Master thesis

The effect of developmental ablation of TAG-1-expressing pyramidal neurons in the mouse brain

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Περίληψη

Είναι γνωστό ότι οι νευρώνες που προβάλουν σε υποφλοιικές περιοχές, όπως ο θάλαμος και το ραβδωτό, εκφράζουν την πρωτεΐνη κυτταρικής συνάφειας της οικογένειας των ανοσοσφαιρινών, την CNTN2/TAG-1. Η TAG-1 εμπλέκεται σε σημαντικές αναπτυξιακές διαδικασίες, όπως η έκφυση νευριτικών προεκβολών και η δεσμιδοποίηση των αξόνων. Επίσης, οι άξονες των νευρώνων που εκφράζουν TAG-1 εμπλέκονται σε βασικές αναπτυξιακές διαδικασίες, μεταξύ των οποίων η μετανάστευση. Χρησιμοποιήσαμε μια νέα σειρά ποντικιών που δημιουργήθηκε στο εργαστήριό μας και μας δίνει τη δυνατότητα να μάθουμε ποιοί ακριβώς νευρώνες εκφράζουν TAG-1 και να απαλείψουμε την πλειοψηφία τους κατά τη διάρκεια της ανάπτυξης. Αυτό είχε ως αποτέλεσμα να δημιουργηθούν δυσπλασίες σε μεγάλες δεσμίδες αξόνων όπως το μεσολόβιο και η πρόσθια δεσμίδα. Επίσης παρατηρήσαμε μείωση στον αριθμό των διάμεσων ανασταλτικών νευρώνων στο φλοιό. Τέλος, παρατηρήσαμε βελτίωση της ικανότητας για μάθηση αλλά χειρότερη χωρική μνήμη.

Abstract

Corticofugal axons projecting to the thalamus, stiatum and palidum are known to express (CNTN2/TAG-1), a neuronal recognition molecule of the immunoglobulin superfamily involved in neurogenesis, neurite outgrowth and fasciculation. TAG-1, which is expressed transiently by cortical pyramidal neurons during embryonic development, has been shown to be fundamental for axonal recognition and cellular migration in the developing cortex. Our lab generated a novel mouse line that enables us to track the neurons expressing Tag-1 and, upon crossing with the $EMX1^{cre}$ line, to ablate the vast majority of Tag-1 expressing neurons in the cortex. This model revealed to us that Tag-1-expressing neurons are also located in superficial layers projecting to other cortical areas and contributing to the formation of the corpus callosum. The ablation of Tag-1-expressing cells resulted in defective development of major fiber tracts such as the corpus callosum and anterior commissure. Furthermore, we observed reduced number of interneurons in the cortex. $Tag\text{-}1^{DTA}$ mutant mice displayed better learning abilities but defective spatial memory.

Introduction

Cortical neurogenesis and generation of layers

During development, the most rostral structure of the neural tube is the telencephalon. The telencephalic vesicle is divided in two major parts. The dorsal one which is called pallium and will give rise to cortical hemispheres and hippocampus and the ventral, termed subpallium, which is the precursor of striatum, pallidum and telencephalic stalk (Rubenstein, 2003).

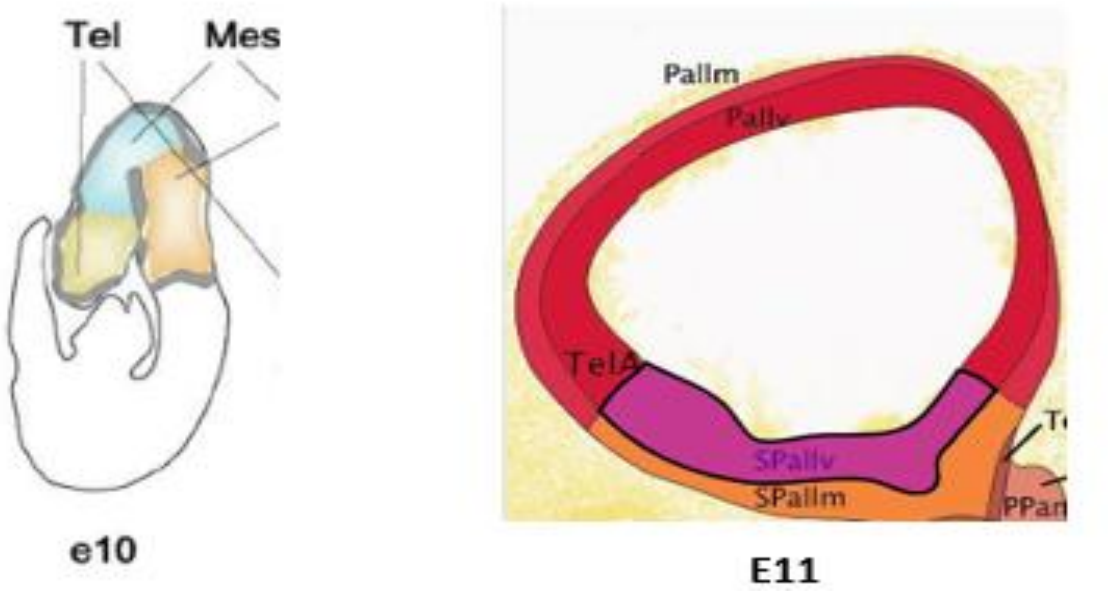


Figure 1:Developing telencephalon during embryonic days E10 and E11. Note the formation of distinct pallium mantle (Pallm) and ventricular zone of pallium (Pallv) and subpallium mantle (SPallm) and ventricular zone (SPallv) during E11. Adapted by Allen brain atlas

Roughly around embryonic day 10 (E10), the developing neuroepithelial cells reside in the space above the future lateral ventricles thus creating a pseudostratified neurogenic epithelium the ventricular zone of which is the primary proliferative zone of the embryonic brain (Mukhtar & Taylor, 2018). Neuroepithelial cells display an apico-basal polarization, being attached apically to the ventricular (through a structure termed primary cilium) and basally to the pial surface (via the basal process). Before the initiation of neurogenesis, neuroepithelial cells divide symmetrically to expand their pool (Laguesse, Peyre, & Nguyen, 2014). During the progression of their cell cycle, neuroepithelial cells display a specific pattern of nucleokinesis. Their nucleus is located on the apical surface of ventricular zone

during mitosis and it migrates towards the basal surface during G1, when it reaches the basal surface of VZ neuroepithelial cells. When these cells undergo S phase and during G2, it migrates back to the apical surface of VZ to undergo mitosis and repeat the oscillation, in a process termed interkinetic nuclear migration (INM) (Ayala, Shu, & Tsai, 2007). As soon as neuroepithelial cells increase their number to a certain point they switch from symmetric to asymmetric divisions. That switch signals the onset of neurogenesis.

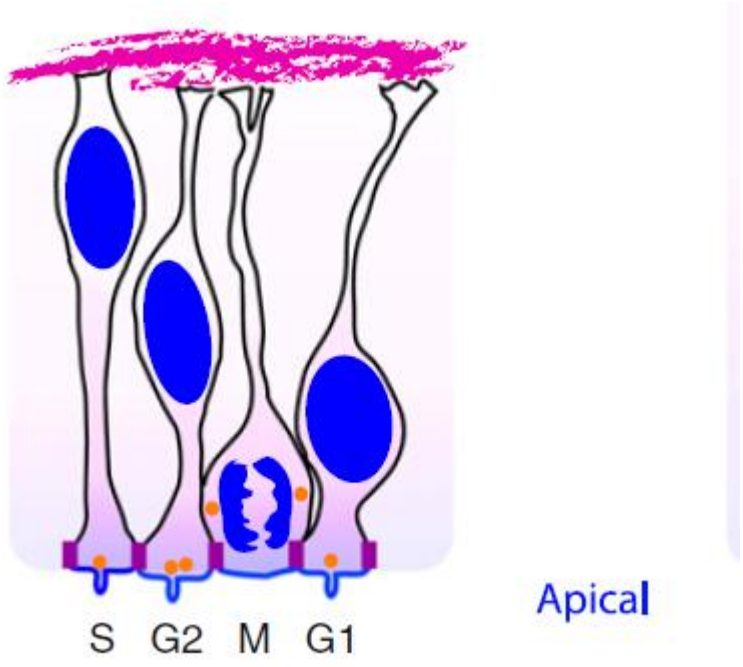


Figure 2: Interkinetic nuclear migration (INM) of neuroepithelial cells' nuclei creates a pseudostratified epithelium. Neuroepithelial cells are attached to ventricular zone and pial surface and their nuclei are oscillating depending on their cell cycle. Once they increase their number adequately they switch to asymmetrical divisions giving rise to other progenitor populations such as radial glial cells. Adapted by (Taverna & Huttner, 2010)

As cortical development proceeds, between E10 and E12, most neuroepithelial cells change their transcriptional profile thus differentiating to a distinct population of apical neural progenitors termed Radial Glial cells (RGs) (Götz, Barde, Landstr, & Munich, 2005). RGs are very similar yet not identical to NEs since they also project a basal process attached to the pial surface elongating along with the cortical thickening and a primary cilium attached to the apical surface of the ventricular zone during the interphase of their cell cycle. RGs also undergo INM but their nucleus does not move beyond the ventricular zone. Unlike NEs, RGs have their cytoplasm full of glycogen granules and express a distinct set of markers such as Nestin, Pax6, Vimentin, the astroglial marker BLBP, Glutamate transporter GLAST, and S100β (Götz et al., 2005).

RGs also differ from NEs when it comes to proliferation and differentiation properties. While both NEs and RGs can divide symmetrically to increase their numbers, NEs can divide asymmetrically to give rise to many different types of neural progenitors including RGs and others, which will be further discussed below. However, RGs asymmetrical division can only give a more differentiated progenitor type, a post-mitotic neuron or glial cells (astrocytes or oligodendrocytes). Except their role as neural progenitors, RGs function as a ladder for post-mitotic neurons to climb upon when they migrate to their proper laminar position (Laguesse et al., 2014).

It is important to note that not all NEs differentiate to RGs at the same time point. NEs differentiate to give various progenitor cells as cortical development proceeds, and these progenitors coexist with RGs throughout the developing cortex. They are named basal intermediate progenitor cells due to their localization at the basal side of the VZ (Laguesse et al., 2014).

Basal intermediate progenitor cells (BIPs) can arise either directly from NEs, which happens even before the expression of first astroglial markers in the VZ and thus the appearance of RGs, or indirectly from RGs. Except from their basal positioning they have distinct morphological and molecular characteristics. BIPs do not have an apical process attached to the apical side of the marginal zone but do preserve their basal process. They are also molecularly distinct since they express Tbr2 and do not express Pax6. The increase of this population generates the second proliferative zone of the developing cortex, called the sub-ventricular zone (SVZ). BIPs mostly divide symmetrically to produce two post-mitotic neurons but there are some which can make one extra symmetrical division to BIPs to double the final number of neurons created (Florio & Huttner, 2014).

Outer radial glial cells comprise another, distinct from BIPs, population of basal progenitors residing in the SVZ. They originate from RGs and divide symmetrically to increase their number or asymmetrically to their selves and a post-mitotic neuron.

Back in the VZ there is one more population of neural progenitors co-existing with RGs named short neural progenitors (SNPs). They are similar to RGs in terms of their apico-basal polarity and the expression of transcription factor Pax6. Compared to RGs, SNPs have a longer cell cycle and smaller self-renewal capacity. Instead, they tend to divide symmetrically and give rise to neurons (Laguesse et al., 2014).

Neurons populating the telencephalon originate from different proliferative areas. Cortical projection neurons originate from the pallial proliferative zones (VZ and later SVZ) and migrate radially to populate the developing cortex, while cortical GABAergic interneurons originate from subpallial proliferative zones (medial, lateral, caudal, ganglionic eminences and pre-optic area) migrate tangentially to enter intermediate or marginal zone and then switch to radial migration to enter the developing cortical plate and integrate to functional networks (Nguyen & Hippenmeyer, n.d.).

The first neurons that result from asymmetrical divisions of NEs and migrate radially out of the VZ roughly around E11-12, even before the formation of SVZ by BIPs, form a distinct cell layer, the preplate. The preplate is separated from VZ by the intermediate zone (IZ). The second wave of post mitotic neurons migrates also radially and split the preplate thus creating the cortical plate in the middle and the subplate on the ventral and the marginal zone on the dorsal side of the cortical plate (Rubenstein, 2003).

Cajal-Retzius cells, which are born at the time the preplate is formed, remain under the pial surface in the marginal zone, whereas the rest of the primordial cells constitute the subplate (Me, Bernard, & Cedex, 2006).

Subsequent waves of migrating neurons populate the cortical plate increasing its size as cortical development proceeds, and generating layers II-VI. The newly born migrating neurons originating from VZ and SVZ settle past the earlier born in such a way that deeper layers are populated by “older” neurons than superficial ones (Atanaka, Hu, & Origoe, 2016) (Nguyen & Hippenmeyer, n.d.).

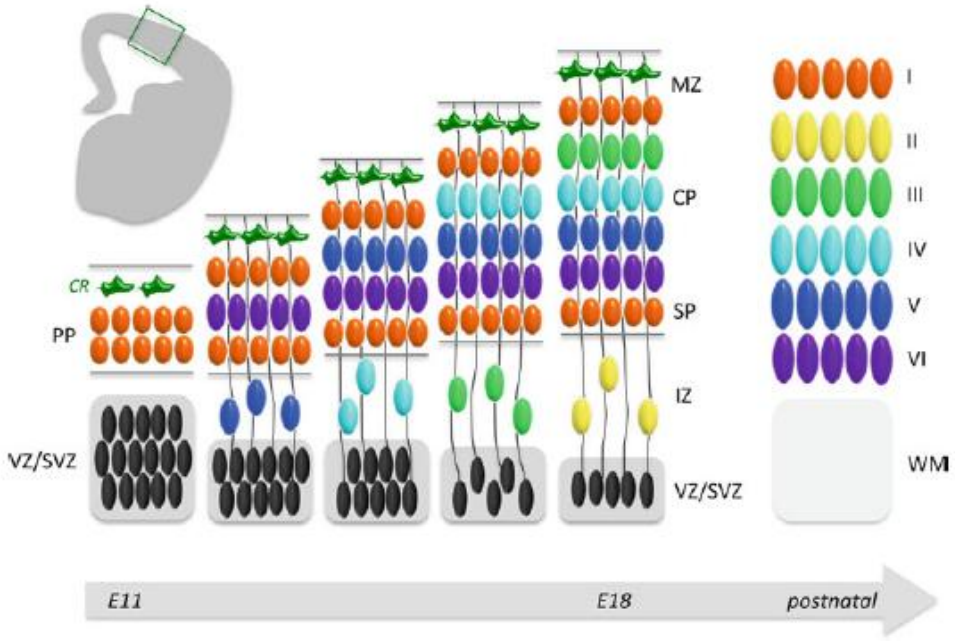


Figure 3: Inside-out layering of migrating neurons. Younger neurons migrate past the older one towards Cajal-Retzius cells residing in the marginal zone due to reelin signaling. The outcome is a six layer cortex in which deeper layers are occupied by earlier born and the more superficial by later born neurons. Adapted by (Nguyen & Hippenmeyer, n.d.)

