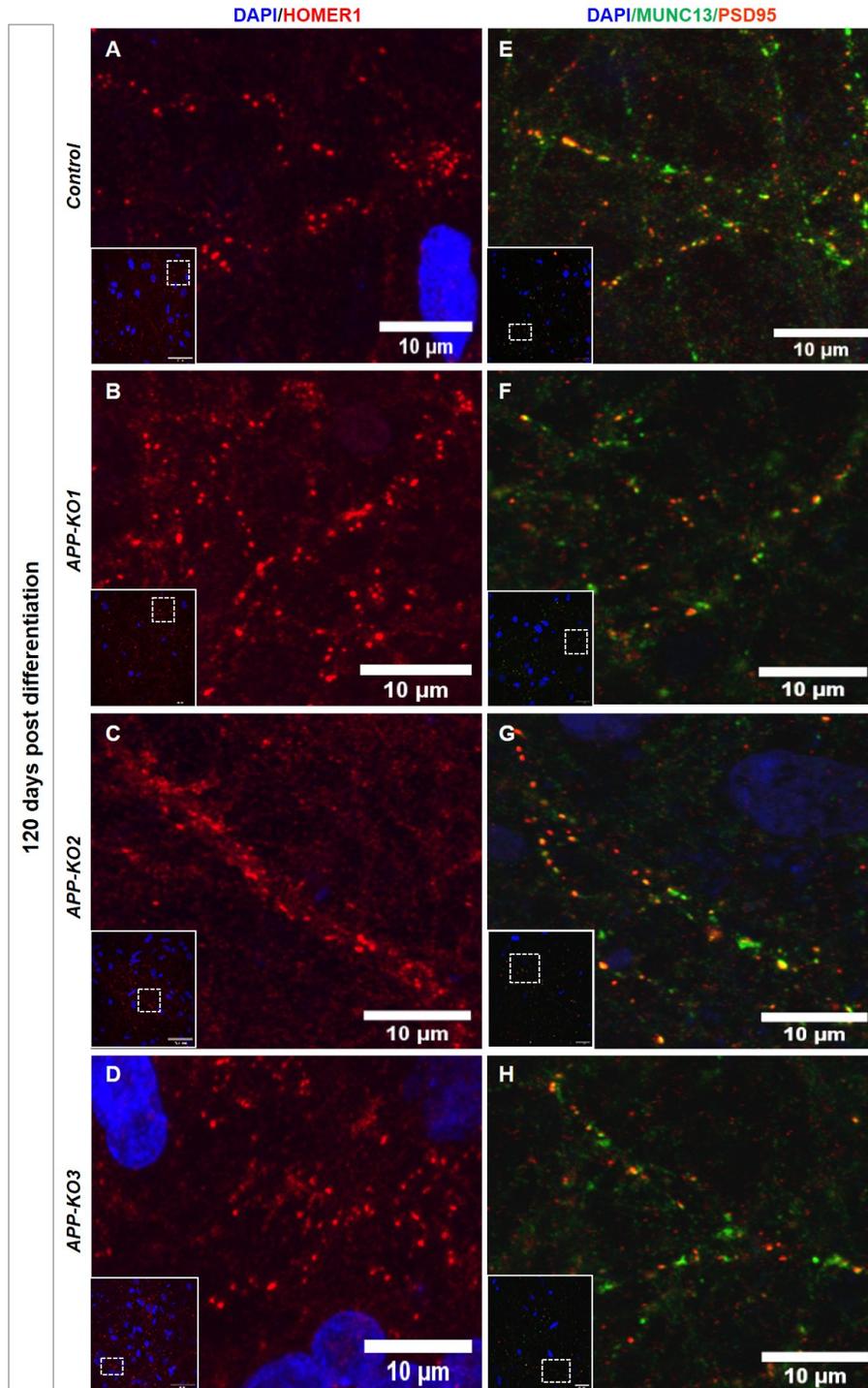


Fig. S6. Pre- and post- synaptic markers are expressed 120 days post differentiation in both APP-KO and controls.

A-D, HOMER1 (synapse marker of glutamatergic neuron), E-H, MUNC13 (presynaptic marker) and PSD95 (postsynaptic marker) show the same pattern 120 days post differentiation in controls and three *APP-KOs* (scale bar 10μm).

