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"Analysis of a novel transgenic mouse line with defective fiber tracts"

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Ευχαριστίες

Πρώτα από όλα θα ήθελα να ευχαριστήσω την επικεφαλή του εργαστηρίου μου κ. Καραγωγέως Δόμνα, για την ευκαιρία που μου έδωσε να συνεργαστώ μαζί της και με τα μέλη του εργαστηρίου της, καθώς και να πραγματοποιήσω αυτή τη μελέτη.

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ΠΕΡΙΛΗΨΗ

Η λειτουργία του εγκεφαλικού φλοιού βασίζεται σε στερεοτυπικές συνδέσεις μεταξύ διακριτών περιοχών, που δημιουργούνται από άξονες οι οποίοι προέρχονται από διεγερτικούς πυραμιδικούς νευρώνες του φλοιού. Αυτοί οι νευρώνες αποτελούν τη πλειοψηφία των νευρικών κυττάρων στο νεοφλοιό και διακρίνονται από την έκφραση διαφορετικών μεταγραφικών παραγόντων αλλά και τη τοποθέτησή τους κατά μήκος του φλοιού.

Οι φλοιοθαλαμικοί άξονες (corticothalamic axons ή CTAs), οι οποίοι συνδέουν το φλοιό με το θάλαμο εκφράζουν σε υψηλά επίπεδα την πρωτεΐνη που είναι γνωστή ως Transient Axonal Glycoprotein-1 ή Contactin-2 (TAG-1/Cntn2). Πρόκειται για ένα νευρωνικό μόριο αναγνώρισης της υπεροικογένειας των ανοσοσφαιρινών και εμπλέκεται στη νευρογένεση, την έκφυση νευριτών και την δεσμίδωση των αξόνων. Μεταξύ άλλων νευρωνικών υποπληθυσμών, εκφράζεται νωρίς κατά την εμβρυογένεση στους νευρώνες γνωστούς και ως πρωτοπόρους (pioneer neurons) στην προφλοιική πλάκα (Preplate-PP) και αργότερα στην επιχείλια ζώνη (Marginal Zone-MZ) και στην υποφλοιική πλάκα (Subplate-SP) του αναπτυσσόμενου εγκεφαλικού φλοιού.

Για τη μελέτη της ανάπτυξης των διαφόρων ομάδων νευραξόνων στο κεντρικό νευρικό σύστημα, στο εργαστήριό μας δημιουργήθηκε η διαγονιδιακή σειρά *Tag1*$^{\text{loxP-GFP-loxP-DTA}}$. Σε αυτή τη σειρά εκφράζεται η GFP πρωτεΐνη υπό τον *Tag-1* υποκινητή και είναι παρούσα επίσης η κωδική αλληλουχία της υπομονάδας Α της τοξίνης της διφθέριας (Diptheria Toxin subunit A (DTA)) υπό σίγηση. Ύστερα από διασταύρωση αυτού του διαγονιδιακού ζώου με ένα ζώο το οποίο εκφράζει ιστοειδικά στο νεοφλοιό το ένζυμο Cre-recombinase, έχουμε απαλοιφή της GFP, και η αλληλουχία της τοξίνης βρίσκεται πιο κοντά στον υποκινητή με αποτέλεσμα την έκφρασή της και το θάνατο των TAG-1+ νευρώνων.

Προβλήματα στην πλαστικότητα και στην συνδεσιμότητα των νευραξόνων, αποτελούν τη βάση πληθώρας νευρικών διαταραχών στον άνθρωπο. Το *Emx1*::Cre;*Tag-1*::DTA διαγονιακό μοντέλο έχει μειωμένους φλοιοθαλαμικούς άξονες και η ανάλυσή τους κατά την εμβρυογένεση αλλά και αργότερα στην ανάπτυξη αποτελεί πηγή χρήσιμων πληροφοριών για τον ρόλο των αξόνων αυτών την ανάπτυξη του εγκεφάλου.

ABSTRACT

The function of the cerebral cortex relies on several stereotypical long-distance projections, which originate from excitatory projection neurons that represent the largest portion of all cortical neurons. Axonal tracts, originating from projection neurons connect areas essential for brain function and they are also important for the final structure of the central nervous system.

Cortical efferents (corticothalamic axons or CTAs) are expressing high levels of Transient Axonal Glycoprotein-1 or Contactin-2 (TAG-1/Cntn2), a neuronal recognition molecule of the immunoglobulin superfamily which is involved in neurogenesis, neurite outgrowth and fasciculation. Among other neuronal subpopulations, it is expressed early by pioneer neurons in the preplate and later on in the marginal zone and subplate of the developing cortex.

To study the formation of several axonal tracts in the mouse central nervous system, we generated the transgenic mouse line *Tag1*$^{\text{loxP-GFP-loxP-DTA}}$. These mice express GFP under the *Tag-1* promoter, also encompassing the coding sequence of Diptheria Toxin subunit A (DTA) under quiescence. Upon crossing with the neocortex-specific *Emx1*::Cre line, GFP expression is eliminated and the toxin is expressed in TAG-1+ neurons cells resulting in their death.

Cortical lamination deficits and aberrant axonal connectivity in the brain is a main cause of neurodevelopmental disorders in human. *Emx1*::Cre;*Tag-1*::DTA mice have reduced CTAs and their analysis in embryonic and postnatal stages will give useful information for the role of these axons in the brain development.

1. INTRODUCTION

1.1. Neocortical development

The mammalian neocortex is responsible for higher cognitive functions, sensory perception, voluntary motor control, and consciousness. All these functions depend on a complex, yet highly organized, six-layered structure composed of hundreds of different neuronal subtypes, in addition to diverse glial populations that offer trophic and functional support to the neurons of the cortex (Peters & Jones, 1984; Ramon y Cajal 1995). The identity of newborn neurons within the six-layered neocortex is ensured by their positioning. Defective neuronal migration results in a plethora of neurodevelopmental disorders, ranging from gross brain malformations such as lissencephaly to neurobehavioral disorders such as autism, underscoring the importance of this process in proper cortical development and function (Gressens, 2006; Suzuki, 2007).