Modes and mechanisms of migration in the cortex.

There are two distinct types of radial migration. The first is somal translocation which is observed in the earliest born neurons including the neurons forming the preplate (Kriegstein & Noctor, 2004) (Nguyen & Hippenmeyer, n.d.). During somal translocation, neurons migrating from the VZ lose their apical attachment to the VZ but not their basal attachment to the pial surface. In such a way they shorten their basal process and their soma is moving toward the pial surface. TAG-1, seems to be necessary for basal attachment as will be discussed later (Okamoto et al., 2013).

The other type of radial migration is glia-guided locomotion (Rubenstein, 2003) (Nguyen & Hippenmeyer, n.d.). Later differentiating neurons from both VZ and SVZ migrate in this manner, by using radial glial cells as their guide. More specifically, when radial glial cells undergo mitosis (always in the apical side of VZ) their basal process is not retracted but moves to one of the daughter cells. If it undergoes an asymmetric division to give rise to a post mitotic neuron, the other cell tends to use the basal process of its sister cell as a guide to migrate. They also tend to make saltatory movements towards the dorsal side, interspaced by long pauses and change their shape from bipolar (when moving) to multipolar (when pausing) perhaps to sense their micro-environment or integrate migratory cues. Some cells often change their migration mode from radial to tangential and radial again until they find their spot on the developing cortex. When they reach the pial surface they project a basal process and attach to it switching to somal translocation for their final settlement (Atanaka et al., 2016).

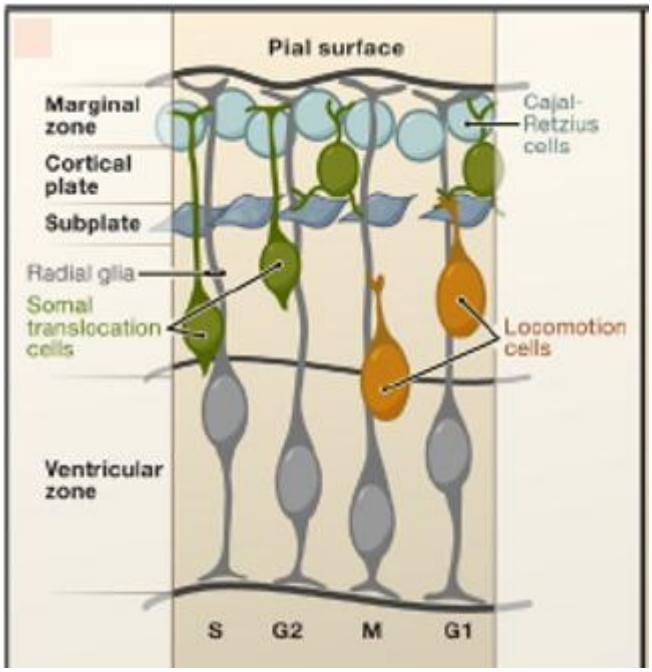


Figure 4: Distinct types of radial migration. Newborn neurons can migrate from the proliferating zones (VZ, SVZ) to the proper layer of the developing cortex in two distinct manners somal translocation (green cells) and glial guided locomotion (orange cells). Adapted by (Ayala et al., 2007)

Neuronal migration is a very complex process which incorporates both intrinsic and extrinsic developmental regulation. Many molecular mechanisms are implicated in regulation of neurogenesis and neuronal cell death to ensure that there is the correct number of neurons in the cortex, the correct proportion of excitatory and inhibitory neurons, and the correct migration to ensure proper layering. Provided that neurons have grown their axons, they should also find their way in the cortex or extra-cortical areas in order to orient to the correct area and connect to the correct target. Mechanisms that govern axon guidance and migration are still rather unclear.

We will now briefly go through the most important molecular mechanisms involved in migration during telencephalic development.

Migration could be divided in two discrete categories, radial and tangential migration, depending on the relative direction of the migrating neuron to the cortex. A migrating neuron can switch direction from tangential to radial and vice versa(Ruiz-reig & Studer, 2017) (Nguyen & Hippenmeyer, n.d.).

The direction of movement is mostly dependent on molecular cues, either repulsive or attractive which bind to their receptors expressed by the migrating neurons. The receptors in turn transduce the signal and trigger transcriptional responses and cytoskeleton (actin and microtubules) rearrangements which results initially in a leading process extension and afterwards in nuclear translocation (Ayala et al., 2007) (Neurochemistry, 2017).

Besides molecular guidance cues, in most cases (except somal translocation) migrating neurons need a scaffold to migrate on. Radially migrating locomoting neurons in the cortex mostly use the processes of radial glial cells to migrate on, while tangentially migrating interneurons from the subpallium seem to follow TAG-1-positive corticofugal axons that express the cell adhesion molecule TAG-1 (Denaxa, Chan, Schachner, Parnavelas, & Karagogeos, 2001). However, data supporting this hypothesis are not conclusive since one of the major migratory routes, intermediate zone, is axon sparse. Moreover ablation of TAG-1 does not affect the number of interneurons in the cortex (Denaxa et al., 2005) and even ablation of most of TAG-1 expressing axons do not prevent cortical interneurons from invading the cortex as will be further discussed later.

Specific pathways governing response to migration cues have been extensively studied, yet many details remain unclear.

Several families of chemotropic molecules, including Netrins, Semaphorins, and Slits that were identified as the guidance cues for axonal navigation have also been found to guide neuronal migration. Netrin acts through its receptor DCC and has repulsive or attractive effect on the migrating neurons (repulsive in the cerebellar granule and striatal neurons and attractive on the pontine neurons of hindbrain). Different

effects are mediated through different intracellular pathways resulting in regulation of actin and microtubule dynamics.

Slits acting via their receptor roundabout (ROBO) have also been shown to play a role in both tangential and radial migration. Acting through a rho GTPase activating protein (GAP), the binding of slit 2 to ROBO also results in regulation of actin dynamics.

Semaphorins 3A and 3F binding to their receptor neuropilin and plexin respectively regulate the migration of cortical and striatal interneurons born in the MGE.

Integrating guidance cues is not sufficient for a neuron to achieve successful migration. For this to happen, cytoskeleton rearrangement is the key in order to extend the leading process and then move the nucleus. The most well studied complex involved in this is LIS1/NDEL1 complex. This complex is essential for bonding the nucleus (through nuclear membrane proteins Syne 1/2, SUN 1/2) to cytoplasmic dynein and in this manner move the nucleus towards the centrosome located in the leading process. NDEL1 in a higher degree but also LIS1 bear many post translational modifications such as palmytoilation and activating phosphorylation and are targets of kinases such as CDK5 and Aurora A which are thought to be signal transducers involved in cortical neuron migration signaling (Nguyen & Hippenmeyer, n.d.). Consistent with the above, inhibition of normal LIS1 function has a serious effect in neuronal polarity and migration "trapping" cortical neurons in a multipolar shape in the embryonic SVZ, in a dose dependent manner while NDEL1 is also necessary for multipolar to bipolar transition and cortical plate invasion (Hippenmeyer et al., 2010).

Transcription is also tightly regulated during development. There are key transcription factors dictating the fate every cell will adopt during development, its laminar position in response to the signals it receives from its surrounding cells and extra cellular matrix. In particular cortical projection neurons and interneurons do deploy specific transcriptional programs controlling their migration and settlement in the correct layer. Superficial versus deep layer positioning of late born versus early born neurons is being controlled by specific transcriptional programs.

Transcription factor SOX5 is expressed in early born subplate neurons, neurons of layer VI and a subset of neurons of layer V and is seemingly necessary for somal translocation of deep layer residing. early born neurons and to the development of their axonal projections (Muhchyi, Juliandi, Matsuda, & Nakashima, 2013). In sox5 $^{-/-}$ mutant mice, early born neurons fail to split the preplate and display a phenotype similar to reeler mouse (Hoerder-suabedissen & Molnár, 2015)(Guy & Staiger, 2017).

As far as the later born neurons are concerned, transcription factors POU3F2 and POU3F3 seem to play a critical role in their migration. They are co-expressed by the VZ progenitors and (until they reach layers II-V ;) by cortical projection neurons in layers II-V. Double mutants are able to migrate out of the VZ but fail to migrate past the subplate and remain stashed beneath it, resembling the phenotype of CDK5

mutants. Interestingly among POU3F2/3 transcriptional targets are DAB1 and p35 and p39 which are known to activate CDK5 (Nguyen & Hippenmeyer, n.d.).

Discrete steps of migration such as detachment from the apical surface of VZ, multi-to-bipolar transition and migration past the subplate are also specifically regulated by step-specific transcription factors. Proneural transcription factors such as neurogenin 1/2 and ASCL1, besides their key role in neurogenesis, also have a critical role in neuronal migration, inducing the expression of transcription factors Scratch1 and Scratch2. Those in turn mediate the downregulation of E-Cadherin which is necessary for the adhesion of apical process on the ventricular zone, in the beginning of radial migration of cortical projection neurons. Transcriptional targets of neurogenin1/2 are not restricted to genes acting in the beginning of migration but involve genes acting in following steps as well such as RhoA, Rnd2, Dcx and P35. ASCL1 also regulates the transcription of genes important for later stages of migration such as Rnd3. RND2 is inhibiting RhoA signaling allowing multipolar to bipolar transition in IZ while RND3 also inhibits RhoA-dependent actin polymerization to promote glial guided locomotion inside the cortical plate. RP58 is another target on neurogenin2 that represses neurogenin2 expression in a negative feedback loop ensuring the temporal limitation of the multipolar state of migrating neurons. Fox1g is another transcription factor implicated in cortical projection neuronal migration. It is expressed to support migration but downregulated to allow the expression of UNC5D which is essential for multi to bipolar transition. After this transition is completed, Foxg1 is again upregulated to induce multi to bipolar transition and migration to the cortical plate (Nguyen & Hippenmeyer, n.d.).

Neurons residing in different layers tend to project to different areas. VI neurons project to the thalamus, V layer neurons project to other subcortical areas including spinal cord and pons. Layer II/III and IV neurons project to other cortical areas including cross hemisphere projections most important of which the corpus callosum (Muhchyi et al., 2013).

Different projection targets are linked to specific layer identity defining transcription factors chromatin remodeling proteins and transcriptional repressors. Subplate neurons and layer VI neurons express SOX5 and TBR1 which, to a great extend seem to be responsible for their laminar identity. They repress a zinc finger transcription factor Fezf2, expression of which is needed for the induction of another zinc finger transcription factor Ctip2. Both FEZF2 and CTIP2 are able to induce guidance of axons in subcortical targets when ectopically expressed. In upper layers Fezf2 and subsequently Ctip2 never seem to be expressed. Satb2 is expressed instead which in collaboration with histone modifying enzymes seem to repress Ctip2 expression, in upper layers. SATB2 is expressed in all callosal neurons in layers II-V and promote subcortical targeting of axons. In the absence of Satb2, Ctip2 is expressed resulting in extra cortical targeting of layer II-IV projections (Dennis et al., 2017) (Guy & Staiger, 2017).

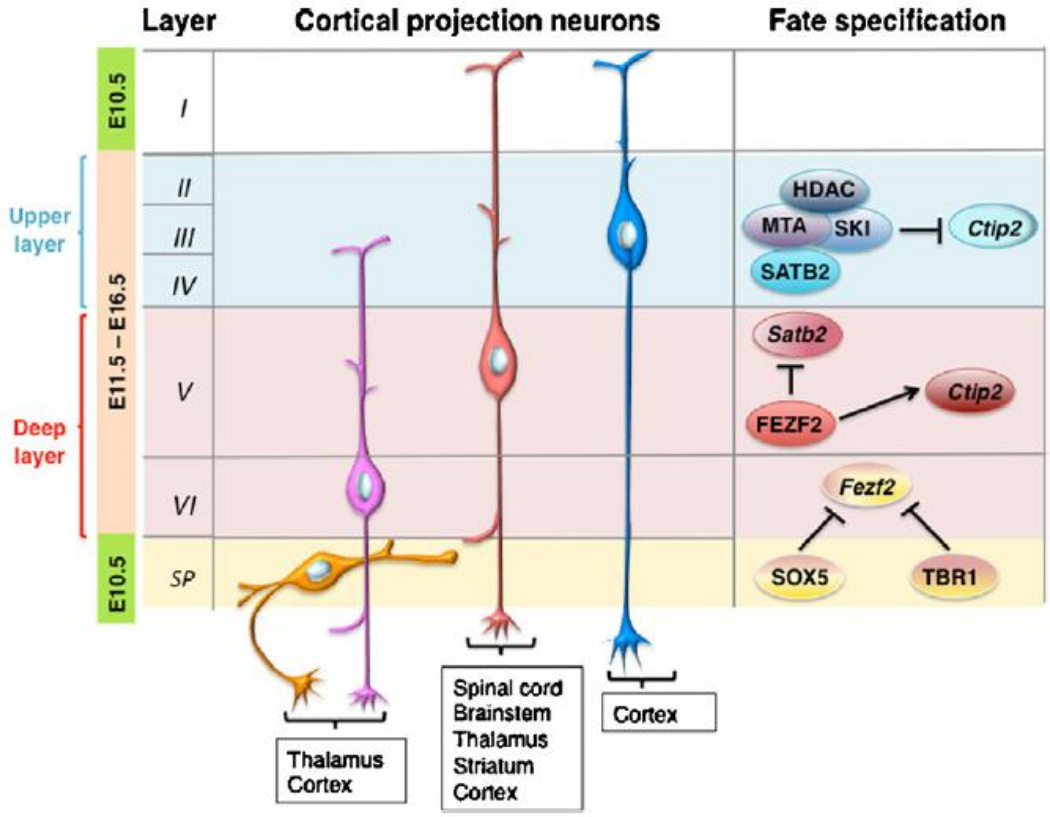


Figure 5: Transcriptional control of layering identity. Layers tightly regulate the transcriptional landscape in order to maintain correct projection pattern. In layer six expression of Tbr1 and Sox5. In layer five fezf2 is de-repressed and Ctip2 is expressed. In more superficial layers the expression of Satb2 leads to repression of Ctip2. Adapted by: (Muhchyi et al., 2013)

Callosal axon guidance and extention

The corpus callosum (CC) is the largest fiber tract in the central nervous system, connecting the right with the left hemisphere. The development of CC happens in descrete stages. After the migration and committment of neurons to upper layers and expression of CTIP2, they extend their axons towards the midline. After that the growth cone senses guidance cues and reaches the midline (Lo, 2013). After crossing the midline callosal axons travel through the IZ and the invade the cortical plate to form synapses with their targets. After crossing the midline, in some cases (motor, visual, parietal cortex) axons wait in the IZ or subplate for a period of time and then migrate to the dorsal side of the cortex probably using radial glial processes (Donahoo, Prof, & Hons, 2009).