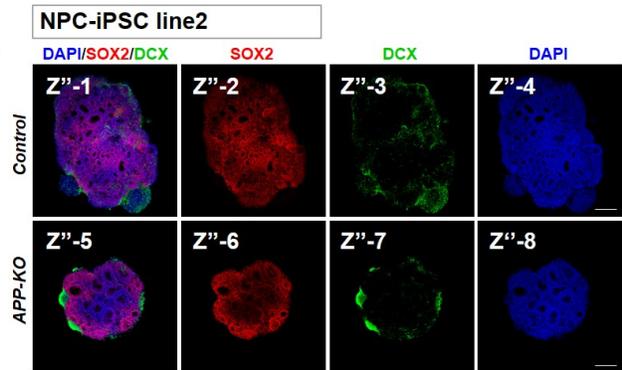
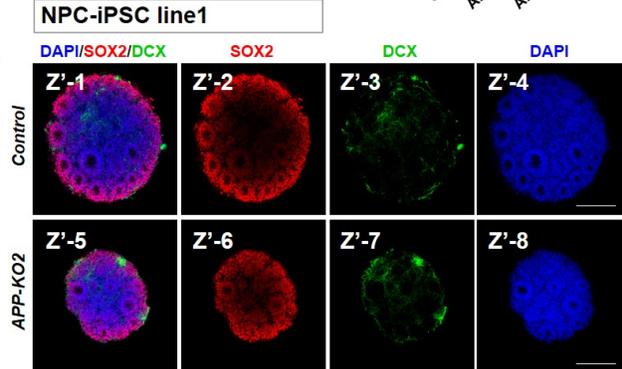
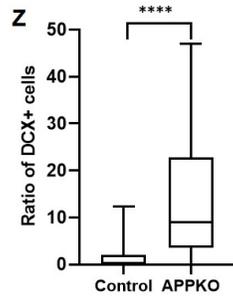
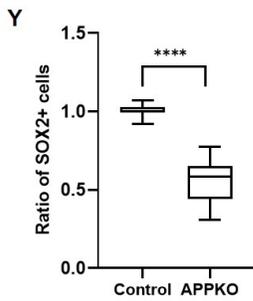
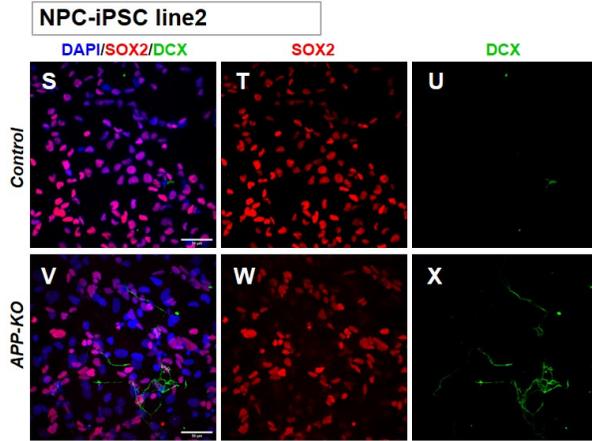
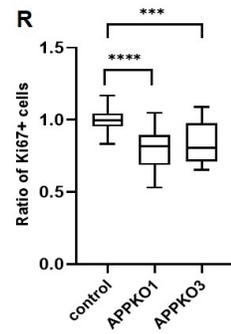
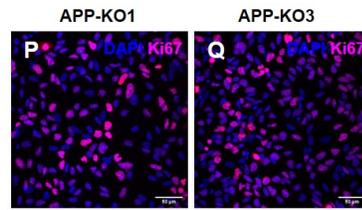
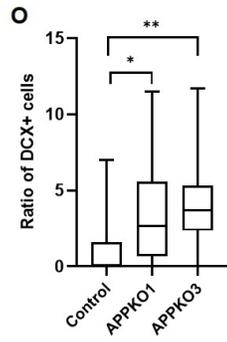
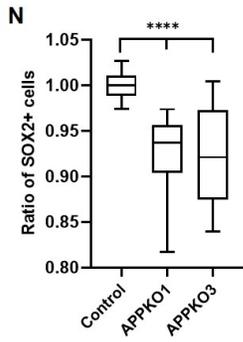
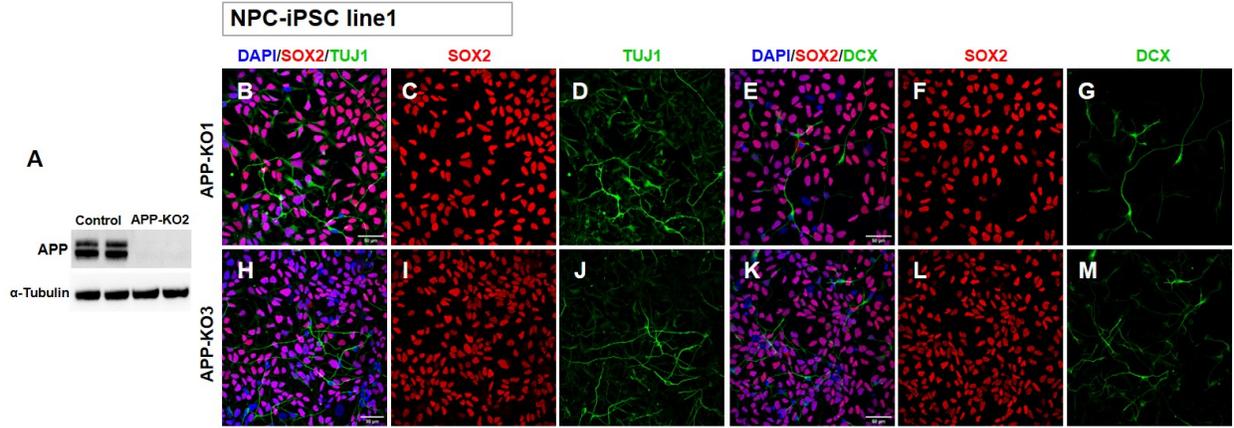


Fig. S7. Loss of APP causes increased differentiation in 3D organoids independent of genetic background.

A, APP expression in control was confirmed in NPCs by western blot. **B-M**, NPCs from *APP-KO1* and *APP-KO3* stained for SOX2/TUJ1 and SOX2/DCX and **N**, Quantification of SOX2+ cells and **O**, DCX+ in neural precursor cells (n=3, ordinary one-way ANOVA, $p < 0.0001$ for SOX2, $p = 0.0183$ and $p = 0.0038$ for DCX of *APP-KO1* and *APP-KO3*, respectively, scale bar 50 μ m). **P-R**, *APP-KO1* and *APP-KO3* NPCs stained for Ki67 and their quantification (n=3, ordinary one-way ANOVA, $p \leq 0.0001$, scale bar 50 μ m). **S-Z**, The results were reproducible in iPSC line 2 (n=3, unpaired t-test, $p < 0.0001$, scale bar 50 μ m). **Z'1-Z'8**, Cortical organoid for control and *APP-KO2* from iPSC line 1 stained for SOX2/DCX (n=3 organoids, scale bar 100 μ m). **Z''1-Z''8**, Cortical organoid for control and *APP-KO* from iPSC line 2 stained for SOX2/DCX (n=3 organoids, scale bar 200 μ m), showing clusters of more intense DCX+ cells (see also supplementary movies 1-4).