Corical neurons can be subdivided into two main classes: interneurons, usually inhibitory GABAergic non-pyramidal neurons that have local connections; and projection neurons, glutamatergic excitatory pyramidal neurons that extend distant axons to intracortical, subcortical, or subcerebral targets. The latter neuronal group is responsible for the transmission of information within the neocortex, or from the neocortex to other regions of the central nervous system (CNS) (Molyneaux, et al., 2007). Interneurons and projection neurons originate from different areas during embryogenesis. Interneurons are generated primarily from progenitors in the ventral (subpallial) telencephalon, and migrate tangentially over long distances to their final location within the neocortex (Wonders & Anderson, 2006). Projection neurons are generated from progenitors of the neocortical germinal zone located in the dorsolateral (pallial) wall of the telencephalon, and migrate radially to their final neocortical position (Rakic, 1972; Tan, et al., 1998; Ware, et al., 1999; Anderson, et al., 2002; Gorski, et al., 2002; Molyneaux, et al., 2007; Leone, et al., 2008, 2015).

Pyramidal neurons arise in overlapping temporal waves, with 80-90% of them originating from undifferentiated neuroepithelial progenitor cells in the ventricular zone (VZ) while the remaining percentage comes from the subventricular zone (SVZ) of the telencephalon (Ayala, et al., 2007; Bystron, et al., 2008; Gotz & Huttner, 2005; Higginbotham, et al., 2011; Kriegstein, et al., 2006). The generation of pyramidal neurons is ongoing from embryonic day (E) 11.5 to E17.5 in the mouse (Martynoga, et al., 2012; Molyneaux, et al., 2007; Angevine & Sidman, 1961; Caviness & Takahashi, 1995; Rakic, 1974). At the initiation of corticogenesis, progenitor cells undergo symmetrical divisions in the VZ, thereby increasing their numbers, and by the time neurogenesis begins, the majority in the VZ divides asymmetrically (Higginbotham et al., 2011; Noctor, 2001; Molyneaux et al., 2007; Angevine & Sidman, 1961; Caviness & Takahashi, 1995; Rakic, 1974). Thus, the fraction of cells that differentiate increases, whereas the remaining progenitors decrease in numbers.

Newly born projection neurons, which appear around E10.5 in the mouse, and migrate radially, form a layer at the outer surface of the cortex termed the preplate (PP). The next group of neurons that migrate in the cortex at E13 form the cortical plate (CP), which divide the PP into the marginal zone (MZ or layer I) and the deeply located subplate (SP)(Ayala, et al., 2007; Molyneaux, et al., 2007). All cortical layers will develop in an inside-out manner between the SP and MZ, such that later born neurons arriving at the cortical plate migrate past earlier born neurons (Molyneaux et al., 2007; Bayer & Altman, 1991; Angevine & Sidman, 1961; Caviness & Takahashi, 1995; Rakic, 1974). At the same time, axons with horizontal orientation, migrating radialy forming the intermediate zone (IZ), while subventricular zone (SVZ) starts to form (Molyneaux, et al., 2007).

1.2. Types of projection neurons

Neurons are derived from progenitors in the dorsal telencephalon and can be classified into subtypes based on: (i) their location within different cortical layers and areas, (ii) their axonal projections to distinct targets; and (iii) the combinatorial expression of different transcription factors or markers (Leyva-Diaz & Lopez-Bendito, 2013).

Based on the second classification system, three broad classes of projection neurons exist and can be discriminated (Figure 1):

1) **Corticofugal projection neurons**, which are located exclusively in deeper layers, and extent their axons away from the cortex to subcortical (e.g. striatum, thalamus) and subcerebral targets (e.g. brainstrem and spinal cord). These can be further subdivided into:

- Corticothalamic neurons (CTAs): Primarily located in layer VI, with a small percentage residing in layer V, that project subcortically to different nuclei of the thalamus.

- Subcerebral projection neurons or corticospinal (CSTs): They include pyramidal neurons of the largest size, which are located in layer V and extend projections to the brainstem and spinal cord.

2) **Commissural projection neurons** or callosal projection neurons, located in layers II/III, V and VI, that extend an axon to the contralateral hemisphere; and

3) **Intracortical projection neurons**, which extend their axon within a single cortical hemisphere (Molyneaux, et al., 2007; Fame, 2011; Leyva-Diaz & Lopez-Bendito, 2013).