Each of the above steps is very closely regulated in order for the correct formation of CC to be completed.

Callosal axons tend to inervate neurons the same layer and the same area as where their somata reside (Fenlon & Richards, 2015). There is also evidence that some callosal axons branch before or after crossing the midline and target other areas

(such as the striatum or other cortical layers) except for their contralateral layer. The first step is the fusion of telencephalic midline and the elimination or extrusion of leptomininges from the cortical plate. Shortly afterwards the pioneer axons af the CC, originating from neurons residing in the most medial part of the cortex (the cingulate cortex) extend towards the midline and the reach it between embryonic day 14-15. By E17, axons grown from the rest of the neocortex follow the path of the pioneer axons which probably provide a structural framework for neocortical axons to be guided probably through signaling by neuropilin-1 receptor and reach the midline as well enlarging the CC. Npn-1 – semaphorin 3C signaling is shown to be inportant in guidance of the pionner axons to the midline. Npn-1 is also expressed by neocortical neurons (Donahoo et al., 2009).

Whether or not pioneer axons from the cingulate cortex actively regulate the formation and/or guidance of the CC has yet no definitive answer but data suggest that the same kind of mechanisms exist in other major fiber tracts (such as the retinal, spinal and olfactory systems) and that damage or malformation of cingulate cortex does indeed affect the targeting of callosal axons in humans (Lj, Plachez, & Mechanisms, 2004).

At the midline, several cell populations namely indisium grisium, glial wedge, subcallosal sling and hippocampal commisures in the caudal part of the cortex, assist the guidance of pioneer and neocortical axons across the midline (Nishikimi, Oishi, & Nakajima, 2013). The glial wedge is a cell population comprised of astrocytes and neurons residing medialy of the latteral ventricles and ventraly of the CC. Due to its anatomical position and to the fact that it expresses the repulsive guidance cue SLIT-2, it is believed to play a role in preventing ventral expansion of the CC, the axons of which express ROBO (the receptor for slit-2). Indusium Griseum is believed to have a similar function, preventing the dorsal expansion of CC again by expressing Slit-2 (Lo, 2013) .

The environment of the developing cortex is not fixed along the rostrocaudal axis susggesting that different cell populations and/or fiber tracts dictate callosal guidance in different rostrocudal levels. Guidance of the caudal part of the CC, the splenium, could be assisted by the hippocampal commissure which is formed approximately 24h before CC and lies exactly ventral to it. There is a correlation between hippocampal commissural and CC defects but no mechanism explaining them is yet described.

After crossing the midline callosal axons travel under the subplate until they reach their target area and then dorsally to form synapses with the target layer.

Moreover, neurons tend to form contralateral callosal conections during development which are retracted (pruned) by the time adulthood is reached (Fenlon & Richards, 2015) (Donahoo et al., 2009). The role of these conections is still unclear but it has been observed that callosal neurons innervate the subplate in a maner similar to thalamocortical axons and the cortex in areas that do not remain

innervated until adulthood. There is also evidence that more axons innervate areas that remain innervated in adulthood but progressively the number of those axons deminishes. The developmental role (if any) of those transient synapses has yet to be elucidated. The activity of the target area seem to play a role in the selection of axons that will be maintained in comparison to those that will be pruned but the mechanism underlying activity-dependent targeting has yet to be elucidated (Donahoo et al., 2009).

There is also evidence that the position in the callossal fiber tract is important for contralateral targeting of the axons contained inside it, whereas the positioning of the neuronal soma is not, pointing towards a mechanism that directs axons in the cortical plate depending on their relative positioning in the fiber tract (Zhou et al., 2013).

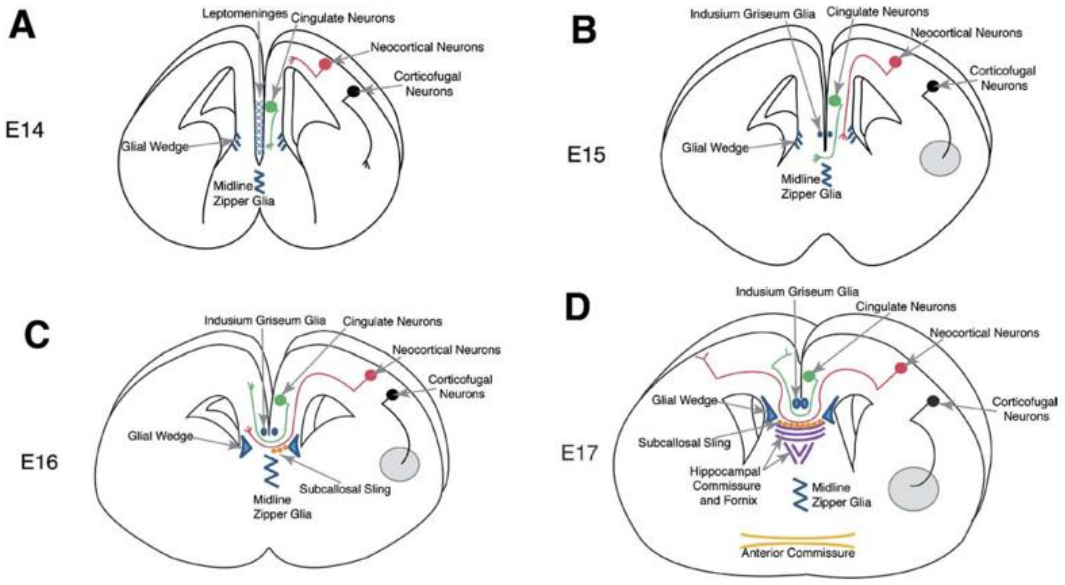


Figure 6: Development of corpus callosum. Corpus callosum development in the mouse. (A) Callosal formation firstly requires the establishment of a substrate, which is achieved through the fusion of the telencephalic midline. This involves either the elimination or exclusion of the leptomeninges found between the hemispheres. (B) The pioneers of the corpus callosum originate from the cingulate cortex (medial-most part of the cortex) and reach the midline between embryonic day14 (E14) and E15. (C) Axons from the neocortex then grow along the pathway defined by the pioneers, expanding the corpus callosum by E17. (C) On arrival of callosal axons at the midline, midline cellular populations (glial wedge, indusium Griseum, and the subcallosal sling), and the extracellular cues that they secrete, assist in the turning and channeling of these axons across the midline. (D) Axon guidance of the caudal corpus callosum may also be facilitated by the hippocampal commissure. After the axons have entered the contralateral hemisphere, they traverse dorsolaterally before innervating homotopic areas of the contralateral cortex. Adapted by : (Donahoo et al., 2009)

Developmental cell death in the cortex

Apart from neurogenenesis, migration and axon guidance, cell death also plays an important role in the development of functional networks in the developing cortex. Different cell types and populations undergo cell death to a differend extent.

Pyramidal neuron numbers are reduced via apoptotic cell death by aproximately 13% throughout the cortex and their cell death peaks between post natal days P2 and P5. The proportion of pyramidal neurons undergoing apoptosis diversifies between different areas of the cortex (more cells die in the motor cortex but in the somatosensory cortex there is minimum cell death) and between different layers of the same area. The most significant factor regulating apoptosis seems to be neuronal activity. Pyramidal neurons seem to regulate their survival in an activity-dependent manner since increase of activity in an area through increase of extracellular K^+ concentration decreases the rate of apoptosis while blocking of activity with tetrodotoxin (TTX), an antagonist of voltage-dependent Na+ channels, increases significantly the rate of apoptosis. The exact mechanism mediating activity dependent survival remains unclear but the phosphorylation of AKT kinase by PIK3 and the subsequent increase of intracellular Ca^{2+} concentration seems to be implicated in this process.

Cortical interneurons also undergo apoptosis postnataly, during the first two weeks after birth, decreasing their number up to 40%. They reach the peak of apoptosis rather later than pyramidal neyrons between post natal day P5 and P10. Cortical interneurons seem to have an intrinsic timer and a set period of time from their birth to when they enter the critical period in which they have to “decide” if they will undergo apoptosis or not. A critical role in this decission plays the synaptic input they get from pyramidal neurons. The more active the pyramidal neurons of a network the more probable is for interneurons of this network to survive. This activity-dependent survival could be regulated by the levels of the phosphatase PTEN which acts as an inhibitor of PIK3 and as a consequence downregulates AKT and calcium-dependent survival. Increase of pyramidal neuron activity in a network leads to an decrease of PTEN levels in interneurons, thus promoting their survival (Denaxa et al., 2018).

Cajal-Retzius cells also undergo programmed cell death but much more extensively than pyramidal neurons and interneurons. By P10 all of them have died with their death time period varying depending on their origin (cortical hem, pallial-subpallial boundary or pallial septum). Interestingly they seem to undergo caspase-independent cell death. The survival of Cajal-Retzius cells also seem to be activity-dependent, but unlike pyramidal cells and interneurons, increased activity leads to increased cell death. This response seems to be mediated by glutamatergic receptor signaling either directly or indirectly by increased activation of interneurons and increased GABA signaling (which leads to depolarization due to very low resting potential of these cells) to the Cajal-Retzius cells.

Subplate neurons function as an intermediate station for thalamocortical axons making transient synapses with them and the migrating pyramidal neurons in the cortex. After pyramidal neurons have settled to their layers, subplate neurons (at least a subpopulation of them) seem to undergo programmed cell death as well but the data supporting it are not conclusive. It is not yet clear if subplate neurons die developmentaly and the subplate is subsequently repopulated, if they migrate to layer 6b or if their population is just reduced as it happens with pyramidal neurons.

Developmental cell death is therefore a regulatory homeostatic mechanism functioning as a quality control (highly aneuploid cells are more likely to die during critical periods of cell death), as a control mechanism to ensure the correct ratio of pyramidal neurons and interneurons and therefor the functionality of networks, and is also required for the removal of missconnected cells (Marin, Rev, Dev, & Downloaded, 2019).

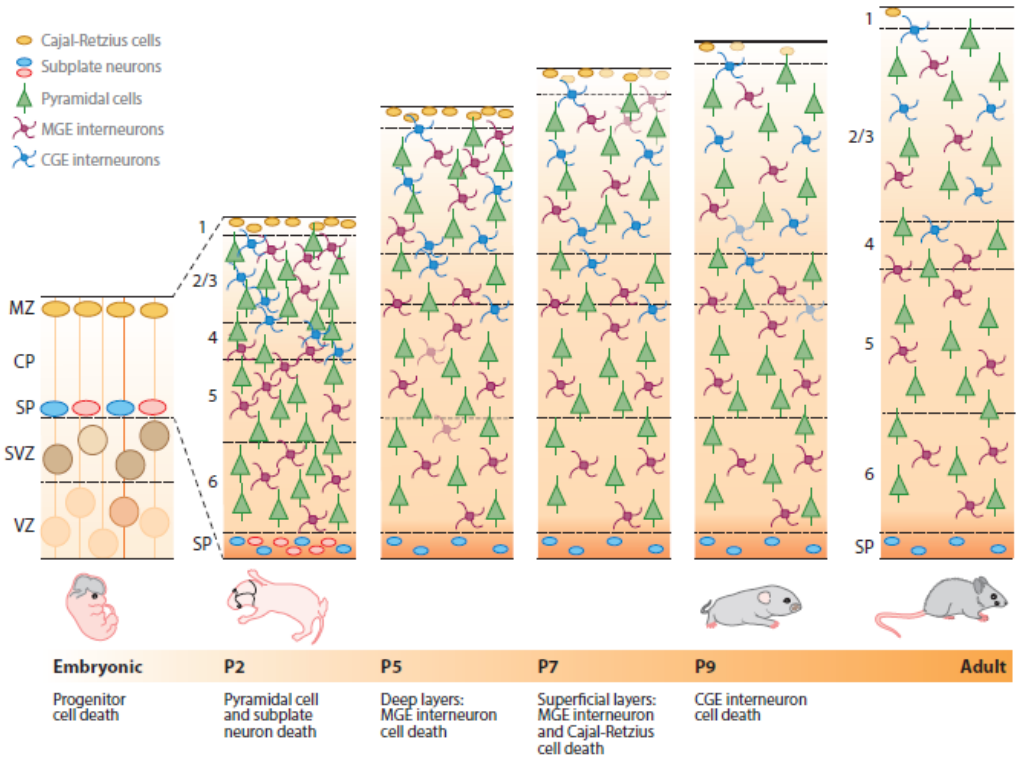


Figure 7: Cell death in the developing cortex. Developmental timeline of programmed cell death in the mouse neocortex. During embryonic development, programmed cell death is particularly abundant among progenitor cells in the ventricular zone (VZ) and subventricular zone (SVZ). Apoptotic progenitors are shown in red. During postnatal development, pyramidal cells (green) and subplate neurons (light blue) undergo programmed cell death starting from postnatal day 2 (P2), followed by infragranular medial ganglionic eminence (MGE) interneurons (magenta) at P5, supragranular MGE interneurons (magenta) and Cajal-Retzius cells (gold) at P7, and caudal ganglionic eminence (CGE) interneurons (blue) at P9. Other abbreviations: CP, cortical plate; MZ, marginal zone; SP, subplate. The numbers 1–6 refer to the layers in the postnatal cerebral cortex. Adapted by: (Marin et al., 2019)

Generation of Tag-1^{EGFP} EMX1^{cre} ; Tag-1^{DTA45}

The cell adhesion molecule TAG-1 has multiple roles in neuronal development. It is involved in neurite extention (Furley, Morton, Dodd, & Jessell, 1990), guidance (Stoeckli, 2018), tangential migration (Denaxa et al., 2001) among other functions. Nevertheless Tag-1 KO mice do not exhibit any major defects pointing towards a rather redundant function of Tag-1. In the cortex, TAG-1 labels strongly the corticothalamic axons (Wolfer, Henehan-beatty, Stoeckli, Sonderegger, & Lipp, 1994). In an effort to identify the neurons expressing TAG-1 we inserted a construct expressing floxed GFP sequence under the promoter of TAG-1 and downstream the subunit A of Diptheria Toxin (DTA) in frame (see figure below). In the absence of Cre recombinase, neurons that express TAG-1 also express GFP and thus this mouse is the first to provide the opportunity of locating the somata of cells expressing TAG-1. In order to understand the role of TAG-1 expressing neurons in cortical development we decided to ablate them from the developing cortex (Kastriti et al., 2019). in order for this to be achieved, Cre recombinase had to be expressed in a very specific spatiotemporal pattern which would result in ablation of all pyramidal neurons expressing TAG-1 only in the cortex.