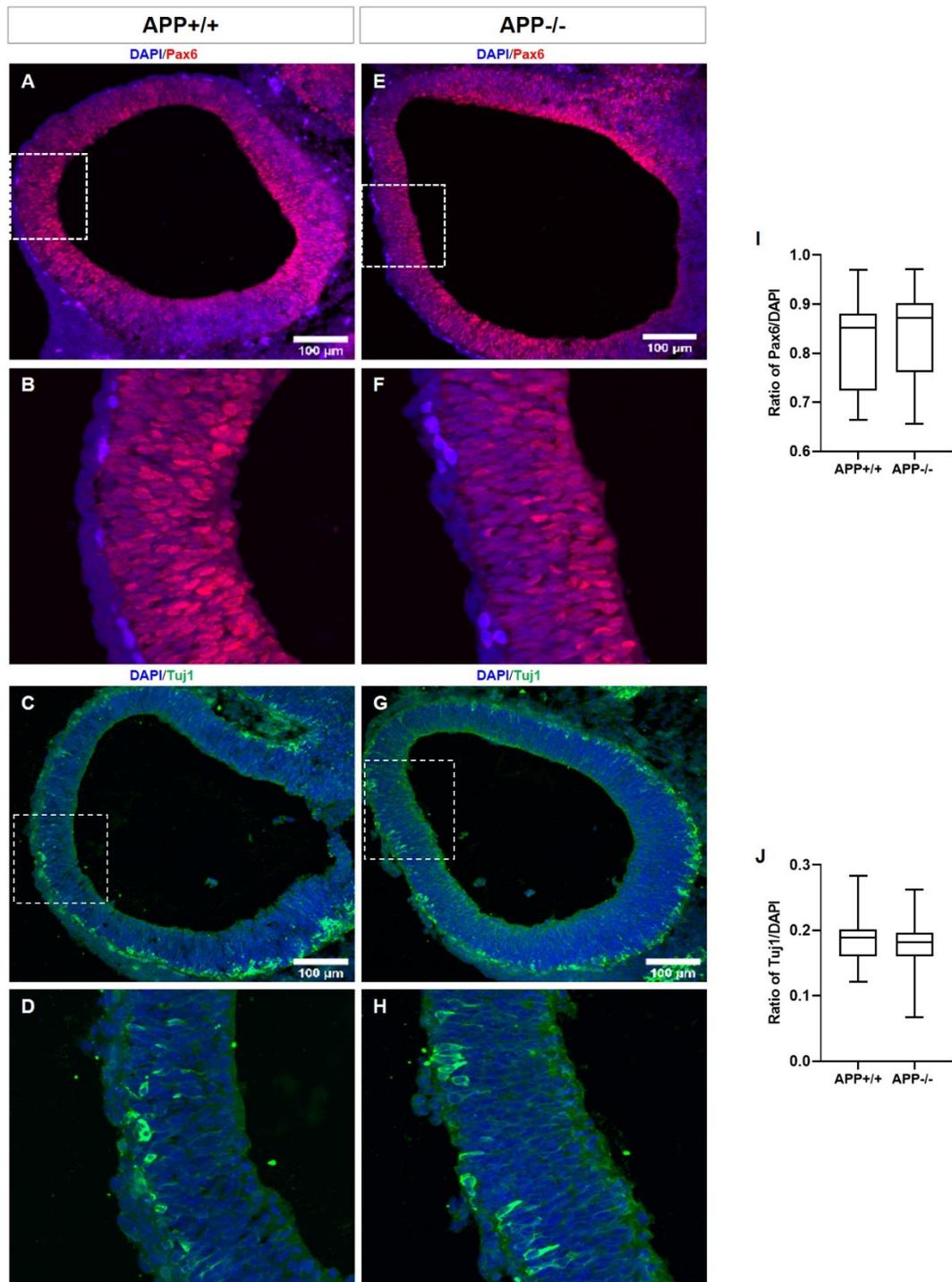


Fig. S8. No difference in Pax6 and Tuj1 expression was observed in in APP^{+/+} and APP^{-/-} E10.5 mouse embryos.

A-H, Brain sections of E10.5 embryo stained for Pax6 and Tuj1. I-J, No difference was observed in the ratio of Pax6/DAPI and Tuj1/DAPI in APP^{+/+} and APP^{-/-} embryo (n=3 embryos, unpaired t-test).

Fig. S9. UMAP and heatmap representations of different clusters from single cell RNA sequencing.

A-C, UMAP of different clusters in **A**, control **B**, *APP-KO2* and **C**, control and *APP-KO2* together. Heatmaps of **D**, five clusters in control and **E**, seven clusters in *APP-KO2*. **F**, Dotplots representing the expression levels and frequency of top marker genes that identify the control and *APP-KO2* clusters (CT=control, KO=*APP-KO2*). Color indicates expression level while the size of the circle indicates percentage of cells expressing the marker. Common markers have grey background, CT-specific markers have blue, and *APP-KO*-specific markers cream color background.

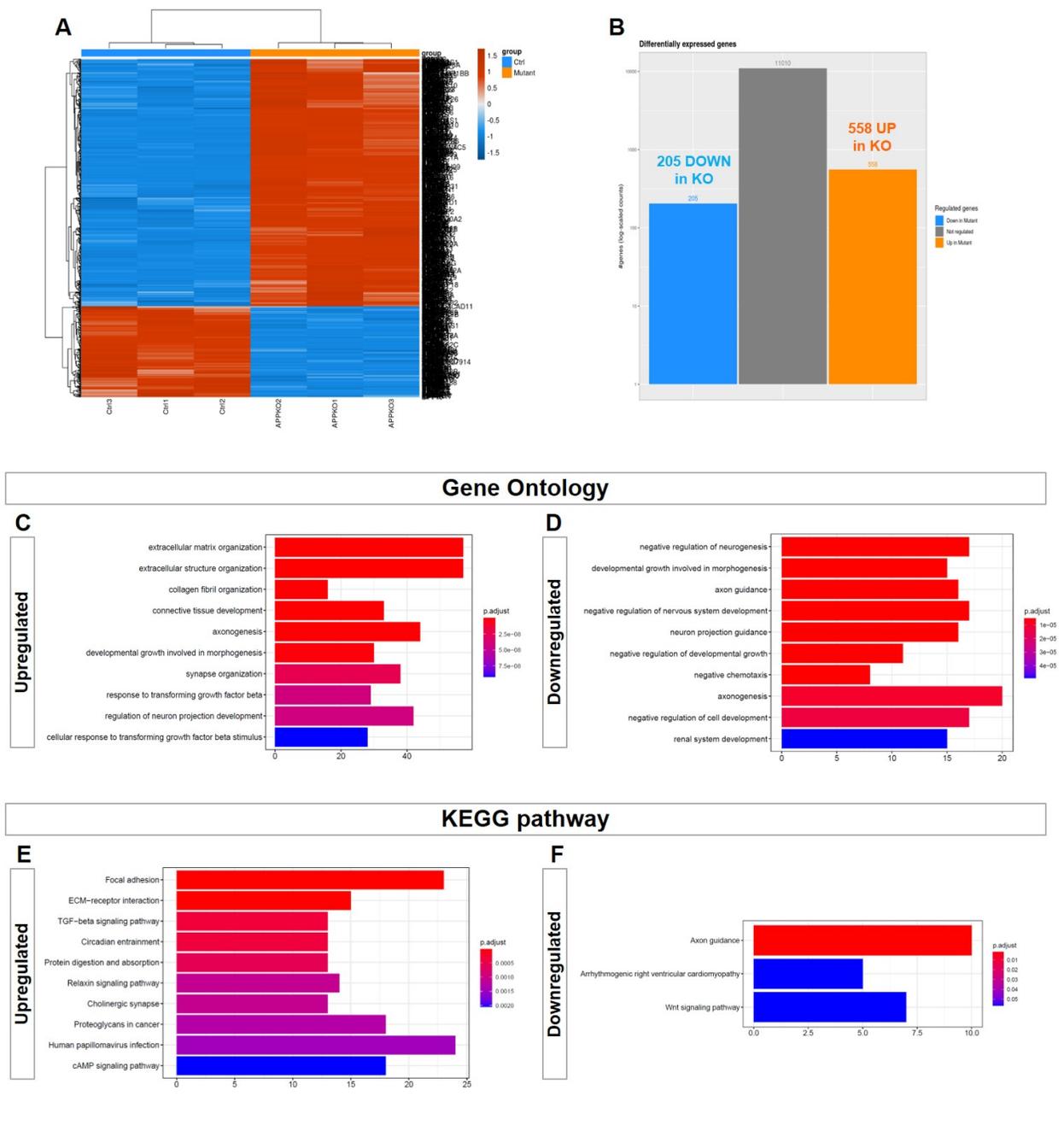


Fig. S10. Gene ontology and pathway analysis for upregulated and downregulated genes.