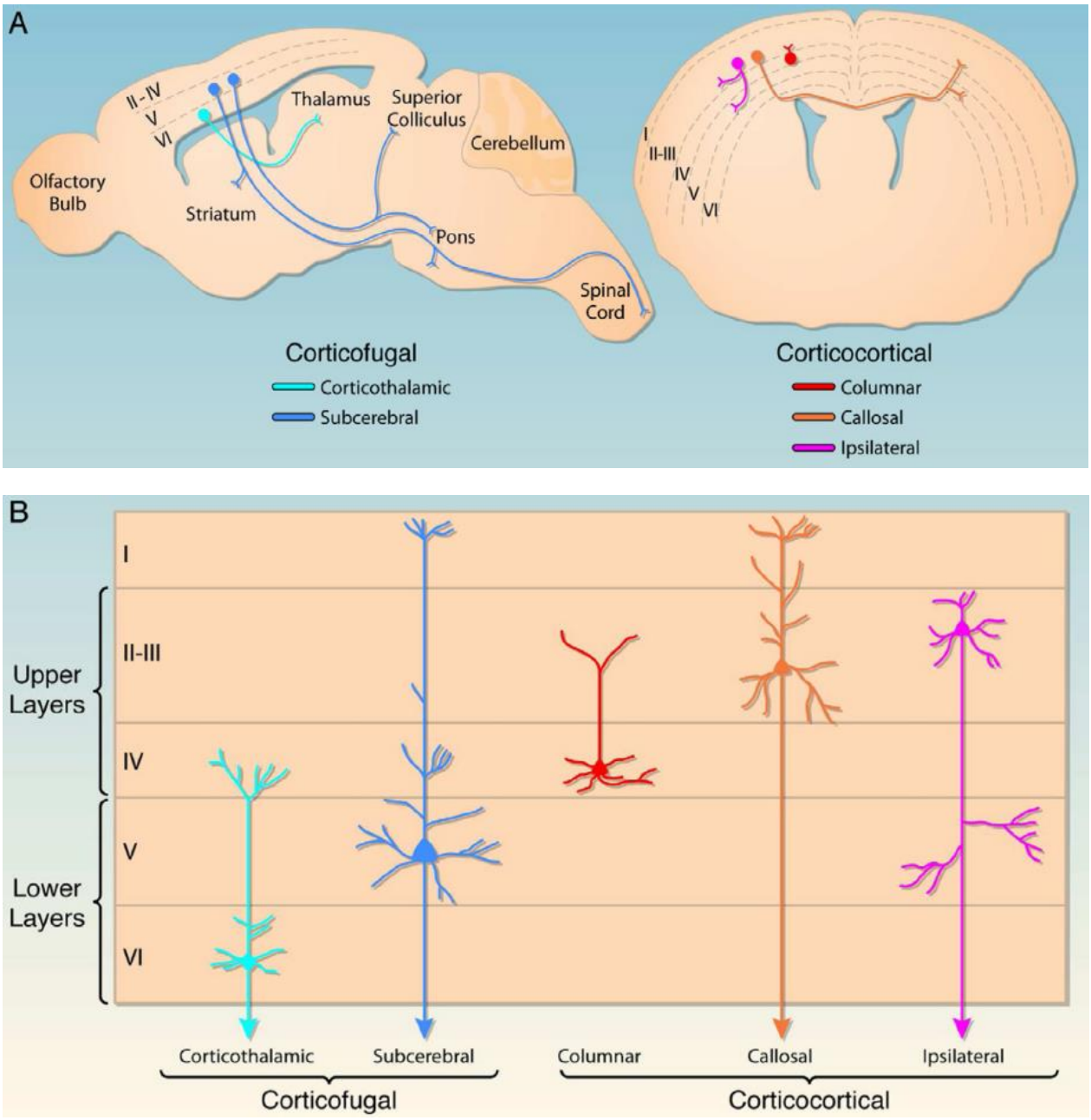

Figure 1 : Major subtypes of neocortical projection neurons, classified by projection pattern and laminar position. (A) Saggital view of corticofugal projection neurons in deep layers. CTAs are found mostly in layer VI and project to the thalamus, while CSP are located in layer V and send primary projections to the spinal cord, pons and superior collilulus. Right in the picture, a coronal view of corticocortical projection neurons in upper layers. Most ipsilateral and callosal projection neurons are located in layer II-III and project within the same hemisphere or to the contralateral hemisphere via the corpus callosum, respectively. Columnar projection neurons are found in layer IV and send short axons locally within a neocortical column. (B) Magnified view of projection neuron subtypes represented in A, detailing the relationship between laminar positions and projection patterns (Franco & Muller, 2013).

1.3. Molecular identification of the projection neurons

The identification of the cortical VZ and SVZ as the germinal zone for the generation of projection neurons and astrocytes has raised important questions in regard to the composition of the neuronal progenitor pool. At the initial stages of cortical neurogenesis (approximately E11.5 in the mouse), individual progenitors are able to give rise to pyramidal neurons across all the layers of the neocortex (Molyneaux et al., 2007; Reid & Walsh, 2002; Tan et al., 1998). As development progresses, progenitors become progressively restricted in their competence states. Several modes of asymmetric cell division are recognized within the VZ: a) neurogenic division, b) asymmetric progenitor division c) final gliogenic division (Kriegstein et al 2006; Noctor et al. 2004, 2008). Two alternative models have been proposed as the mechanism by which this temporal order is established (Figure 2). According to the first model, the potential fate of a common progenitor might change over time to generate the different subtypes of projection neurons and astrocytes in a defined temporal order (Figure 2A). Alternatively, multiple progenitor types may co-exist, each of which is intrinsically programmed or extrinsically triggered to generate a specific subclass of neurons or astrocytes on a progenitor-specific time line (Figure 2B)(Franco & Muller, 2013; Evsyukova, et al., 2013).