Transcription factors such as EMX1/2, have an indispensable role in brain development. Distinct areas of the developing telencephalon have distinct transcriptional programs governed by major transcription factors (Schuurmans & Guillemot, n.d.). EMX1 is expressed in the dorsal telencephalon seemingly being regulated by Gli3. Fate mapping studies using the EMX-cre/ lacz model demonstrated that in the adult cortex and hippocampus 88% of neurons in the cortex come from Emx1+ progeny with less than 2% of GABAergic interneurons being β-gal positive (Gorski et al., 2002). Emx1 also seems to induce transcription of Nrp1, an axon guidance receptor which plays a critical role in callosal axons finding their way across the midline (J. W. C. Lim et al., 2015).

Therefore crossing Tag-1^{EGFP} with Emx1^{cre} line would eventually result in ablation of pyramidal neurons expressing TAG-1 that have migrated to the cortex before E14, since EMX1 (and cre recombinase) is expressed between E11-E13,5. Moreover upon crossing with EMX1^{cre} line, the GFP sequence gets floxed out and DTA sequence comes in frame and it is being expressed under the promoter of Tag-1. DTA expression results in death of TAG-1 expressing neurons in the developing cortex. of note, Tag-1^{DTA} mice do not exhibit complete ablation of TAG-1-expressing cells but the vast majority is being ablated.

In our mouse model the escapee cells expressing TAG-1 in the cortex could correspond to that 12 percent. Those cells could have migrated from extrapallial areas, outside Emx1 expression zone (J. W. C. Lim et al., 2015).

The model described above is an indispensable tool for identifying the layer identity of neurons expressing TAG-1 and their role in diferent aspects of development such

as major fiber tract formation migration of interneurons in the cortex and the behavioral phenotype of Tag-1^{DTA} mutants compared to Tag-1^{EGFP} controls.

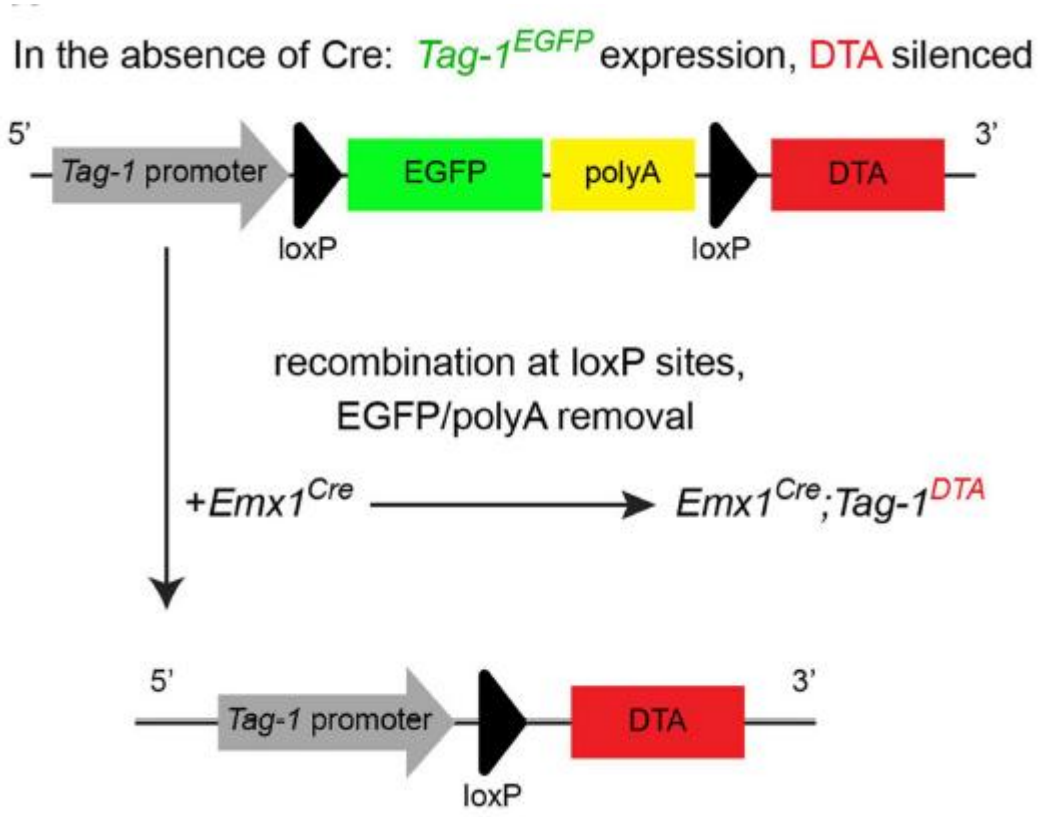


Figure 8: Tag-1$^{loxP-EGFP-loxP-DTA}$ construct. In the absence of Cre recombinase TAG-1-expressing cells are expressing EGFP. Upon crossing with Emx1^{Cre} Gfp is floxed out in cells expressing Emx1 and DTA is expressed leading cortical pyramidal neurons to apoptosis. Adapted by:(Kastriti et al., 2019)

Aim of study

In the present study we used the Emx1^{cre};Tag1^{DTA45} mice, previously generated in our lab, which exhibit extensive cell death in the developing cortex, in order to understand the role of TAG-1 expressing axons in different aspects of cortical development starting from formation and guidance of thalamocortical axons and of major fiber tract such as the CC and anterior commissure (AC). Yet we got the opportunity to study a system in which extensive developmental cell death in the cortex results in a viable phenotype. In these terms we wanted to examine the anatomical and behavioral results of the ablation of TAG-1 expressing neurons in the cortex. In particular we examined the effect of the ablation of TAG-1 expressing cortical pyramidal neurons in the formation of major fiber tracts such as CC and AC. Moreover we checked if ablation of pyramidal neurons expressing Tag-1 from the cortex has any indirect effect concerning the population of cortical interneurons. Finally we were interested in the behavioral phenotype of Tag-1^{DTA45} mice concerning learning, spatial and temporal memory.

Results

Reduced cortical size and major defects in corpus callosum and anterior commissure

Satb2 expressing neurons residing in cortical layers II/III are known to project mostly to the respective cortical areas of the contralateral hemisphere forming the largest in volume fiber tract, the CC (Seuntjens et al., 2009). Even though most neurons of layer V project to subcortical areas, some neurons of upper layer V do also contribute to the formation of CC (Fenlon & Richards, 2015) (Petreanu, Huber, Sobczyk, & Svoboda, 2007). We stained brain cryoslices taken by Tag-1^{EGFP} for GFP to check what layers are populated by Tag-1 expressing neurons. We observed that GFP expression is notable in layers II/III and also in layer V and layer VI. In Tag1^{egfp} mice and there is an observable reduction in cells of those layers in Emx^{cre} ; Tag1^{DTA45} mice (**figure 9).**

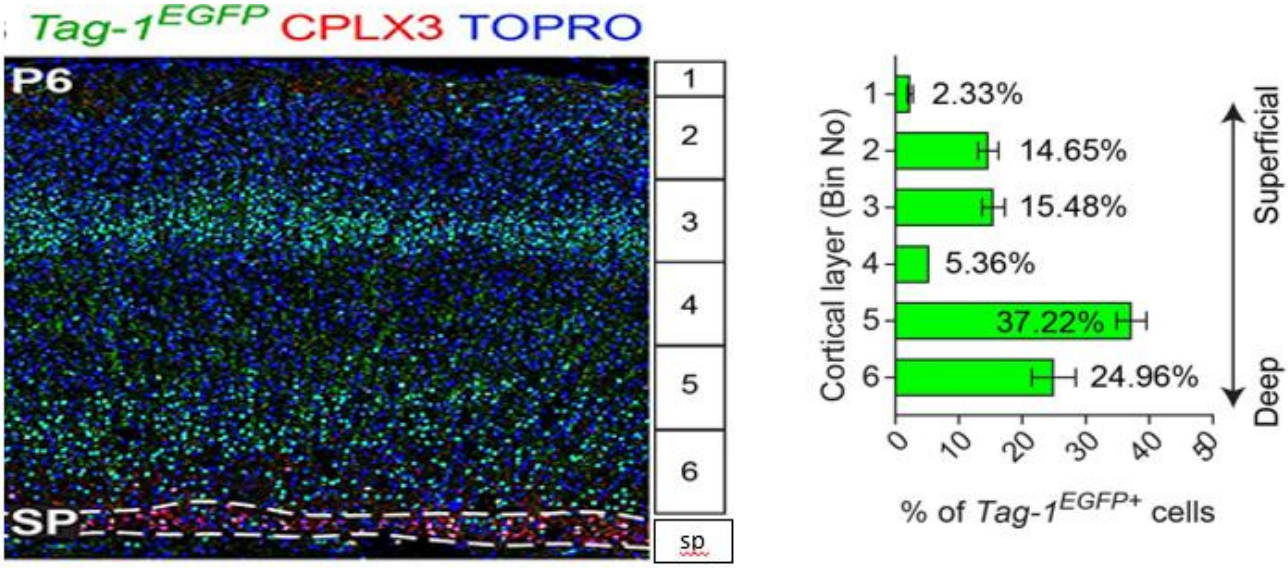


Figure 9: expression pattern of Tag-1^{EGFP} in the barrel cortex of mice. We can observe a strong representation of Tag-1 expressing neurons in layers II/III and V which account for the formation of CC. n=2 controls and 2 mutants

The ablation of TAG-1 expressing neurons in the cortex as a result of expression of DTA in the Emx^{cre}; Tag1^{DTA45} resulted in ablation of the vast majority of TAG-1 expressing neurons (**figure 10**).

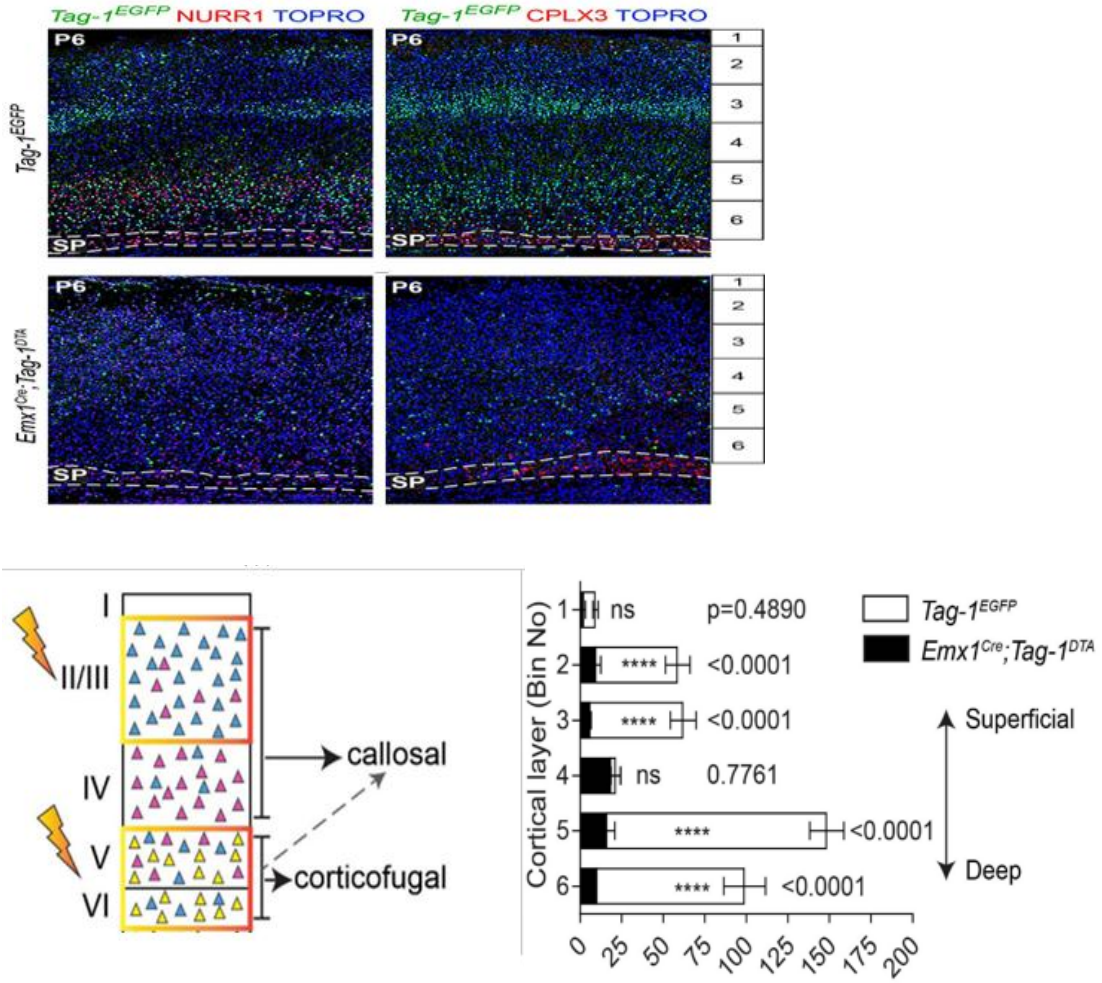


Figure 10: DTA expression by the promoter of Tag-1 led to ablation of the vast majority of cortical TAG-1 expressing neurons. N=2 controls and 2 mutants

Given the fact that the vast majority of neurons expressing TAG-1 was ablated and the fact that in controls these neurons populate layers II/III and V which contribute a great deal to the formation of callosal axons, we checked the effect of DTA-mediated ablation of TAG-1 neurons in the formation of the CC.