A, Heatmap of differentially expressed genes shows strikingly different patterns in control and *APP-KO2* NPCs. (*Ctrl1,2,3* correspond to 3 technical repeats of control and *APP-KO1,2,3* correspond to 3 technical repeats of *APP-KO2*). **B**, 763 differentially expressed genes in *APP-KO2* NPCs compared to control which 558 genes are upregulated and 205 genes are downregulated. **C-D**, Gene ontology **E-F**, Pathway analysis of upregulated and downregulated genes.

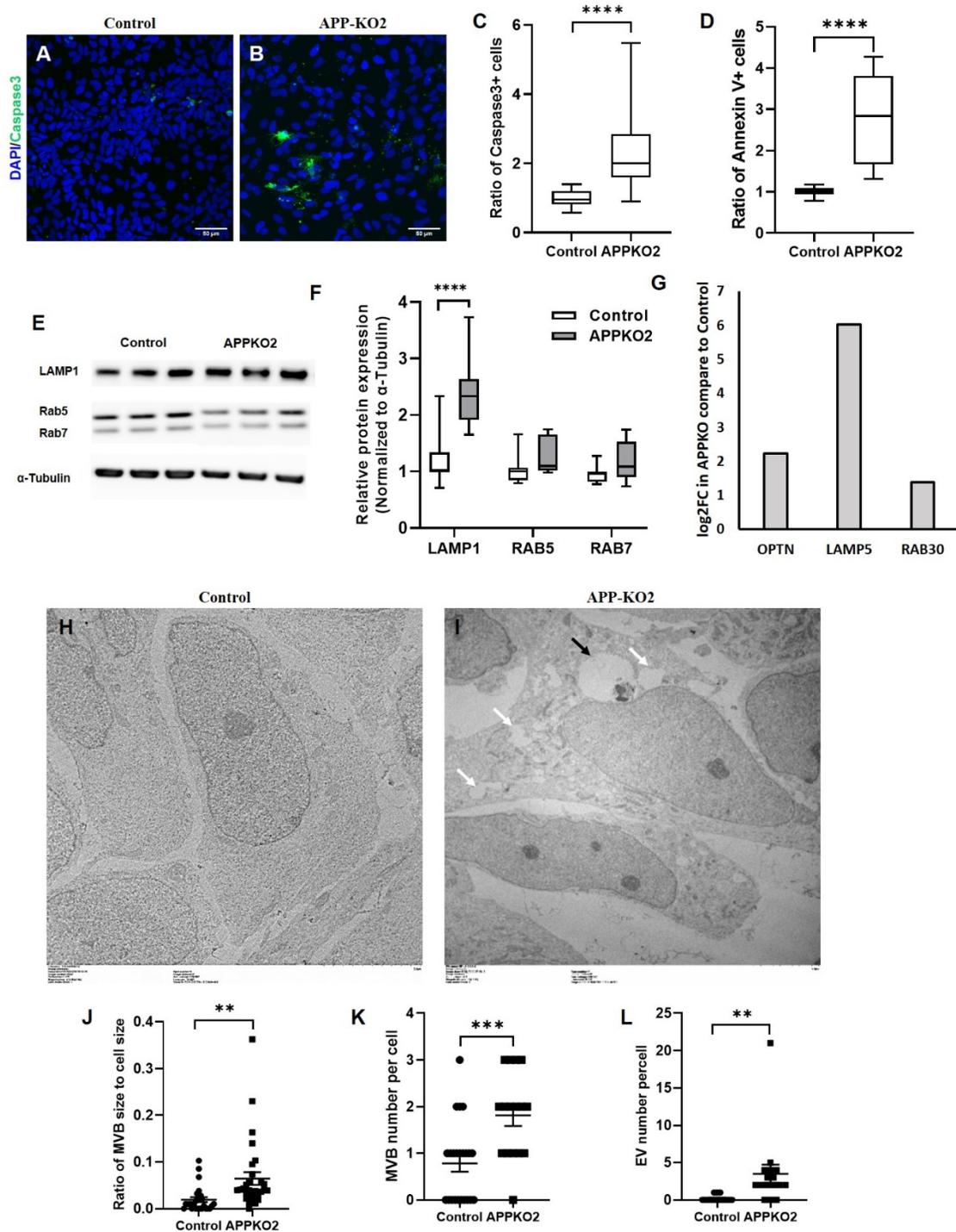


Fig. S11. APP-KO NPCs show increased apoptosis and lysosomal defects.

A-D, increased cleaved caspase 3 (n=6, unpaired t-test, $p < 0.0001$, scale bar 50 μ m) and Annexin V (n=4, unpaired t-test, $p < 0.0001$) showing more cell death in *APP-KO* NPC compared to control. **E-F**, LAMP1 is

significantly increase in *APP-KO* background while RAB5 and RAB7 remain unchanged (n=4, two-way ANOVA, $p < 0.0001$ for LAMP1). **G**, LAMP5, a brain specific lysosomal protein, shows approximately 6 Log2FC increase in the absence of APP. **H-I**, EM images of *APP-KO* and control NPC shows multivesicular bodies (MVB, black arrow) and empty vacuoles (EV, white arrow) structure. **J-L**, Significant increase in the ratio of MVB size to cell size (unpaired t-test, $p = 0.0043$), MVB number (unpaired t-test, $p = 0.0009$) and number of EVs in *APP-KO* compared to control (unpaired t-test, $p = 0.0021$).

Supplementary Tables

Supplementary Table 1- Number of cells per time point

Number of cells			
	Day0-Progenitor (SOX2+)	Day7-Progenitor (SOX2+)	Day7-Neuron(TUJ1+)
Control	70	560	254
APP-KO1	52	52	808
APP-KO-2	60	106	1786
APPK-KO3	79	82	1786

Supplementary Table 2-single cell RNA seq data (excel file)

Supplementary Table 3-bulk RNA seq data (excel file)

Supplementary Table 4- Upregulated genes that have AP1 as their top transcription factor

	Cell type	Gene sign	Top 10 TF
Progenitors	bRGC	TNC	AP-1 ATF-2 c-Jun NF-kappaB1 Sp1 STAT3
	IPC	BCL6	AP-1 STAT5A
		DCX	AP-1
Excitatory Neuron	SP	DKK1	AP-1 ATF-2 c-Jun GATA-6 NCX Nkx2-5
	Deep	HIVEP3	AP-1 AREB6 ATF-2 c-Jun c-Myc Hlf Max Max1 Sox5 SREBP-1b
		CAV1	AP-1 ATF-2 c-Jun IRF-1 MRF-2 Nkx2-5 Sp1 STAT3
	UP	STMN2	AP-1 ATF-2 c-Jun Egr-4 NRSF form 1 NRSF form 2 Pax-5 USF-1
Inhibitory Neuron		GAD1	AP-1 AP-2gamma ATF-2 c-Jun HOXA5 Pax-5
Canonical WNT Inhibitor		DKK1	AP-1 ATF-2 c-Jun GATA-6 NCX Nkx2-5
		DKK3	AP-1 ATF-2
		CDKN2B	AP-1 p53 PPAR-gamma1 PPAR-gamma2 Sp1
Canonical Notch		DNER	AP-1 AP-4 ATF ATF-2 c-Jun MyoD NRSF form 1 NRSF form 2
		NOTCH1	AP-1 ATF-2 c-Fos c-Jun NF-kappaB NF-kappaB1
		HEY1	AP-1 ATF-2 c-Jun c-Myc C/EBPalpha Evi-1 GATA-1 Max p53
Stress response		NR3C1	AP-1