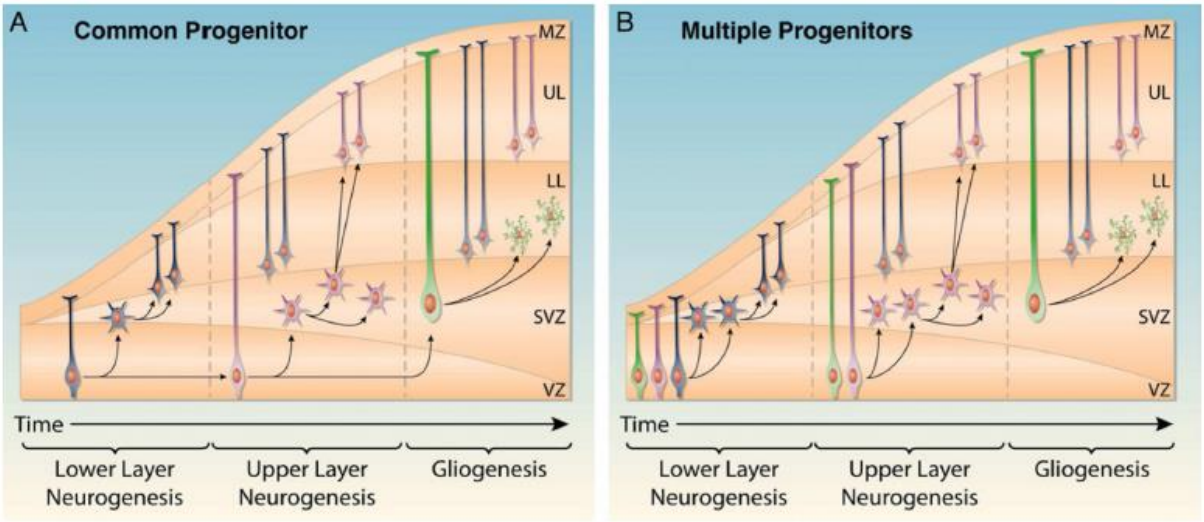

Figure 2 : Different models of projection neuron cell-type specification in the developing neocortex. (A) A single kind of radial glial cells progenitors sequentially generates subtypes of projection neurons and macroglia. (B) Distinct subtypes of radial glial progenitors co-existi and are pre-specified to generate different subtypes of projection neurons and macroglia (Franco & Muller, 2013)

Different subpopulations of projection neurons are born in overlapping but successive temporal waves, between E14.5 until E18.5, with newly born projection neurons migrating past the earlier born neurons to form a superficial layer (Hatten, 1999; Marin & Rubenstein, 2003; Ayala et al., 2007; Molyneaux, et al., 2007). This results in a progressive establishment of the six-layered mature neocortex in an "inside-out" manner, with layers VI and V generated first (E14.5-E16.5), followed by layers IV, III and II (E16.5-Adult) (Angevine & Sidman, 1961; Molyneaux, et al., 2007; Leone, 2008, 2015).

Within the mature neocortex, distinct populations of projection neurons are located in different cortical layers and areas (with many subtypes intermingled in each), have unique morphological features, express and are controlled by different combinations of transcription factors, and ultimately serve different functions. The complexity and diversity of these subtypes makes any classification scheme difficult, but the most accurate system includes a combination of hodology, morphology, electrophysiological properties, and combinatorial patterns of gene expression that correspond to discrete functions (Peters, A. & Jones, 1984; Migliore & Shepherd, 2005; Molyneaux et al., 2007).

Transplantation studies support the principle of extrinsic factors on defining neurogenic and non-neurogenic regions in the adult neocortex, and the potential fate of neural progenitors (Gage, et al., 1995; Suhonen, et al., 1996; Shihabuddin, et al., 2000). Studies have shown that early-born, but not late-stage progenitor cells are capable of sensing local microenvironmental cues that drive them to establish their layer fate (Kriegstein & Noctor, 2004). Hence, not only do the signals that direct the neurons to specific layers identity change, but also the neuronal response to those signals changes as development proceeds (Ayala, et al., 2007).

Recent studies have identified transcription factors involved in the sequential generation of neuronal subtypes during different stages of cortical neurogenesis (Figure 3). These factors are exerting their function through gradients of expression and drive neuronal differentiation. A central role in the specification of neurons located in layer V of the cortex and projecting out of the cortex is played by the zinc finger protein Fezf2 and its target gene Ctip2 (Figure 3C,D) (Arlotta, et al., 2005; Molyneaux, et al., 2005). Repression of Ctip2 is required for the specification of both earlier-born and later-born neurons, as Tbr1 represses Ctip2 to promote generation of layer VI neurons early, whereas the homeobox protein Satb2 represses Ctip2 later to promote layer II/III neurogenesis (Figure 3C,D). In contrast, part of Fezf2's function in promoting layer V neuron production is to inhibit precocious expression of the Satb2 (Figure 3C,D) (Britanova, et al., 2008; Chen et al., 2008; McKenna et al., 2011). Thus, a network of mutually cross-repressive cell-intrinsic regulators controls temporally the cortical laminar fate specification in the developing cortex (Martynoga, et al., 2012; Molyneaux, et al., 2007).

In addition, matrix and cellular membrane bound molecules directly instruct neuronal migration. Protein which have the ability to bound molecules are, Reelin (Rln), a large extracellular glycoprotein (Rice & Curran, 2001), EphB2 and its ligand EphrinB2, and heparin sulfate glycosaminoglycans (Conover et al, 2000; Hu, 2001). The signaling pathway involving Rln is one of the most characterized mechanisms involved in the cytoarchitecture of the cerebral cortex (Rice & Curran, 2001). Rln deficient neurons show defects in the first-migrating neurons to split the preplate, and later on in the migration of cells along radial glia (Magdaleno, et al., 2002). Similar neuronal migration defects display all mouse mutants of Rln, indicating that they all fuction in a linear signaling pathway. Several studies have demonstrated that Rln signaling regulates the extension and orientation of the processes of radial glia, aprerequisite for laminar formation during mammalian brain development (Nomura, et al., 2008; Hartfuss, et al.,

2003; Zhao, et al., 2004). Nomura et al proposed that this Rln-dependent directed growth substantially contributes to the mammalian-specific, highly polarized "pyramidal" shape of neurons. Additionally, Rln directly affects migrating neurons themselves (Nomura, et al., 2008; Trommsdorff, et al., 1999; Howell, et al., 1997), and/or another cell intrinsic/extrinsic mechanisms controlling establishment of neuronal polarity (Arimura, et al., 2007).