We stained P6 brain cryoslices for L1, which is a cell adhesion molecule localized in axons, and GFP, to stain TAG-1 expressing neurons. We observed that the ablation of TAG-1-expressing cells had a great impact on the formation of the CC. We observe

that this structure is smaller and that the splenium (the caudal part) is mostly affected since it cannot even cross the midline (**figure 11**).

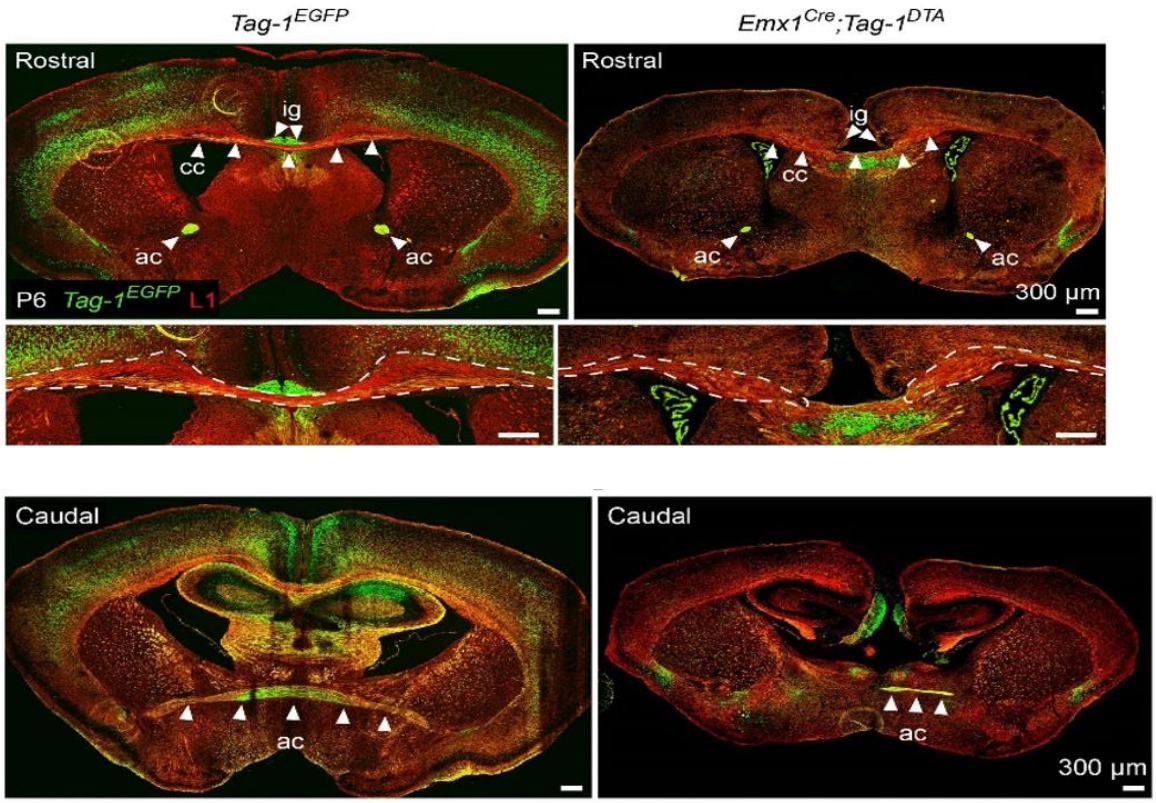


Figure 11: immunostaining for L1 (an axonal marker) and GFP. Note the reduction of the size of the CC rostrally especially on the midline. Also note that caudally corpus callosum is more severely impacted and fails to cross the midline.

In order to get a 3D view of the CC we performed micro computed tomography. The results confirmed the phenotype already observed by immunostaining and displayed that the overall volume of CC is almost 4 times smaller in EMXcre; $Tag1^{DTA45}$ mutants compared to $Tag1^{EGFP}$ controls (1,31mm^3 vs 5,26mm^3 for controls) **figure 12**. The phenotype seems to be more intense caudally in the splenium, where the CC of mutant mice does not even cross the midline than rostrally in the genu. Finally, no Probst bundles (large, bilateral, barrel-shaped axonal structures that do not cross the midline, often observed due to lack of guidance cues by midline glial populations) have been detected in mutant mice (Nishikimi et al., 2013).

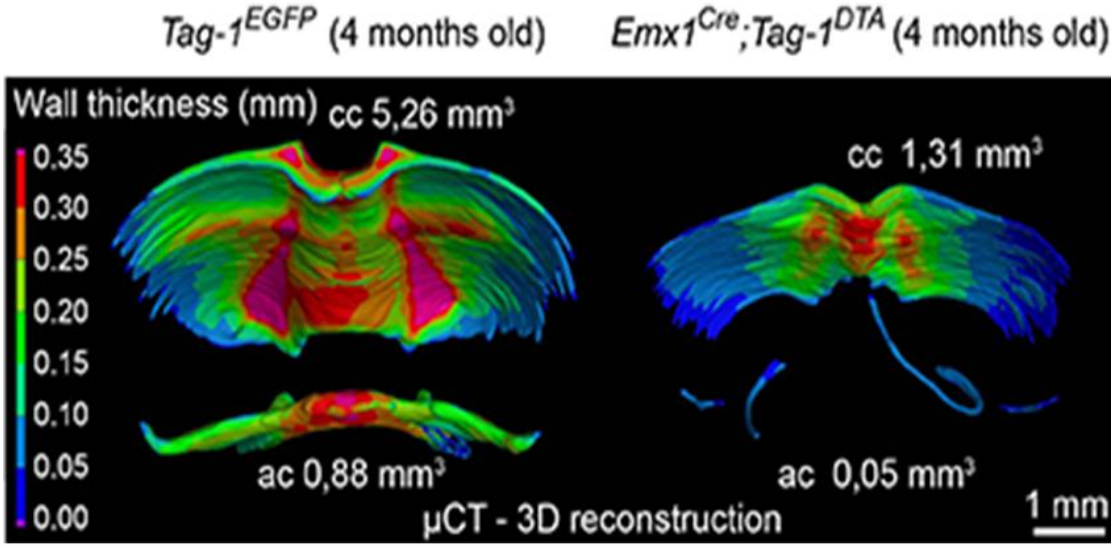


Figure 12: micro computed tomography of control compared to mutant mice. Note the overall volume reduction of the CC and the absence of the rostral part. Also note the severe malformation of anterior commissure and the failure to cross the midline. N=2 controls and 2 mutants (but all data from IHC agree with the phenotype from CT scans)

Anterior commissure (AC) is the second largest fiber tract of the mammalian brain, which is composed of a rostral and a caudal component and is formed by the axons of neurons of the anterior olfactory nuclei, the olfactory tubercle, perirhinal cortex, agranular insular area and temporal cortex, and amygdaloid nuclei and the piriform cortex of the temporal lobes (Livy et al., 1997). Previous knowledge of TAG-1 expression by the AC, and specifically neurons of the AON, prompted us to examine *Tag-1*^{EGFP} expression by the AC. Indeed, we detected EGFP+ axons in both components of the AC during late embryonic and early postnatal development (Bastakis, Savvaki, Stamatakis, Vidaki, & Karagogeos, 2015) (Wolfer et al., 1994). Additionally, we observed a decrease in the thickness of the AC in early postnatal *Emx1*^{Cre}*;Tag-1*^{DTA} mice. Furthermore, DiI tracing from the AON clearly depicted the anterior AC in the brain of newborn control mice extending towards the midline, but failed to do so in the TAG-1-ablated brain, confirming our previous observations. Analysis of adult brain using µCT showed an even more dramatic phenotype of misguidance of the AC.

Thus, we conclude that *Tag-1*^{EGFP} mimics the reported expression for TAG-1 in the AC and that *Emx1*^{Cre}-induced ablation of TAG-1+ neurons results in a significant reduction of the TAG-1+ neuronal population that contributes to the AC, with a small part of the tract still detectable.

Indusium Griseum is not affected

Indusium Griseum is a mixed glial-neuronal population residing in the midline above the CC and has an important role in the guidance of pioneer callosal axons past the midline (Donahoo et al., 2009). In those terms, we wanted to examine if Indusium Griseum was affected by DTA-mediated ablation of TAG-1+ neurons in the cortex and if so it could contribute to the defective formation of the CC. Since Indusium Griseum is a mixed population composed of astrocytes and neurons we stained for NeuN (a pan-neuronal marker), GFP (to see if neurons composing the Indusium Griseum express Tag-1) and BLBP (a marker for astrocytes) to check the integrity of the structure. GFP staining showed that neurons in Indusium Griseum do express Tag-1 in Tag-1^{EGFP} mice their ablation does not affect the integrity of Indusium Griseum (**figure 13**).

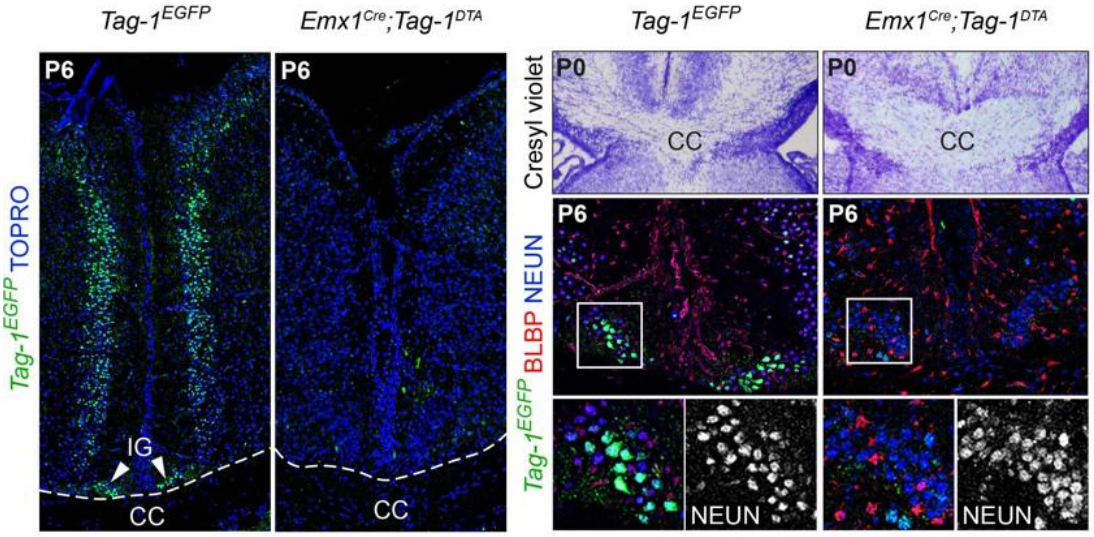


Figure 13: Tag-1 expressing neurons contribute to the formation of Indusium Griseum in control mice. Even after the ablation of TAG-1-expressing cells in Tag-1^{DTA45} mice Indusium Griseum seems to remain intact, thus it is improbable that defective formation of the CC is caused by malfunctions in Indusium Griseum. N=2 controls and 2 mutants

Reduction in the subplate cells

Deeper layer cells are known to project to the thalamus. These corticothalamic projections are also known to serve as a substrate for thalamocortical axons to be guided towards the cortex. Even though in our model there was massive reduction of corticothalamic axons due to the DTA-mediated ablation of TAG-1 expressing neurons, we did not achieve a complete ablation. The remaining corticothalamic axons were enough for the correct formation and guidance of thalamocortical axons to the cortex even if a slight defasciculation was evident.

In order to explain the unexpectedly correct development of corticothalamic axons, we checked the percentage of ablation in subplate neurons since the subplate is known to serve as a target and a "waiting hub" for the thalamocortical axons making transient synapses with them before they infiltrate the cortex (Kanold, Deng, & Meng, 2019). Furthermore, subplate axons also projecting across the pallium-subpallium boundary to different subcortical areas, including the thalamus are followed by TCAs in order for the last to reach the subplate. Approximately at E18, TCAs infiltrate the cortex using subplate neurites as a scaffold (Hoerder-suabedissen & Molnár, 2015). During early post-natal period, P0-P2 subplate neurites extend to the marginal zone and by P6 they retract to reach layer IV followed by TCAs. BY P8 subplate neuritis retract further below the "barrel hollow" of layer IV while TCAs do not retract further (Luhmann, Kirischuk, & Kilb, 2018).

In order to identify subplate cells and to check if DTA mediated ablation affected them, we stained against two different markers namely NURR1 and Complexin 3 (Hoerder-suabedissen, Oeschger, Krishnan, Belgard, & Zhi, 2013). These markers are shown to be expressed by subplate neurons born between E11.5 –E12.5, largely coinciding temporally with EMX1 expression. We found out that, even though there is less reduction in subplate neurons positive for NURR1 and Complexin 3 relatively to other subplate populations in wild type mice, we observed a decrease of both marker-expressing cells in mutants compared to wild type mice (**figure 14**).