Supplementary Table 5- List of mutations in iPSC clones

16 clones homozygous mutation									
Deletion (13)							Insertion (2)		Transition (1)
6 clones	2 clones	1 clone	1 clone	1 clone	1 clone	1 clone	1 clone	1 clone	1 clone
1bp	1bp	2bp	3bp	20bp	33bp	22bp	1bp	2bp	G to A
Stop codon after 40aa	Stop codon after 40 aa	Stop codon after 20aa 2 stop codon	Stop codon almost at the end of protein after 693 aa	Stop codon after 18 aa	Stop codon after 18 aa 2 stop codon	Stop codon after 18 aa	Stop codon after 22aa 2 stop codon	Stop codon after 40 aa	Mutation in 5'-UTR, no early stop codon

Supplementary Table 6- List of Antibodies

	Antibody	Dilution	Company, Ref
Immunofluorescence	Ki67	1:250	Abcam ab16667
	OCT4	1:1000	Abcam,
	SOX2	1:500	Millipore, AB5603
	SOX2	1:20	R&D Systems, AF2018
	PAX6	1:200	Biologend, PRB-278P
	Nestin	1:300	Abcam, abcam 22035
	SOX1	1:10	R&D Systems, AF3369
	TUJ1	1:1000	Biologend 802001
	TUJ1	1:500	Biologend 801202
	DCX	1:2000	Millipore MAB2253
	SATB2	1:25	Abcam, ab51502
	CTIP2	1:1000	Abcam, 25B6
	Homer-1	1:500	Synaptic Systems 160 011
	MUNC-13-1	1:500	Synaptic Systems 126103,
	PSD-95	1:500	Abcam, ab2723
	Neurogenein2	1:200	Cell signaling Technology_ mAb 13144S
	GFP	1:1000	Ab13970
ISLET1	1:100	Abcam ab109517	
Cleaved Caspae3	1:400	Cell signaling technology, 96615	
Western blot	APP	1:10,000	Gift from Bart de Strooper lab
	α -Tubulin	1:2500	Sigma (T6199)
	Phospho JUN	1:1000	Cell signaling Thecnology 9261
	LAMP1	1:200	SantaCruz (sc-18821)
	RAB5	1:1000	Abcam, ab18211
	RAB7	1:2000	Abcam, ab50533

Supplementary Table 7- PCR and qPCR primers

Gene	Forward Primer	Reverse primer	application
APP	5'-GGCTCCGTCAGTTTCCTCGG-3'	5'-CCCGGCTTCTCTGCATTAAAGA-3'	PCR
APP	5'-CAGAATGGGAAGTGGGATTCA-3'	5'-CAGTTCAGGGTAGACTTCTTGG-3'	qPCR
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'	qPCR



Chapter III-General Discussion

Loss of APP shows human cortex specific phenotype

The approximately 16 billion neurons of the human neocortex are derived from a relatively limited number of cortical neural progenitor cells (NPCs). During embryogenesis, human cortical NPCs initially generate neurons at a particularly slow rate while preserving their progenitor state for a relatively long time, in part contributing to increased human cortical size. How this balance between the progenitor state and neurogenic state is regulated, and whether it contributes to species-specific brain patterning, is poorly understood. APP is expressed during human cortex development but its function is unclear. Here, we present evidence supporting a specific requirement for APP during human cortical development. Loss of APP caused a striking increase in the generation of neurons in a very short time window and it even caused auto-differentiation within the population of human cortical NPC. This suggests human cortex development may be particularly sensitive to loss of APP based on three observations:

First, both *in vivo* and *in vitro* studies on mouse NPCs lacking APP have shown no consistent, even contradictory, evidence of altered neurogenesis. Based on our results, loss of APP does not affect the differentiation rate of mouse neural progenitor cells *in vivo*. Bergman et al showed that triple knock out of the APP family (*App/Aplp1/Aplp2*) does not cause defects in critical neurogenesis processes. Triple KO NPCs formed equally pure neuronal cultures, had unaltered *in vitro* migratory capacities, and had a similar acquisition of polarity *in vitro* compare to control. Hu et al. showed neural stem/progenitor cells (NSPCs) derived from APP knock-out mice had reduced differentiation rates compared to NSPCs from the corresponding background strain (*C57Bl/6*). In contrast, Ma et al. showed that NPC derived from APPKO mouse show more TUJ1+ and MAP2+ cell within 7-8 days *in vitro* culture (Ma et al., 2008; Bergmans et al., 2010; Hu et al., 2013).

Second, we did not observe any defect on human motor neurogenesis due to loss of APP, suggesting that human cortical neurogenesis is particularly sensitive to loss of APP. The question that arises here is that why human cortical NPCs show sensitivity to loss of APP, while this sensitivity is not detectable in the differentiation of human motor neuron and mouse corticogenesis. One possible answer could be related to pace of development. Human cortical neurogenesis occurs during 3-4 month while human motor neuron generation happens in 2-3 weeks and mouse cortical neurogenesis only needs 2 weeks. We propose that APP is a

homeostatic regulator of the fine balance between proliferation and differentiation in processes where the pace of development is slower (human cortical neurogenesis). APP keeps this balance in two ways: activation of canonical WNT signaling which is one of the key pathway for NPC proliferation and downregulation of proneural genes such as NEUROG2, differentiation promoters such as DNER, JAG2 and DLL3 and CDKN2B - a well-known marker for cell cycle exit-. However, it is still possible that loss of APP cause subtle defect in processes where the pace of development is faster (human motor neurons and mouse cortical neurons) but we are not able to detect them due to low resolution of our techniques. Since we only assess human cortical and motor neurons, it is worth to test other type of human neurons to screen for any defects. In conclusion, we speculate that this greater requirement for APP in the human cortex is not due to changes in the highly conserved APP protein itself but to the context of human cortical neurogenesis, especially the evolutionary pressure to maintain human cortical progenitors in a proliferative undifferentiated state for a relatively long time during embryogenesis.