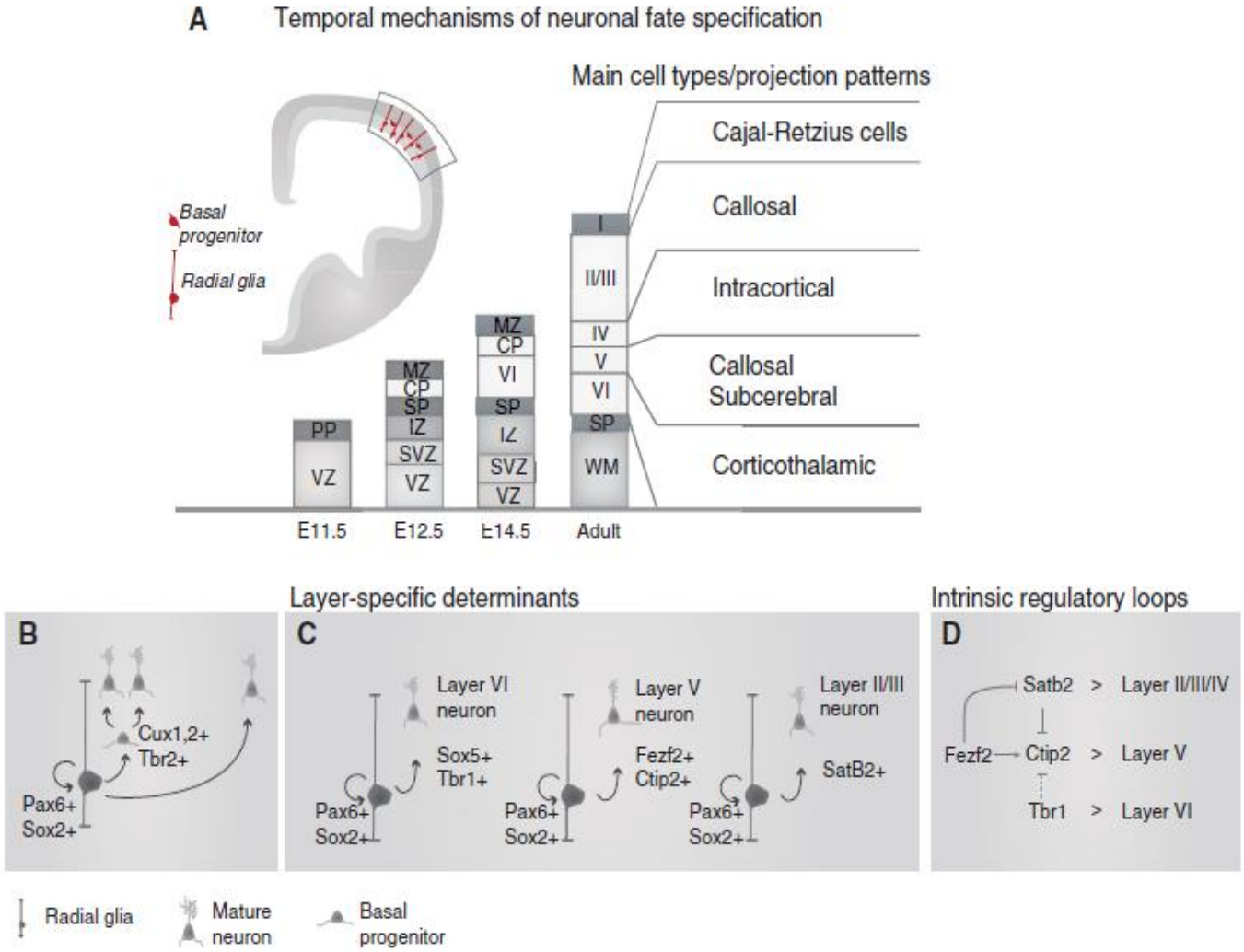

Figure 3 : Temporal mechanisms of neuronal fate specification. (A) Generation of distict neuronal subtypes (B) Cortical projection (CP) neurons generation (C) The transcriprion factors that specify the identity of the distinct classes of projection neurons (Molyneaux, et al., 2007) (D) Layer diterminant transcription factors (Martynoga, et al., 2012)

1.4. The handshake hypothesis

The function of the cerebral cortex relies on several stereotypical reciprocal long-distance projections, which provide critical feedback and feedforward loops in major neural circuits. Reciprocal connections between the thalamus and the neocortex are formed by thalamocortical (TCAs) and CTAs that convey sensory and motor information essential for cortical functioning. Several questions have aroused on how axon guidance of these two axonal tracts is controlled.

The trajectory of CTAs and TCAs is controlled by a combination of various guidance factors acting at different points along their route (Figure 4). Early axon guidance cues are likely to be common for all corticofugal projecting axons as they course laterally toward the Internal Capsule (IC) towards the TCAs (Bagnard, 1998). TCAs and CTAs are part of the IC, a large axonal bundle navigating through the basal ganglia or subpallium. The IC, also comprises of output CSAs en route to the cerebral peduncle and pyramidal tract (Auladell, et al., 2000; Price, et al., 2006). The IC plays a crucial role for the formation of these projections and many studies are focused on the mechanisms governing its development as well as, on its role on CTA and CSA specification and the function of subpallial guidepost cells in TCA pathfinding.

The " handshake hypothesis" was formulated by Blakemore and Molnar (1990) to explain how ascending TCAs navigate towards their cortical targets through interaction with reciprocal descending cortical axons (Molnar & Blakemore, 1990,1995).