Nevertheless thalamocortical guidance is mostly complete until P6-P7 and thus remains unaffected.

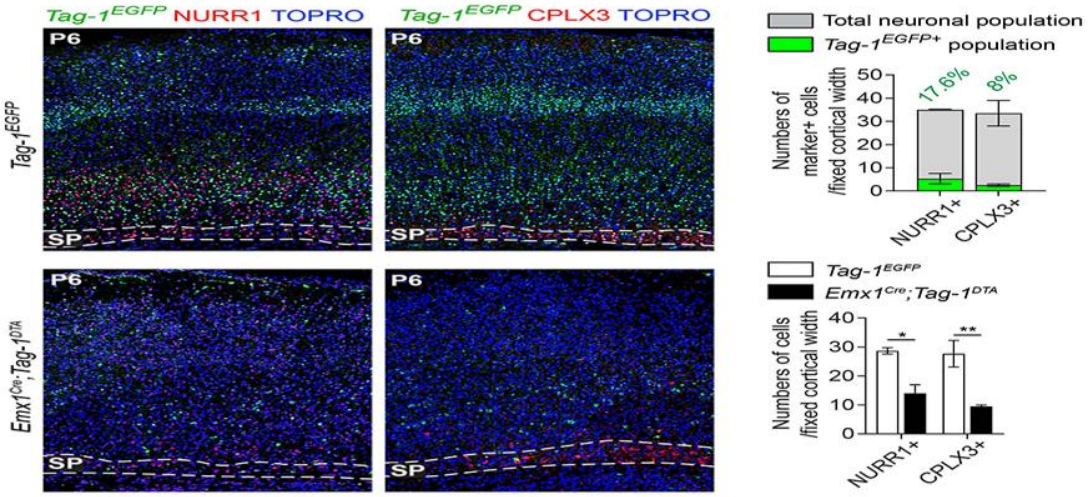


Figure 14: reduction of subplate neurons in Tag-1^{DTA45} compared to Tag-1^{EGFP} controls. Note that despite of reduction of subplate neurons thalamocortical axons are correctly guided to the cortex. N=2 controls and 2 mutants

Reduction of interneurons in the cortex

Cortical interneurons are born outside the cortex in distinct ganglionic eminences of the subpallium, namely the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE) and pre-optic area (Kessaris, Magno, & Rubin, n.d.) (Hu, Vogt, Sandberg, & Rubenstein, 2018). In order to populate the cortex they migrate through the marginal zone and the intermediate zone/subplate (Network, 2015). They begin with tangential migration while inside the migratory routes and then they switch to radial migration in order to infiltrate the cortex and reach the correct layer (L. Lim & Llorca, 2018). Interestingly, despite being born in different places they follow the layering pattern of pyramidal neurons born at the same period of time and thus also generate an inside-out model of layering (Bartolini & Ciceri, 2013). There is evidence that TAG-1 is necessary for

proper migration of interneurons from the MGE to the developing cortex, since blocking of TAG-1 with antibodies in brain slices resulted in reduced numbers of interneurons in the developing cortex (Denaxa et al., 2001). The above observation together with the expression of TAG-1 by corticofugal axons, led to the conclusion that TAG-1-expressing axons serve as a scaffold to guide migration of interneurons to the cortex. It was reasonable to expect that such a drastic reduction of TAG-1 expressing neurons (and their axons) would lead to impaired migration of interneurons to the cortex.

We performed in situ hybridization for different markers of interneurons (namely: SST, GAD67, NPY) and immunostaining for PVA, and counted interneurons in the barrel cortex of $Tag1^{EGFP}$ versus $Tag1^{DTA45}$ in juvenile mice (p21-p31). We observed a significant decrease in all cells expressing the interneuron markers we stained for in p21-p31 mutant mice (**figure 15)**.

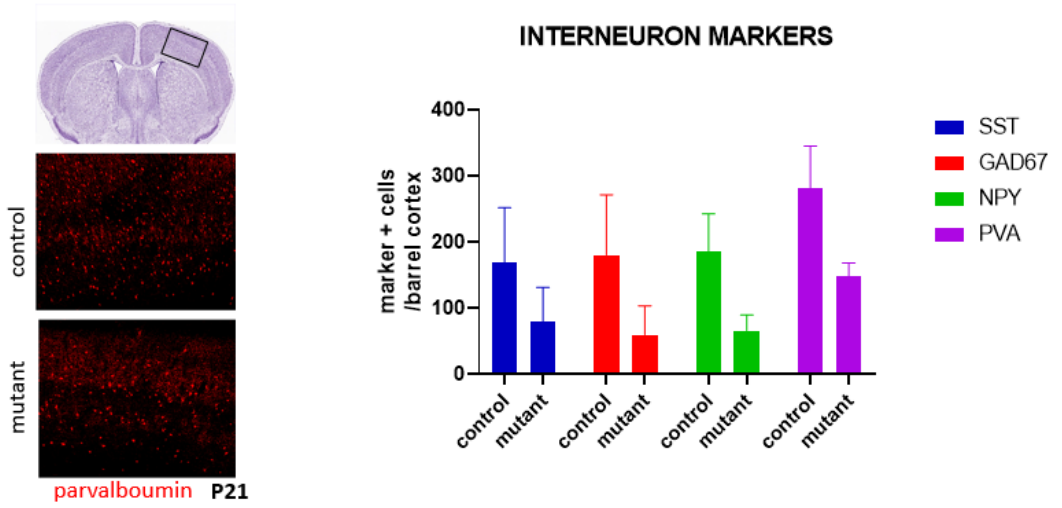


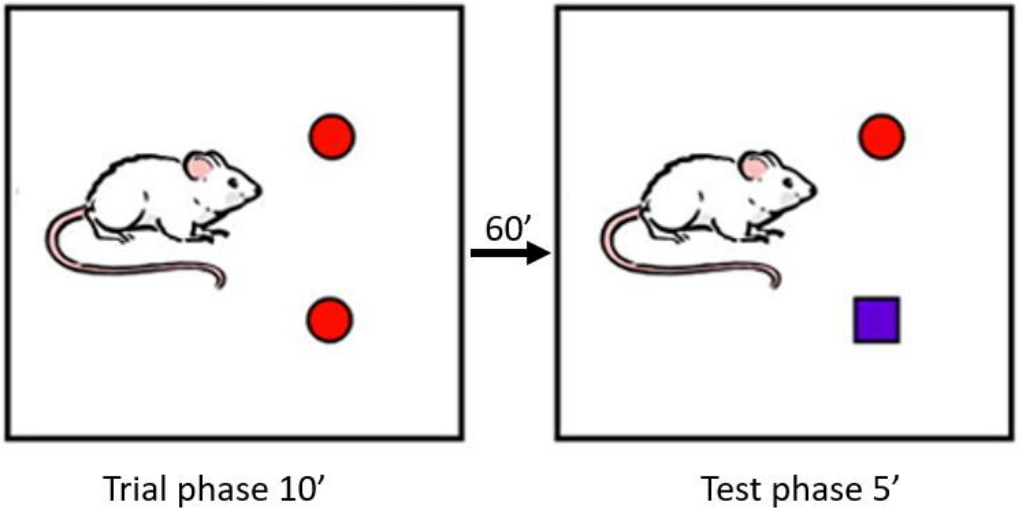
Figure 15: ISH and immunostaining for somatostatin GAD67 neuropeptide Y and parvalbumin. Note the reduction of cortical interneurons in the barrel cortex of Tag-1^{DTA} mutants compared to TAG-1^{EGFP} controls. For sst N=13 controls and 12 mutants, for NPY n =6 controls and 6 mutants, for PVA n=3 controls and 3 mutants for Gad67 n=10 controls and 9 mutants.

Behavioral effects of DTA mediated ablation of TAG1 expressing pyramidal neurons in the cortex.

Behavior is the output of brain function, therefore we wanted to examine how behavior is impacted from the extensive reduction of pyramidal neurons in all cortical layers except layer IV, the subsequent reduction of cortical interneurons and the deformities of major fiber tracts.

Driven by reduced cortical size and smaller hippocampal volume (not quantified) we wanted to assess learning, spatial memory and short term memory as well as decision making. We also examined motor learning and coordination.

We used object recognition tests, which depend on the innate tendency of the mouse to spend more time exploring novel objects than familiar ones (Ennaceur, 2010). For learning we used the novel object recognition test, which assesses the ability of the mouse to learn an object which has already spent time to familiarize with and therefore spend more time exploring the novel object compared to the familiar one (Lueptow, 2017). The mouse is left ten minutes with two same objects and after one hour one object is replaced with a different one regarding shape and color. Spending more time exploring the novel object is associated with learning. To our surprise, mutant mice displayed increased learning ability compared to controls as well as increased exploratory behavior (**figure 16**).



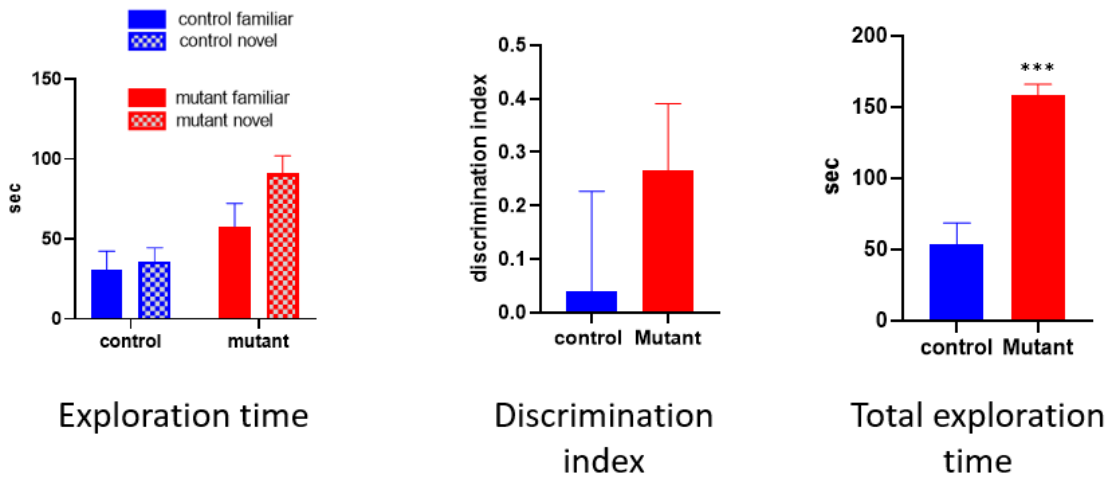


Figure 16: novel object recognition test for learning evaluation. Mutants spend more time exploring the novel object than the familiar one compare to controls. Discrimination index is a normalized index for learning and it is equal to the difference of time spent exploring the novel object compared to the time spent exploring the familiar one divided by the total exploration time. N=5 controls (1 was excluded) and 5 mutants

In order to examine spatial memory we used the object location memory task. In that test we conditioned the mice that were familiarized with two objects located in fixed places in the open field cube. After one hour, one object was moved in a new place. The mice are supposed to explore the moved object more time compared to the object remaining to the same place (Ennaceur, 2010). Control mice actually did so while mutant mice failed in that task and explored the stationary object for longer times. These results indicate that the almost complete ablation of TAG-1-expressing neurons in the cortex and/or the reduction of cortical interneurons caused impaired spatial memory (**figure 17**)

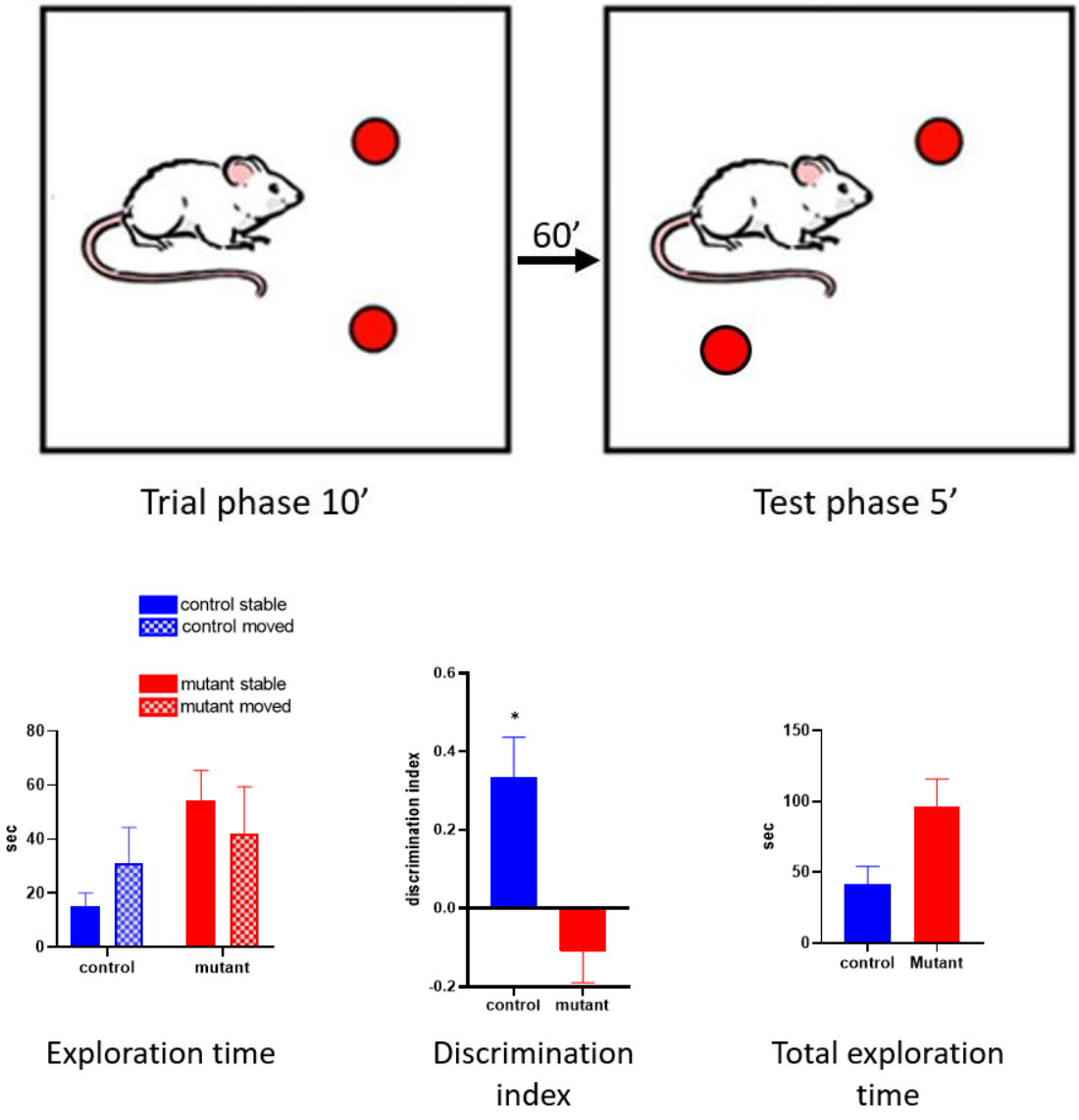


Figure 17: object location recognition test. Mice are supposed to explore moved object more time compare to stationary ones. We show that mutant mice fail to effectively learn objects location and thus spent more time exploring the object that has not been moved. Exploration time is increased in that test as well. N=5 controls (1 was excluded) and 5 mutants

Furthermore we wanted to assess the short term memory of Emx^{cre} ; $Tag1^{DTA}$ mice we performed the temporal object recognition test. This task comprised two sample phases and one test. In each sample phase, the mice were allowed to explore two copies of an identical object for a total of 4 minutes. Different objects were used for sample phases 1 and 2, with a delay between the sample phases of 1 hour. In the test phase the mice were exposed to one object from the first sample phase and one from the other, placed in the same place as in the sample phases (Ennaceur, 2010). The mice are supposed to spend more time exploring the object they saw first than the object they saw more recently. Indeed, control mice explore the last seen object less time than the first object but mutant mice performed even better.