Third, loss of APP affects the expression of genes located within human accelerated regions (HARs) which are conserved genomic loci with elevated divergence in humans. They are enriched for neural regulatory elements and they seem to play an important role in exceptionally developed human brain (Levchenko *et al.*, 2018). Doan *et al.*, 2016, reported HARs regions related to cognitive disorders such as autism spectrum disorders (ASD) (Doan *et al.*, 2016). By comparing to their data set, we observed an upregulation ranging from 1.1 to 6.1 log₂FC in some of the HARs- related genes in *APP-KO* NPC such as ADAMTS14, AHRR, BNC2, CCDC102B, CDH11, CHST7, COL6A3, CPEB2, CYP1B1, DLC1, DOK5, EPHB3, FAM196B, FAM198B, FST, ITGA11, KCNIP2, KCTD12, LRRTM2, MASP1, MEIS2, MPPED2, NFIA, NFIB, NFIX, NTM, PDGFRB, POU2F2, SAMD4A, SAMD5, SOCS2, TGFB2, ZIC3. These results show that presence of APP is necessary for maintaining the specific features of human brain/cortex acquired during evolution.

APP regulates the balance between self-renewal and differentiation of NPC

We propose that *APP* is a homeostatic regulator for maintaining the balance between proliferation and differentiation of NPC, which may contribute to human-specific patterns of

neurogenesis. Loss of APP cell-autonomously drives human cortical NPCs towards a neurogenic state and reduced proliferation, without changing the temporal order of neuronal fate acquisition or the rate of neuronal maturation. In the following, I will discuss molecular signatures of reduced- proliferation and increased-neurogenic stages.

Reduced proliferation

Several studies uncovered that canonical WNT signaling is one of the well-known pathway involve in maintaining and expanding pool of NECs and RGCs during cortical development. Increasing β -catenin –downstream of Wnt signaling pathway- from early corticogenesis strikingly expands the NEC population by decreasing their cell cycle exit and by delaying the neurogenic period (Chenn and Walsh, 2002; Machon et al., 2007; Wrobel et al., 2007). We find 20% fewer proliferative cells in APP-KO NPC compare to control due to downregulation of canonical Wnt pathway where bulk RNA seq data could support this phenotype: I) Upregulation of well-known canonical WNT inhibitors such as DKK1, DKK3, APCDD1 and ALPK2. II) Downregulation (-1.70 logFC) of WNT7a which is one of the canonical WNT ligands. Qu et al. showed that Wnt7a is essential for neural stem cell self-renewal and neural progenitor cell cycle progression in adult mouse brains. Loss of Wnt7a expression dramatically reduced the neural stem cell population and increased the rate of cell cycle exit in neural progenitors in the hippocampal dentate gyrus of adult mice (Qu et al., 2013). III) In unpublished data in our lab, Liu et al showed that App is a receptor for canonical Wnt ligand, Wnt3a. Therefore, it is likely that part of reduced proliferation is due to loss of canonical Wnt receptor, App.

Besides to downregulation of canonical Wnt, other evidences in bulk RNA seq supports less proliferation in APP-KO NPCs. MIR100HG, a long noncoding RNA (lnc RNA) involve in the regulation of cell cycle progression, shows 2.09 log₂FC increase in APPKO-NPC. MIR100HG produces spliced and stable lncRNAs that display elevated levels during the G1 phase of the cell cycle. Depletion of MIR100HG-encoded lncRNAs in human cells results in aberrant cell cycle progression where levels of G2/M population elevated with a concomitant reduction in G1 population (Sun et al., 2018). We also observed that level of a well-known cell cycle inhibitor cyclin-dependent kinase inhibitor 2B (CDKN2B) increase 2.7 log₂FC which push APP-KO NPC to exit cell cycle.

One question that arises here is whether loss of APP affect cell cycle length. Is proliferation reduced due to protracted cell cycle length? It can be studied via dual labeling approaches for BrdU versus EdU incorporation to measure the length of different phases of the cell cycle. However, since in mammalian cells, the length of S and G2+M phase remains relatively constant while the length of G1 regulates proliferation (DiSalvo, Zhang and Jacobberger, 1995) and we observed upregulation of MIR100HG in bulk RNA seq, I assume that APP-KO NPCs may have protracted G1 phase.

Neurogenic state

We observe appearance of a new cluster called neurogenic progenitors (NPs) in single cell RNA seq, which express progenitor and differentiation marker concomitantly. This cluster accounts for 13% of the cells in the population of *APP-KO* NPC while it did not appear in control. Moreover, 15-fold more NPCs express *NEUROG2* in *APP-KO* population, which could explain why *APP-KO* NPC are highly prone to differentiation. The increased neurogenic state of *APP-KO* NPC is not only due to presence of neurogenic progenitors but it is even obvious in other clusters. There is a clear shift toward neural fate in all the *APP-KO* clusters in pseudo time analysis; i.e even RGC in *APP-KO* background has higher potential to differentiate compare to RGC from control. This shift toward neural fate is also detectable in bulk RNA seq data. We observed a decrease in aRG marker (*PAX6*, *CDON*), increase in basal radial glial fate (*BLBP*, *MOXD1*, *GLI1*, *ACSBG1*, *TNC*) and Intermediate progenitors (*NEUROG2*, *BCL6*, *ASCL1*, *NHLH1*, *DCX*). All together, these data point to the fact that the pool of aRGs is consumed in favor of producing bRG and IPC, which are both closer to neural fate in the developmental trajectory. Several studies classified markers corresponding to different type of neurons during human cortical development (Pollen *et al.*, 2015; Nowakowski and Pollen, 2016; Nowakowski *et al.*, 2017; Polioudakis *et al.*, 2019). By comparing our data to theirs, we noticed upregulation of pan neural genes (*GRIA2*, *STMN2*), markers of sub plate neurons (*ST18*, *DKK1*, *HAS2*), early born neurons (*NFIA*, *OPTN*, *MYT1L*, *TSHZ1*, *BCL11A*, *HIVEP3*, *CAV1*, *B3GALT2*, *CHRNA3*), late born neurons (*STMN2*, *CUX2*, *INHBA*, *HCN1*, *SEZ6L*). All these data suggest that there is an accumulated neural potential in *APP-KO* NPC that push cells toward neural fate.

Although the elevated neurogenic state originates from NPC, we noticed that for the first time once the differentiation starts. In viral approach experiment, we showed that the neuron-to-progenitor ratio was strikingly higher in all three *APP-KO* clones compared to controls at day 7, but was not significantly different by day 30. The question that raises here is if the pool of aRGs are consumed very fast in *APP-KO* background, how we obtain the same number of neurons by day 30 in both *APP-KO* and control. One possible answer is that the loss of the aRG pool is compensated by producing more bRGs, which ultimately lead to the same number of neurons in *APP-KO* and control. This increase in basal progenitors especially bRG can be supported by some evidences such as increase in bRGC markers BLBP, MOXD1, GLI1, ACSBG1, TNC with 1.2, 2.3, 1.6, 1.4 and 4.2 log₂FC in RNA level respectively. Pollen et al showed that genes involve in extracellular matrix organization are enriched in bRG (Pollen *et al.*, 2015). Interestingly, we found in gene ontology/pathway analysis that upregulated genes in *APP-KO* are involved in extracellular matrix (ECM) organization, collagen fibril organization, focal adhesion and ECM-receptor interaction.