Early corticofugal projections or pioneer CTAs (pCTAs) are generated by pyramidal neurons located in the subplate and layer VIb. These pioneer axons seem to provide structural guidance to the majority of CTAs that arise from layer VI. The pCTAs reach and cross the pallial-subpallial boundary (PSPB), a key decision point for the orientation of these axons, before the thalamic projections. They extend through the intermediate zone (IZ) until they reach the lateral IC between E13 and E15.5 (Auladell, 2000; Jacobs, et al 2007), where they briefly pause in a so-called "first waiting period". In contrast, TCA progress through the subpallium to reach the lateral stiatum between E13.5 and E14.5. Thus, pCTAs enter the subpallium at least a day before TCAs and halt their outgrowth allowing the TCAs to reach them. The navigation of TCAs through the subpallium is independent of CTAs and relies on the presence of guidepost corridor neurons (Leyva-Diaz & Lopez-Bendito, 2013), but descending corticofugal axons are important for guiding TCAs across the PSPB (Chen, et al., 2012). When the pCTAs enter the prethalamus, they pause during a so-called "second waiting period" until E17.5 (Molnar & Cordery, 1999, Jacobs, et al., 2007) through a PlexinD1/Sema3E signaling-dependent mechanism (Deck, et al., 2013). Moreover, this waiting period at the striatum is required for the encounter between the CTAs and TCAs and is regulated by temporal modifications in cortical neuron properties. Additionally, thalamic axons are required to guide pCTAs into the corridor and towards the thalamus, since subpallial corridor cells alone are not sufficient to guide CTAs.

Taken all together, the formation of reciprocal connections between the thalamus and cortex relies on a timing of sequential events : the migration of conserved subpallial guidepost cells defines the trajectory of TCA (Lopez-Bendito, et al., 2006; Bielle, et al., 2011a, 2011b), which in turn guide reciprocal CTA (Deck, et al., 2013).

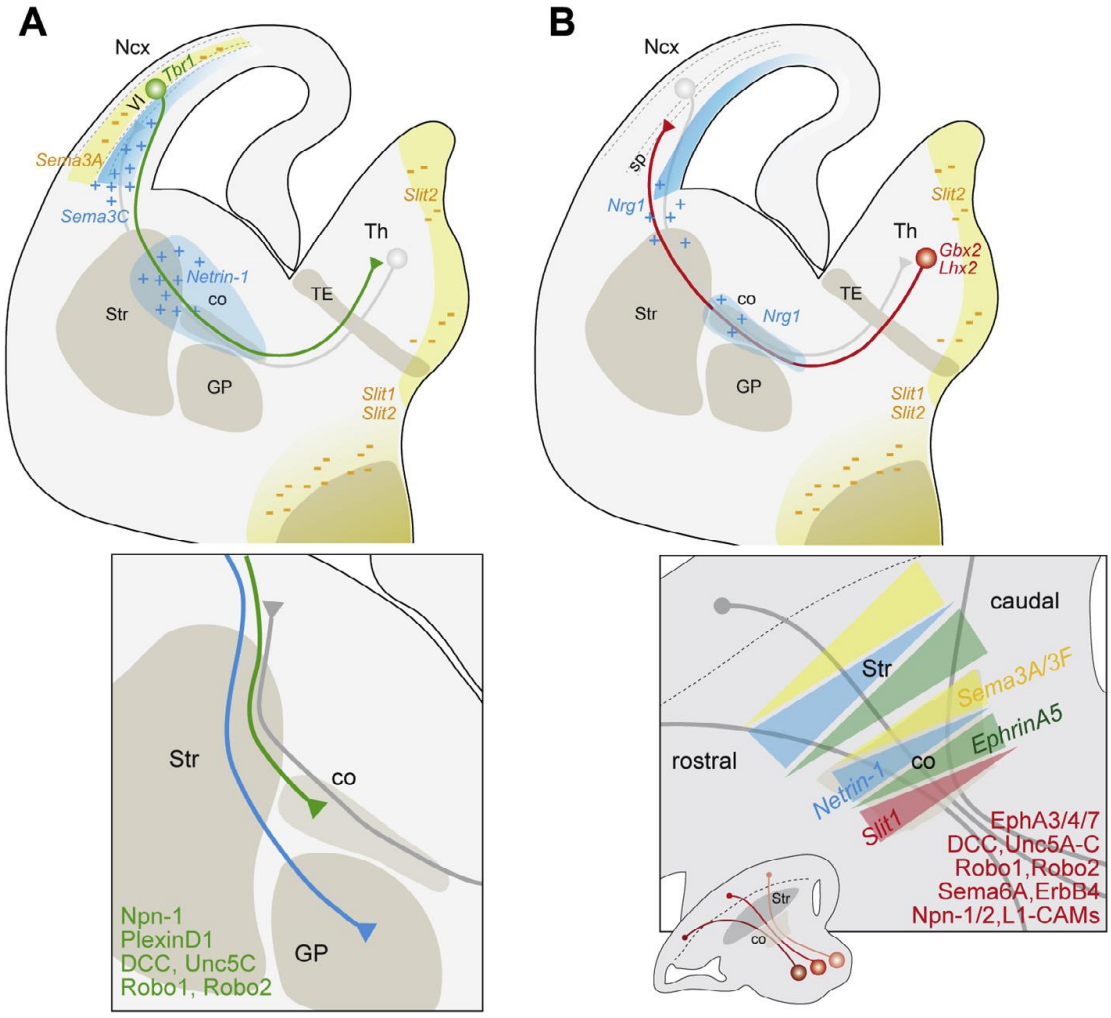

Figure 4 : CTA and TCA guidance in the development brain. (A) Schematic representation of the CTA (in green) in a developing coronal brain section showing the key gradients for their guidance and the close anatomical relation with TCA (in gray). Sebcerebral axons (in blue) have a different trajectory from CTA. (B) Schematic representation of TCAs (in red) and CTAs (in gray) showing repellent and attractant molecules (Leyva-Diaz & Lopez-Bendito, 2013).

1.5. TAG-1 protein and its expression in the CNS

During nervous system development, neurons sense the local microenvironment and migrate, extend axons, fasciculate, and form synaptic networks. Adhesion molecules, found on the surface of cells, are key players in all of the above processes, and thus are critical for proper formation of the cortex and generally of the mature nervous system (Katidou, et al., 2008).