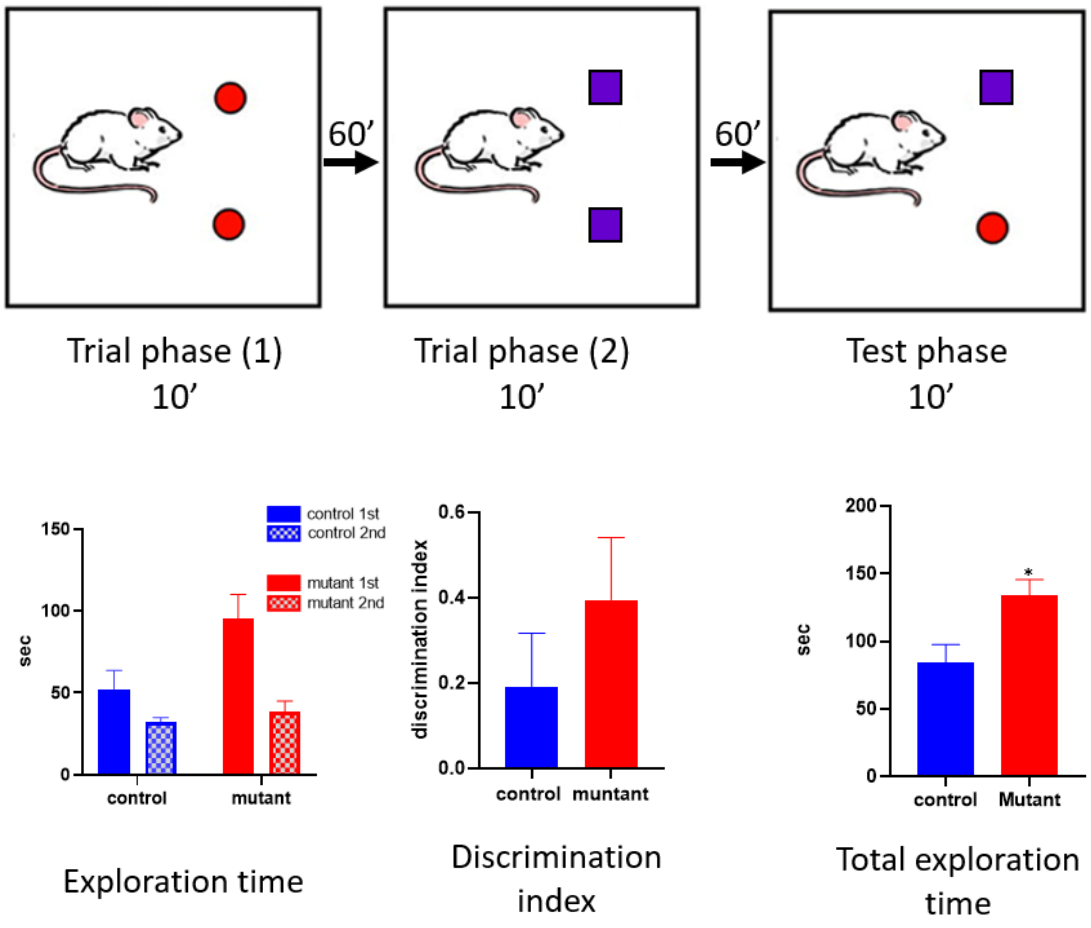


Figure 18: temporal object recognition test. Mutant mice spend more time exploring the object they saw first than the object they saw last compared to controls indicating better short term memory. Total exploration time is again increased. N=5 controls (1 was excluded) and 5 mutants

Discussion

TAG-1 is known to be expressed during development by deeper layer cortical neurons that project to subcortical areas such as the striatum and the thalamus. The axonal distribution of TAG-1 combined with its secretion made it difficult to pinpoint the exact populations of neurons expressing TAG-1 at different developmental stages.

The Tag-1^{EGFP} construct gave us the opportunity to observe the spatiotemporal expression pattern of TAG-1 in the cortex. To our surprise and in contrast with previous knowledge we found out that TAG-1 is also expressed in superficial layers projecting to other cortical areas mostly through the corpus callosum. Upon crossing with EMX1^{cre} we attempted to ablate all cortical neurons expressing TAG-1.

Crossing Tag-1^{EGFP} mice with Emx1^{cre} floxed out the Egfp sequence and resulted in the expression of DTA and therefore induction of apoptosis in the neurons expressing both EMX1 and TAG-1. Even though we did not achieve a complete ablation we managed to reduce the number of neurons in all layers except layer IV. Even though this resulted in a great reduction of corticothalamic axons, to our surprise, thalamocortical axons were mostly unaffected except for a partial defasciculation. On the other hand, the reduced number of neurons in layers II/III and V which comprise the majority of callosal axons most probably was what resulted in the great reduction of the CC, since Indusium Griseum, a mixed glial-and neuronal population, was not severely affected despite the fact that in Tag-1^{EGFP} mice a great portion of the neurons comprising Indusium Griseum was GFP positive. TAG-1 is implicated in the guidance and fasciculation of fiber tracts and therefore the

ablation of the majority of the axons expressing it could be also a contributing factor to the CC and AC size reduction.

DTA mediated ablation of TAG-1 resulted in a remarkable reduction of subplate cells in Tag-1DTA45 mutants compared to Tag-1^{EGFP} as shown with immunostaining for complexin-3 and NURR1.

Subplate cells are known to be a heterogeneous population regarding their birthplace, their neurotransmitter and their morphology. Indeed some subplate neurons have extracortical origin since they originate from the rostromedial telencephalic wall (RTMW) and others originate from the SVZ (Pedraza, Hoerder-suabedissen, Albert-maestro, Molnár, & Carlos, 2014). Given the fact that EMX1 and therefore Cre recombinase and DTA toxin is expressed in the dorsal telencephalon, and the fact that corticothalamic axons are normally guided to the cortex, we would not expect such an extensive reduction of subplate in mutant mice (Cecchi & Boncinelli, 2000). We hypothesize that the ablation we observed at p6-P7 was a secondary effect caused by TAG-1+ axonal ablation. The fact that subplate cells undergo cell death in the early post natal period and then the subplate is partially repopulated is another fact pointing towards a problem in repopulation rather than direct DTA mediated ablation.

The novel transgenic Emx1^{cre}; Tag1^{DTA45} mouse line that was generated in our lab, except for the role of TAG-1 expressing neurons in cortical development, is a great tool for one to examine the plasticity of the developing brain, and the homeostatic mechanisms employed to respond to early developmental ablation of such a big number of cortical pyramidal neurons (Network, 2015).

We have already shown a significant reduction in cortical interneurons, probably to maintain a functional ratio with excitatory neurons at least in the somatosensory cortex. One should keep in mind that subcortical origin of interneurons implies that they do not express EMX1 and Cre recombinase and therefore DTA (Gorski et al., 2002). Thus, DTA expression cannot be directly responsible for the reduction of interneurons in the cortex. The reduction could be attributed to either defective

migration of interneurons to the cortex or to increased apoptosis in the critical period for interneuron survival due to the decreased number of pyramidal neurons. Of note, the number of interneurons per area is not significantly different between control and knock out mice thus pointing towards the second scenario.

Whether the reduction of cortical interneurons is attributed to increased apoptosis during the critical period (P5-P10) in order maintain excitation-inhibition equilibrium or due to defective migration from the ganglionic eminences to the cortex remains to be elucidated with future experiments.

The probability of defective migration due to ablation of the majority of TAG-1-expressing neurons can also be further investigated with a series of experiments. These include in situ hybridization for early interneuron markers during embryonic day E13-E15 (such as LHX6 or NKX2) which will show reduced numbers of interneurons in the migratory routes and in the cortex of mutants compared to that of controls.

If this is the case then explants from mutant MGEs to control brain slices should display normal migratory behavior and the same postnatal number of interneurons to the cortex after the critical period compared to mutant MGE explants to mutant brain slices.

Early postnatal (before and after the critical period of interneuron cell death) immunocytochemistry for interneuron markers (GABA or GAD67) and neuronal markers such as NEUN would indicate whether the reduction of cortical interneurons is due to apoptotic cell death during the critical period. If this is the case, follow up experiments of co-immunostaining for Caspases and interneuron markers during the critical period will show the increased apoptosis of interneurons and solidify our conclusion.

Behavioral assessment of $Emx1^{cre}$; $Tag\text{-}1^{DTA45}$ mice showed that they display improved learning and increased exploratory behavior but impaired spatial memory compared to $Tag\text{-}1^{EGFP}$ controls.

The increased exploratory behavior of Tag-1^{DTA45} mice compared to Tag-1^{EGFP} controls could also indicate increased curiosity and/or impaired ability of risk assessment.

Performance in novel object recognition is associated with learning ability. The surprising finding that mutant mice perform better than controls in this task, leads to the conclusion that reduced numbers of neurons in the cortex might be beneficial for cognitive performance, at least in early adulthood.

Performance in the temporal object recognition task and the temporal memory is associated with the prefrontal cortex functionality. Since mutant mice perform better in that task compared to controls we hypothesize that the prefrontal cortex also functions better with less neurons. We have not yet checked for anatomical differences in the prefrontal cortex of mutant mice compared to controls and it would be very informative if we did.

Spatial memory was evaluated with object to place recognition test, in which mutant mice performed worse than controls. Anatomical changes concerning the hippocampus, which is the primary structure associated with spatial memory and navigation in the rodent brain, of mutant mice compared to that of controls have not yet been quantified.

A primary behavioral evaluation of mutant mice implies that cortex of control mice has more neurons (pyramidal and interneurons) than required for optimal cognitive performance. An explanation for such a counter intuitive observation could be that the juvenile cortex has more neurons than necessary for optimal computational power. This excess of neurons are minimally integrated in neuronal networks and create noise, since synchronization of neurons in high frequencies (which is necessary for higher cognitive functions) is more difficult as the number of neurons increases, because the temporal window of a high frequency oscillating network is more narrow.

The sub-optimal cognitive performance could probably be the evolutionary price that needs to be paid for the delay of age-related cognitive decline. Given the limited regenerative capacity of the brain, maybe the “redundant” neurons are minimally

integrated in networks and serve as a reservoir ready to compensate for age related neuronal cell death caused by starvation, dehydration, hypoxia, oxidative stress, mechanical traumas or excitotoxicity. If this is indeed the case, mutant mice should be more vulnerable to age-related cognitive decline as compared to controls. This prediction can be easily tested by performing the same tests in older mice.

In any case one should keep in mind that the results of novel and temporal object recognition are not statistically significant. This means that more mice should be behaviorally evaluated in order to be sure that the results are not random.

Further behavioral evaluation of functions such as motor learning and coordination, spatial navigation, pain tolerance, risk assessment, muscle tone/strength, stress, and social and aggressive behavior would provide a more detailed view of the consequences of such a developmental ablation of cortical neurons.

Finally electrophysiological studies to measure the conductivity of CC in different rostrocaudal planes should be performed to check the velocity and amplitude of action potentials across this structure.

It would be also of great interest to cross Tag1^{DTA} line with different lines expressing Cre recombinase under the promoter of different transcription factors expressed in different developmental time points and examine the effect of ablation of the same population during different times in development as well as examine the results concerning the anatomical function and behavior of mutant mice. Intriguing questions such as: how does the brain's plasticity change with time and until when is such a vast ablation sustainable in the mouse and how is the behavior affected in response to different ablation timepoints.

Materials and methods

Perfusion dissection and freezing

Before dissection brains mice were perfused with 4% PFA/1X PBS, to ensure homogenous fixation of brain tissue. In order for that to happen mice were anesthetized with a mix of 35µl ketamine and 25µl xylazin. When mice were unconscious their chest was cut open and ice cold PBS was pumped to the left ventricle of their hart. Right atrium was sniped in order to avoid excess pressure in vasculatory system. When PBS cleared all the blood, ice cold 4% PFA was perfused in the left ventricle (15-20ml). After that the brains were dissected and incubated in ice cold 4% PFA for 20'. Then the brains are washed twice with 1X PBS and are dehydrated in 10% sucrose/1x PBS overnight. The next day the brains are transferred to 30% sucrose to be further dehydrated. Dehydration is considered to be completed when brains start sinking in 30% sucrose. Proper dehydration is a very important step to avoid mechanical damage of the fixated tissue by water crystal forming by the subsequent freezing of the tissue. When dehydration is complete brains are embedded in gelatin sucrose gel and are let to freeze at 4^{o}C. Then the brains are dipped in methyl butane frozen with dry ice (approximately -40/-50^{o}C) until gelatin sucrose solidifies completely. Frozen brains embedded in blocks of gelatin can be kept in -80^{o}C for a long period of time.