Loss of APP mimics stress and drives premature differentiation

We propose that loss of APP mimics cellular stress and cell-autonomously triggers conversion of cortical NPCs to a neurogenic state through activation of immediate early genes such as AP-1 (JUN and FOS), which ultimately drive neural differentiation.

To investigate how loss of APP induces premature neurogenesis in relation to stress, we need to answer two questions:

Can stress induce neural differentiation?

Several studies suggest that increased stress drives neural differentiation in human embryonic stem cells and rodent neural progenitor cells. Hu et al., showed that oxidative stress induces neural differentiation of human embryonic stem cell (hESC) by MAPK-ERK1/2 signaling pathway. They showed that oxidizing agent paraquat could induce oxidative stress by production of high level of ROS which suppress the expression of

stemness markers (NANOG, OCT4 and TDGF1) whereas it enhanced the spontaneous expression of neuronal differentiation markers (PAX6, NEUROD1, HOXA1, NCAM, GFRA1 and TUJ1). Expression of neuronal markers in the paraquat-treated cells was suppressed by an antioxidant while further enhanced by knocking down Nrf2, a key transcription factor associated with antioxidant signaling (Hu *et al.*, 2018). In another study, Pérez-Estrada *et al.*, showed that stress in particular H₂O₂-induced oxidative stress cause more neural differentiation of rat neural progenitor cells. In their study, H₂O₂ levels within the range measured at neuroinflammatory events were applied to rat NPC cultures for 24 h. The effects were assessed either directly after exposure or in NPCs that were differentiated for 7 days post treatment. Exposed differentiated NPCs showed significantly increased numbers of neurons and oligodendrocytes compared with unexposed controls due to significant increase in both Olig2+ cells and Ascl1+ cells that could contribute to both more neurons and oligodendrocytes (Pérez Estrada *et al.*, 2014). This is in contrast to our finding that stress induced by loss of APP does not affect differentiation of NPC in mice. This difference could be explained by: I) we are assessing neural progenitor cells in developing mouse cortex at E10.5, while they are evaluation adult neural stem cells from 7–8 weeks old rat. II) we showed that loss of APP causes stress by disrupting endo-lysosomal pathway and did not assess oxidative stress in particular; So different type of stress may have different outcomes. III) Our mouse data comes from an *In vivo* model while their data is results of an *In vitro* study.

How is APP related to stress?

First, APP is a conserved stress response protein which is upregulated in brain injury models. It has been suggested that *APP* has neuroprotective function by acting in neural regeneration and repair.

Second, APP is involved in transcriptionally regulation of well-known members of stress response genes belonging to a large family of immediate early genes (IEGs). Hendrickx *et al.*, showed that APP epigenetically down regulates expression of four stress response genes, *Egr1* (early growth response 1), *c-Fos*, *Bdnf* and *Arc* by decreasing acetylation of

histone H4 on the corresponding gene promoter. In the absence of App, *Egr-1* expression together with other stress response genes like *c-Fos*, *Bdnf* and *Arc* were also upregulated in prefrontal cortex of *App*^{-/-} mice compare to *App*^{+/+} mice (Hendrickx *et al.*, 2013, 2014). In parallel to their finding, expression of JUN, FOS and EGR1 is increased (1.3, 2.7, 2.5 Log2FC respectively) in our *APP-KO* NPCs model, which suggest APP also regulate their expression in human context.

Third, some studies showed an important role of APP in the control of JNK/c-Jun signaling, target gene expression and cell death activation in response to stress. Kogel *et al.*, showed APP-dependent transcriptional repression of c-Jun and reduced basal c-Jun N-terminal kinase (JNK) activity in PC12 cells (Kögel *et al.*, 2005). Stress-induced activation of the JNK signaling pathway and subsequent apoptosis were likewise reduced either by over expression of APP or treatment with exogenous sAPP α (Kögel *et al.*, 2005; Copanaki *et al.*, 2010; Eckert *et al.*, 2011). In contrast, overexpression of the Swedish mutant of APP did not inhibit stress-triggered JNK activation and cell death (Kögel *et al.*, 2005). The protective effect of APP/sAPP α could be mimicked by an MLK inhibitor that inhibits upstream JNK-activating kinases of the mixed lineage kinase (MLK) family (Kögel *et al.*, 2005) or by direct inhibitors of the JNK pathway (Eckert *et al.*, 2011).

Fourth, mutations in APP show features corresponding to cellular stress status such as cell death and defect in endo-lysosomal pathway. Based on our results, there is more cell death and defect in endo-lysosomal pathway in *APP-KO* NPC compare to control which also was shown in other studies. Hung *et al.*, using human neuronal models of monogenic Alzheimer's disease showed that APP and PSEN1 mutations disrupt lysosome function and autophagy, leading to impaired lysosomal proteolysis and defective autophagosome clearance (Hung and Livesey, 2018). Kessissoglou *et al.*, in our lab reported that loss of function of Appl (fly homologue of APP) results in the dysregulation of endolysosomal function, in both neurons and glia with a notable enlargement of early endosomal compartment in neurons followed by neuronal cell death. These defects can be rescued by reduction in the levels of the early endosomal regulator *Rab5*, indicating a causal role of endosomal function for cell death (Kessissoglou *et al.*, 2020). In unpublished data in

our lab using mouse primary culture, Liu et al., indicated that App is a receptor for Wnt ligands (Wnt3a and Wnt5a) through its cysteine rich domain (CRD). They showed that Wnt ligands regulate App protein levels by sorting to either the trans Golgi network (TGN) for recycling (Wnt3a), or the lysosome for degradation (Wnt5a).

Although we described the mechanisms involved in premature neurogenesis, several unanswered questions remain. For instance, **which domain of APP correlated to this phenotype? Whether this phenotype is related to full length APP or its processed product is not clear.** To address this question, rescue experiment could be performed by truncated forms of APP with deletion in different domain. However, I showed APP could rescue the phenotype cell autonomously which suggest it may be related to APP intracellular domain (AICD). The next question is **whether this phenotype is correlated to unique feature of human APP protein or it is dependent on human context.** To assess that, rescue experiment can be accomplished by APP homologues in other organisms including mouse App and fly Appl. If the phenotype is not rescued by App and Appl, we can conclude that it is dependent on unique features of human APP protein. However, since APP is highly conserved protein, I assume that APP homologues in other organisms can rescue the phenotype because the phenotype is human context dependent not protein structure dependent. Finally, since loss of APP controls NPC proliferation and differentiation by affecting two different pathways, It is worth doing a double rescue experiment to see whether blocking AP1 and inducing WNT signaling pathway can rescue both phenotype or not.