It has been well documented that cell adhesion/recognition molecules of the immunoglobulin (Ig) superfamily play a crucial role in the formation and maintenance of the nervous system. Among them, TAG-1, also known as Cntn2, has been extensively studied for the last two decades and implicated in key developmental events, including neurogenesis, neuronal migration, neurite outgrowth, fasciculation, axon guidance and myelination (Katidou, et al., 2008; Kyriakopoulou, et al., 2002; Furley, et al., 1990; Denaxa, et al., 2001; Stoeckli, et al., 1991; Fitzli, et al., 2000; Wolman, et al., 2008; Ma, et al., 2008).

TAG-1 expression has been detected early during development (E12.5, Denaxa, et al., 2001), displaying a dynamic pattern that includes commissural fibers and motor neurons of the spinal cord, the dorsal root gaglia (DRG), cerebellum, hippocampus and corticofugal fibers (CTAs and CSPs) (Katidou, et al., 2008; Karagogeos, et al., 2003). TAG-1 is found at the membrane of corticothalamic axons, by the interaction with which TCAs are guided to reach the cortex (Deck, et al, 2013; Molnar & Blakemore, 1995), the suggested "handshake hypothesis" where these reciprocal projections might generally guide each other to reach their final targets.

CTAs provide the predominant synaptic input to the thalamic nuclei responsible for processing sensory information, thus the study of the development of these two tracts is basic for the understanding of brain sensory function (Crandall, 2015). Thus, deciphering the development of CTAs and TCAs will help explain neurodevelopmental disorders such as the L1 syndrome, in which there is agenesis of the corpus callosum and corticofugal tracts, supporting a defect in axon guidance (Engle, 2010).

1.6. Experimental Goals

The general experimental idea is to study the role of corticofugal axons during brain development. TAG-1 is expressed by corticofugal axons (Denaxa, et al., 2003), thus we specifically study TAG-1+ neurons, their role in the development and the role of corticofugal axons.

By generating the *Tag1*$^{\text{loxP-GFP-loxP-DTA}}$ mouse line we aim to study the TAG-1+ neurons, especially CTAs, and their implication in neuronal development. TAG-1 positive neurons cannot be identified using IHC postnatally, probably due to a change in the epitope recognized by the available antibodies. The *Tag-1*::GFP model gives one the opportunity to use the transgenic GFP signal to detect and study TAG-1+ cells embryonically and postnatally.

Crossing the previous mouse line with a Emx1$^{\text{Cre}}$ mouse line, we are able to analyze the phenotype of double transgenic *Emx1*::Cre;*Tag-1*::DTA mice, where there have been the ablation of TAG-1+ neurons. Thus, the function of TAG-1+ neurons and their involvement in the developing neocortex will be elucidated. Moreover, we will acquire important information about the corticofugal tracts, among which are the CTAs and CSP. Especially the ablation or elimination of CT axons is essential for the study of the handshake hypothesis and how these neurons guide the TCAs in the case of TAG-1 reduction. CT and TC circuits are very important for the brain function and parts of their development still remain unknown. Investigate the development of such circuits, neurodevelomental disorders which affects these tracts will be studied more easily. Using any other Cre line specialized for the cortex, the investigation of other developmental systems will be possible.

The core of this project is the thorough characterization of these two mouse models, especially that of the double transgenic, aiming ultimately in using it as a potential model for neurodevelopmental disorders.

2. MATERIALS & METHODS

2.1. Experimental animal

For the needs of this project, the following mouse lines are used:

1) The transgenic (tsg) mouse line *Tag1* $^{\text{loxP-GFP-loxP-DTA}}$ (PhD thesis, Vidaki M.). This mouse line is characterized by the expression of the *e*GFP gene (enhanced GFP) under the control of the *Tag-1* promoter. The eGFP-coding sequence is flanked by two loxP sites, with the second loxP site followed by the DTA (Diptheria Toxin subunit A) coding sequence. There is controlled expression of Cre recombinase, depend on the Cre animal which will be crossed with. In this way the domain between the loxP sites is removed, and DTA gene comes closer in TAG1 promoter and it is expressed.
2) The tsg mouse line *Emx1*$^{\text{Cre}}$, where the expression of Cre-recombinase is under the control of *Emx1* promoter. Thus, cre recombinase is expressed early in the development from almost all mitotic cells in the neocortex (E9.5) (Chan CH et al., 2001).
3) The double tsg mouse *Emx1*$^{\text{Cre}}$;*Tag1*$^{\text{DTA}}$, which is generated after the cross between the two above mentioned mouse lines. In these animals the removal of GFP and the expression of DTA takes place in the pyramidal cells of the neocortex.

2.2. Genotyping

2.2.1 Genomic DNA extraction from tissue

In order to see the genotype of the mice, a small piece of the animal's tail is acquired and used for the isolation of genomic DNA and genotyping as follows:

1. The tail is incubated in 400μl of tail lysis buffer (100mM NaCl, 10mM Tris-HCl, pH 8, 25mM EDTA, pH: 8.0, 0.5% SDS) and 5μl of 20mg/ml ProteinaseK in 55^{o}C for 6-18 hours, until it is dissolved.
2. Addition of 1μl 10mg/ml RNAse and incubation in 37^{o}C for 10min.
3. Addition of one volume of phenol and mild stirring for 10min in RT.
4. Addition of one volume of chloroform and mild stirring for 10min in RT.
5. Centrifugation for 5min, 13000rpm, RT and transfer of the supernatant in a new eppendorf tube.
6. Addition of equal volume of chloroform and mild stirring for 10min, RT.
7. Centrifugation for 5min, 13000rpm, RT and transfer of the supernatant in a new eppendorf tube.
8. Addition of ½ volume ammonium acetate (10M) and 2 volumes of ice cold ethanol.
9. Vortexing followed by centrifugation for 20-25 min, 13000rpm, 4^{o}C.
10. Removal of the supernatant and the pellet of DNA is allowed to air-dry.
11. Redissolving of the DNA in 120-150μl ddH_2O.