Cryoslices

Brain tissue embedded in block of gelatin/sucrose was sliced at -25^{o}C with leica Cryoslicer. The slices for immunostaining were 10-16µm thick and were stored in -20^{o}C.

Immunohistochemistry

Brain cryosections were taken from -20^{o}C and let in room temperature for 20-30' to warm and dry. Then the slide was surrounded by DACOPEN and let dry for additional 10' (or more if necessary). The slides were incubated in acetone for 10' in -20^{o}C. Subsequently washed 3 times 10' each in 1X PBS, at room temperature. After the final wash slides were one by one removed from PBS, put in a humidified chamber and incubated with blocking buffer (5% BSA, 0,1% TRITON-X-100 in 1x PBS) for 60' RT. Subsequently blocking was removed from each slide and substituted with blocking buffer containing primary antibodies in the proper concentrations and left overnight at 4^{o}C. Wash 3 times, 10' each with 1X PBS, RT. Remove each slide from PBS and incubate with the proper secondary antibody for 1:30-2h, RT. After this point exposure of slides in light must be avoided as much as possible. After the secondary antibody wash 3 times in 1X PBS (dark) and then incubate for sharply 3' with TOPRO (1:2000) in 1X PBS, if necessary. Mount with mowiol (70μl) per slide and let it solidify at 4^{o}C before observing it in the microscope.

Confocal imaging and image processing.

Photos were taken using leica SP2 confocal microscope. Briefly, for every image a stack of photos on the Z axis was taken an then the maximum or the transparent projection was taken for each stack. The consecutive and overlapping projection were merge using photoshop CS-6, either automatically (if possible) or manually. Further image processing including setting threshold, contrast and exposure levels were kept to minimum possible.

Primary antibody concentrations

The following antibodies were used:

Goat anti-GFP (1:500, #ab6662, Abcam), mouse antineurofilament (1:100, clone 2H3, DSHB), mouse anti-ISL1 (1:250, clone 39.4D5-s, DSHB), rat monoclonal anti-L1 (1:1,000, Clone 557.B6); (Appel et al., 1995), rat anti-L1 (1:1,000, MAB5272, Millipore), rat anti-GFP (1:1,000, #04404- 84, Nacalai Tesque,), rabbit anti-GFP (1:5,000, Minotech), rabbit anti-neurofilament heavy (1:1,000, #ab8135, Abcam), chicken anti-neurofilament heavy (1:1,000; #ab4680, Abcam), rabbit polyclonal anti-TBR1 (1:600, #ab31940, Abcam), rat anti-BrdU (1:1,000,#OBT0030CX, AbD Serotec), mouse anti- TAG-1 (1:1,000, clone 4D7, DSHB) mouse anti-SATB2 (1:250, #ab51502/100, Abcam), rat anti-CTIP2 (1:1,000, # ab184650, Abcam), rabbit anti-cleaved caspase3 (1:200, # 9661, Cell Signaling), mouse anti-REELIN (1:200, clone G10, DSHB), chicken anti-GFP (1:500, GFP-1020 /Aves Labs Inc), goat anti-NURR1 (1:100, AF2156, R&D), rabbit anti-CPLX3 (1:1,000, Synaptic Systems), rabbit anti-BLBP (1:500 #ab32423, Abcam), rabbit anti-PVA (1:800, PV27, Swant), mouse anti-NEUN (1:100, MAB377, Millipore)

ISH for interneurons

DIG-LABEL IN SITU HYBRIDIZATION

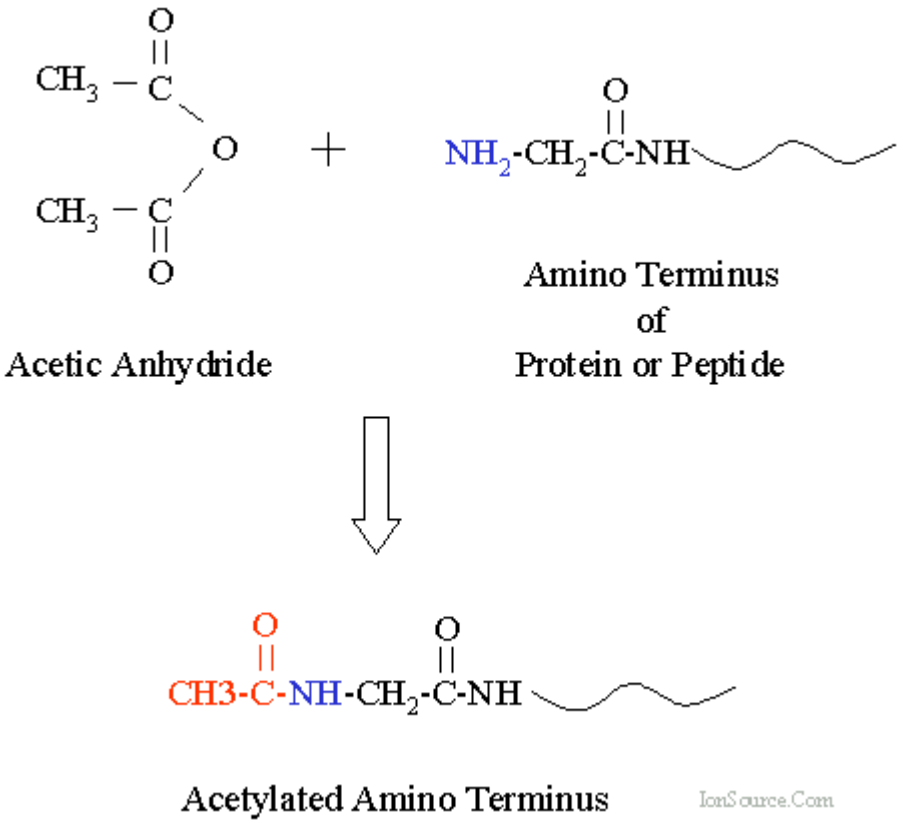
on cryotome sections

1. TISSUE SECTION

Fix embryo O/N at 4 ^{o}C in 4% PFA, 0,1 M PB
Transfer embryo to 30% sucrose in 0,1 M PB for 24 hr at 4 ^{o}C
Mount in 7,5% Gelatin (Sigma, G-2500)/15% Sucrose in PBS
Section with **superfrost plus slides**
After sectioning air dry 20 min (maximum 3 hr)

2. TISSUE PREPARATION

Put **"DAKOPEN"** around the slices
Fix in 4% PFA/PBS for 10 min at RT
Wash 3 times with PBS for 5 min each
Acetylation (to prevent the reaction between the PO_4 groups of the RNA-probe and the amino-terminus of the proteins of the tissue)
Acetylation mix (should be fresh made): 295 ml H_2O, 4 ml triethanolamine, 0,525 ml HCl 37%. Mix well. Add 0,75 ml acetic anhydrite, and mix by dipping slides several times and/or shaking for 10 min, RT.



For fixed tissues permeabilize with PBS+1% Triton for 30 min, RT.
Wash 3 times with PBS for 5-10 min each.

3. PRE-HYBRIDIZATION & HYBRIDIZATION

Place 200 – 300 µl pre-hybridization buffer on slide.
Incubate at RT for 6 hrs (or O/N) or 2hrs at 65 ^{o}C, in a 5x SSC hymidified chamber horizontal without coverslips.
Replace pre-hybridization buffer with 80 - 100 µl hybridization solution containing probe (200 – 400 ng/ml DIG-RNA which was heated to 80 ^{o}C for 5 min and iced).
Coverslip slides with parafilm and place in humidified chamber (5x SSC, 50% formamide)
Incubate O/N at 72 ^{o}C.
*** Heat also (at 72 ^{o}C) sterile dH_2O and glass-baths and/or glass-racks for the next day.

4. WASHES & IMMUNOLOGICAL STAINING

Place slides in rack, submerge in 72 ^{o}C – preheated 5x SSC and carefully remove coverslips (parafilm).
Transfer slides into 0,2x SSC at 72 ^{o}C for 1 hr.
Transfer slides to 0,2x SSC at RT for 5 min.
Transfer (wash) slides in B1 for 5 min at RT.
Place 1-2 ml B1 with 10% FBS on slides for 1 hr at RT.

Incubate with the primary antibody (300 – 400 µl anti – DIG AP, Roche, dilution 1/5.000) in B2 and incubate O/N (12 – 18 hrs) at 4 ^{o}C or 2 hrs RT (B2 = B1 + 1% FBS)
Wash 3 times with B1 for 5-10 min each.
Wash 3 times with B3 for 5-10 min each.
Place 500 – 1000µl B4 on slides.
Incubate at RT in humidified chamber in dark.
After staining of the tissue, stop the reaction with TE or PBS = 0,1% Triton.
Mounting (A) with **gelatin – glycerol:** use a mix of 50% gelatin – 50% glycerol (preheated at 50-55 ^{o}C) and coverslips (Gelatin: from Bovine skin, Type B, ≈ 225 Bloom, Sigma #G-9382)

or

Mounting (B) with **Entellan:**
Dehydration with EtOH/H_2O (50%), 70%, 80%, 90%, 2 x 100% , 5-10 min each
3 x Xylol (100%), 5-10 min each
Mounting with 100 – 150 µl Entellan (per slide) and coverslips
Leave O/N to dry at R.T.

Acetylation Buffer	**for 100 ml**	**for 300 ml**
0,1 M triethanolamine	1, 33 ml	4 ml
0,25% acetic anhydrite	0,25 ml	0,75 ml
0,065% HCl	0,175 ml	0,525 ml
in sterile H_2O	98,3 ml	295 ml

Hybridization and Pre-Hyb solution	**for 20 ml**
50% formamide (Mol Biol Grade!!)	10 ml
5x SSC	5 ml 20x SSC
5x Denharts	2 ml 50x Denharts
250 µg/ml Yeast RNA	0,5 ml Yeast RNA 10mg/ml
500 µg/ml salmon/herring sperm DNA	0,5 ml s/h sperm DNA 20mg/ml
Sterile H_2O	2 ml sterile H_2O

B1:	**for 500 ml**
0,1M Tris pH: 7,5	50 ml Tris pH: 7,5 1M
0,15M NaCl	15 ml NaCl 5M

B2:

B1 + 1% Serum (FCS/FBS)

B3:	**for 300 ml**
0,1M Tris pH: 9,5	30 ml Tris pH: 9,5 1M
0,1M NaCl	6 ml NaCl 5M
50mM $MgCl_2$	15 ml $MgCl_2$ 1M

B4:	**for 12 ml**
4,5 µl/ml NBT (stock 75 mg/ml) 3,5 µl/ml BCIP (stock 50 mg/ml) in B3 buffer	54 µl NBT 75 mg/ml 42 µl BCIP 50 mg/ml

Interneurons counting

Interneurons were counted using the count particle tool of image J after the threshold was set manually to ensure optimal signal/nose ratio. Then two tailed unpaired t-test was performed to calculate the statistical significance of interneurons reduction.

Behavioral evaluation

For behavioral tests mice older than two months old were used. Every mouse was handled for seven days straight before the initiation of behavioral tests. Same uniform should be used for handling and habituation and the subsequent phases of behavioral tests. After the handling mice should be comfortable with people performing the experiments.

Every day for three days before the first object recognition test (in this case novel object recognition), mice were put in the open field for ten minutes each (habituation phase) to get comfortable with the open field.

After every mouse the open field must be thoroughly cleaned with ethanol and let dry. Mice are video recorded in every phase of behavioral assessment including the habituation phase. Every in all phases the lighting was deemed and diffused. For that purpose a led lamp was used but it should not be pointing towards the open field.

During all phases of behavioral experiments there should be as much silence as possible and we left the room for as long as mice were in the open field to minimize distractions. Moreover perfumes and other odors should be avoided. Interaction with mice should be as gentle as possible and abrupt movements should be avoided.

All behavioral experiments should take place at approximately the same time every day. In our case experiments started at 6pm.

Objects used were legos of different shapes, sizes and colors. For every different behavioral test we used legos that have never been used before in other behavioral tests.

For every test 5 mice of each genotype were examined. Of note one mutant mouse fail to meet minimum exploration time (20" for 5' test phase) and was excluded from statistical analysis.

Novel object recognition

For the novel object recognition test the 5 control mice with genotype (Tag-1^{EGFP}) and 5 mutant mice (Tag-1^{DTA45}) were examined. The mice were put for 10' in the open field to familiarize with a set of same objects placed anti-diametrically in the open field and recorded (trial phase). After each mice was removed from the open field the open field was thoroughly cleaned with ethanol before the next mouse was placed in the open field.

1h after the end of trial phase the test phase started. One object of the set was replaced with a novel one. The test phase lasted 5 minutes and was also recorded. Quantifications, measurements and statistical analysis was based on the test phase.

Object to place recognition

For object to place recognition test the same principles were followed but in test phase one object was misplaced compared to the trial phase instead of being replaced with a novel one.

Temporal object recognition

Aim of this test is to assess the temporal memory of mice. There are two trial phases of 10’ and a different set of identical objects are presented to the mice during each trial phase. During test phase, which lasted 5’, one object from the each set is presented to the mice.

Quantification and statistical analysis of behavioral tests

Every trial and test was video recorded. The quantification of exploration times for object, during each test was performed with the help of Jwatcher video analysis software from the analysis of the test phase of experiments. Exploration times were then statistically quantified with two tailed unpaired t-test. Discrimination index is the difference of the time spent exploring the objects divided by total exploration time of the test phase.

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Acknowledgments

Science is always a fascinating journey. During the last year we attempted to push the burdens of knowledge just a little bit further and have fun doing so. I like to believe that science is a team sport and that we have the best team. Due to that team, my work ethic was always high and I was always looking forward to go to the lab despite the volume of work that waited there.

The present study was thus a team effort and many people contributed to the outcome in one way or another. Thus at this point I want to thank all of them for their help and support.

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