Cortex specific requirement of APP in the context of Alzheimer's disease

More than 50 different mutations in the APP gene can cause early-onset Alzheimer disease, of which the Swedish mutation (KM670/671NL) and the London mutation (V717I) are two very well established examples. Of note, there is strongly reduced tolerance (0.42) to complete loss of APP in human genetic data (Lek *et al.*, 2016; Karczewski *et al.*, 2020), which means loss of APP causes mortality in more than 50% of the cases. Here we report enhanced requirement of APP in human cortex development while human motor neurons and mouse cortical neurogenesis does not

show this requirement. The question that arises here is how does the specific requirement for APP in human cortex development fit in the context of AD? Below I consider several possible scenarios:

1) It is possible that early-onset AD mutations in APP have subtle effects on early human cortical development that cause reduced cortical robustness and resistance to a lifetime of neuronal and glial stress eventually contributing to premature neurodegeneration. The reason why APP mutation that is already present from conception, manifest its toxicity only in midlife depends on possible compensatory mechanism in brain which maintain normal cognition for decades and the diseases only appeared in adulthood due to failure of those compensatory mechanisms.

2) Mutation of APP can also affect other risk factor of early-onset AD such as the Tau protein. Muratore et al., showed that London mutation of APP increases levels of total and phosphorylated Tau in addition to altered APP processing (Muratore *et al.*, 2014). Kwak et al., also showed that ratio of A β 42/40 plays a critical role in inducing neurofibrillary tangles (NTFs) in human neurons (Kwak *et al.*, 2020). Interestingly, we also observed an upregulation of the MAPT gene, encoding the other key AD protein Tau, in APP mutant progenitors.

3) APP, in particular soluble APP α , has neuroprotective effects. Since aging is the most reliable risk factor for sporadic AD, changes in the expression, maturation and cleavage of endogenous APP can affect the onset and progression of AD. For instance, AD patients have decreased levels of soluble APP α in their cerebrospinal fluid (Palmert *et al.*, 1990). Kern et al showed that processing of endogenous APP is downregulated during the aging of replicative senescent IMR-90 cells during cell aging; i.e generation of sAPP α and intracellular APP cleavage products C99, C83, and AICD gradually are declined with increasing cellular life span and the maturation of APP is reduced in post mitotic senescent cells. In addition, Level of APP-processing enzymes such as γ -secretase complex progressively reduced during aging, but ADAM10 and BACE protein levels exhibited no age-associated regulation, but, interestingly, BACE enzymatic activity was increased in aged cells (Kern *et al.*, 2006) which is the enzyme involve in production of A β .

In conclusion, accumulation of A β and tau pathology is a slow, gradual process that is tolerated by CNS cells in preclinical stage of the disease. The disease only manifests clinically when cellular

homeostatic mechanisms fail. This leads to impaired clearance of aggregated pathologic protein, increased cellular stress, and a complex break-down of intercellular physiologic functions that ultimately lead to neurodegeneration.

4) AD can be thought of as a developmental disease:

I) while AD is highly prevalent in human, the typical neuropathological lesions are hardly seen in non-primate mammals and even non-human primates develop only an incomplete form of the disease. Interestingly, the main area affected by AD in human brain belongs to highly developmentally complex of the human brain and regions that are particularly prominent in evolution (Arendt, Stieler and Ueberham, 2017a).

II) The Apo lipoprotein E polymorphism, which accounts for 50% risk for AD in many populations is a unique feature of human evolution (Fullerton et al., 2000; McIntosh et al., 2012).

III) During brain development, neurons could contain a DNA amount deviating from the diploid level called DNA content variation, DCV. This is quite frequent, affecting more than 30% of proliferating progenitors but majority of these cells undergo apoptotic clearance during brain development (Rehen et al., 2001; Peterson et al., 2012). Neurons with DCV are preferentially affected by cell death during aging and there are studies that show an increased rate of DCV in AD patients. This clearly identifies DCV as a developmental consequence and molecular signature for impaired viability in the adult brain (Arendt *et al.*, 2010; Fischer *et al.*, 2012; Arendt, Stieler and Ueberham, 2017b).

IV) Braak and Del Tredici (2011) assessed brains of 42 children and young adults between the ages of 4 and 29 for the presence of abnormally phosphorylated Tau protein (pretangle material). They found 38 out of 42 cases indicates pretangle material in nerve cells or in portions of their cellular processes, with the youngest case being only 6 years old. These findings might show that the AD-associated process of pathological tau conversion begins already in early adulthood or even before puberty, assuming that these pre-tangle alterations are not transient and do not regress (Braak and Del Tredici, 2011).

The last important question that arises here is what will be the phenotype of different mutation of APP either pathogenic Swedish mutation (APP KM670/671NL) or protective Icelandic mutation (APP A673T)? Whether iPSC derived from AD patients with APP mutation also show premature differentiation. To address these questions, AD patient derived iPSC and their isogenic control could be used for 2D and 3D differentiation toward cortical fate and assess what kind of phenotype will be obtained.

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Thesis summary

The neocortex is the highly elaborate part of the human brain which is responsible for complex cognitive behavior. During embryogenesis, human cortical neural progenitor cells (NPCs) initially generate neurons at a particularly slow rate while preserving their progenitor state for a relatively long time, in part contributing to increased human cortical size. The Amyloid Precursor Protein (APP), which is responsible for genetic forms of Alzheimer's disease, is highly expressed in early embryonic days in human telencephalic neurospheres, along with differentiation, migration and maturation of cortical neurons. However, the role of APP in human context development is completely unexplored. The general introduction of this thesis summarizes previous findings on cortical neurogenesis and the implication of APP in various aspect of neurobiology with a focus on human cortex development. We hypothesized APP may have a role in the development of human cortex and this project outlines a strategy to unveil the physiological functions of human APP during cortical development. We used human induced pluripotent stem cells as a model. We produced APP knock-out iPSC in two different genetic backgrounds and studied cortical neurogenesis in 2D and 3D. We found that loss of APP causes premature differentiation specifically in human cortical progenitors, but the relatively slow timing of neuronal maturation appears preserved. In contrast, we found no effect on mouse cortical progenitors and the rate of human spinal motor neuron generation. Mechanistically, loss of APP cell-autonomously triggers conversion of cortical NPCs to a neurogenic state through repression of the canonical Wnt pathway and upregulation and activation of AP1 (Jun and Fos), the well-known stress response genes. AP1 activates downstream proneural proteins, like Neurogenin2 and B-cell lymphoma 6 (Bcl6) thus inducing neural progenitors to exit the cell cycle and prematurely differentiate. These phenotypes can be fully rescued either by restoring APP, or by adding exogenous Wnt3a to restore NPC proliferation and inhibiting AP1 activity to inhibit premature differentiation.

In summary, this project reveals a human cortex specific role for APP in regulating neurogenesis and identifies the regulation of cellular stress as a potential intrinsic mechanism for prolonged maintenance of human cortical progenitor fate.

Working model