2.2.2 Polymerase Chain Reaction

Following DNA extraction, PCR was used to determine the genotype of the animals used. Primer and reaction setups are included in the table below:

Primers	PCR setup
DTANcoI-F: 5'-ccatggatcctgatgatgttgttg-3' DTAEcoRI-R1: 5'-gaattctcacaaagatcgcctgaacacg-3' DTA band : 599bp	4 min 94°C 30" 94°C 45" 61°C x33 1 min 72°C 5 min 72°C
TK 142: 5'-atccgaaaagaaaacgttga-3' TK 140: 5'-atccaggttacggatatagt-3' Cre band: 600bp	4 min 94°C 30" 94°C 40" 54°C x32 1 min 72°C 5min 72°C

2.3. Histological Methods

2.3.1. Tissue collection and processing for cryosections

Tissue from embryos was collected after careful dissection and fixed in 4% PFA (parafolmadehyde) in 1xPBS at 4°C for 12-18 h. For mice older than 1 week, we anaesthetized the animal with intraperitoneal injection of pentobarbital (DOLETHAL, 140μg per g of animal weight). After non-responsiveness to painful stimuli was established, the heart was exposed and with the assistance of a peristaltic pump 10-20ml 0.1M PBS (1xPBS) were infused in the left ventricle, followed by 25-30 ml of 4% PFA in 1xPBS. Finally the brain was dissected and post-fixed in 4% PFA in 1xPBS at 4°C for 12-18 h. Following the fixation step, both embryonic and postnatal tissues were washed with 1xPBS and incubated in 30% sucrose solution in 1xPBS to allow cryoprotection. Next, tissues were embedded in a gel consisting of 15% sucrose and 7,5% gelatin in 1xPBS and frozen in isopentane, in -45°C. Tissue blocks were stored at -80°C,prior to cryosectioning (Cryostat, LEICA). Sections of 12-14 um were collected on glass slides and stored at -20°C until further analysed.

2.3.2. Tissue collection and processing for vibratome sections

As explained previously, the brain was dissected in 1xPBS and fixed in 4% PFA in 1xPBS at 4°C for 12-18 hours. Fixed tissue was washed in 1xPBS and embedded in 4% low melting agarose in 1xPBS. The tissue-containing block was placed in the 1xPBS-containing bath of the vibratome (Leica) and cut in 90-100 um-thick sections. Sections were collected in 1xPBS and stored at 4°C prior to DiI labeling.

2.4. Histological techniques

2.4.1. Immunohistochemistry

2.4.1.1. Immunohistochemistry on cryosections from embryonic tissue

i) The slides were removed from -20ºC and the tissue was encircled with Dakopen.
ii) Washes in 1xPBS and 0.1% Triton (1xPBT) for 5 min (3x).
iii) Incubation in 5% Bovine Serum Albumin (BSA) and 0.1% Triton in 1xPBS (blocking solution), 1h, RT, to block unspecific binding of the antibodies.
iv) Incubation of the sections in primary antibody diluted in blocking solution (see Table), 12-18 hours, 4ºC.
v) Washes in 1xPBT, 3x10 min, RT.
vi) Incubation with secondary antibody diluted in blocking solution (see Table), 1-2 hours in the dark, RT.
vii) Washes in 1xPBT, 3x10 min
viii) Mount sections with MOWIOL and store in 4ºC or -20ºC for longer storage.

2.4.1.2. Immunohistochemistry on cryosections from post-natal tissue

i) The slides were removed from -20ºC and the tissue was encircled with Dakopen.
ii) Sections are post-fixed in acetone, 10 min, at -20ºC.
iii) Washes in 1xPBS, 3x5 min, RT.
iv) Incubation in 5% Bovine Serum Albumin (BSA) and 0.1% Triton in 1xPBS (blocking solution), 1 h, RT.
v) Incubation in primary antibody diluted in blocking solution (see Table), 12-18 hours, at 4ºC.
vi) Washes in 1xPBS, 3x10 min, RT.
vii) Incubation in secondary antibody diluted in blocking solution (see Table), for 1-2 h in the dark, at RT.
viii) Washes in 1xPBS, 3x10 min
ix) Incubation in DAPI or TOPRO, diluted 1:1000 or 1:2000 respectively in 1xPBS, for 3 min at RT.
x) Mount sections with MOWIOL and store in 4ºC or -20ºC for longer storage.

Primary Antibodies	**Dilution**	**Isotype**
4D7 (Developmental Studies Hybridoma Bank)	1:1000	Mouse IgM
Anti-GFP (Minotech)	1:5000	Rabbit IgG
Anti-GFP (Nacalai Tesque)	1:1000	Rat IgG
Anti-Tbr1 (Abcam)	1:100	Rabbit IgG
Anti-Satb2	1:250	Mouse IgG
Anti-Ctip2	1:1000	Rat IgG
Anti-TG3	1:2000	Rabbit IgG
Anti-BrdU (Oxford Biotech)	1:1000	Rat IgG

Secondary Antibodies	Dilution
Goat-anti-mouse IgG_ALEXA 555 (Molecular Probes)	1:800
Goat-anti-rabbit IgG_ALEXA 488 (Molecular Probes)	1:800
Goat-anti-rabbit IgG_ALEXA 555 (Molecular Probes)	1:800
Anti-rat IgG_ALEXA 555	1:800
Anti-rat IgG_ALEXA 488	1:800
Anti-rabbit Cy3 555	1:800

2.4.2. 5-Bromo-2'Deoxyuridine (BrdU) staining and immunolocalization

1) Injection of BrdU and tissue preparation

This substance is a synthetic nucleoside that is an thymidine analog. After BrdU injection in a tissue/organism, it is incorporated in the proliferating cells for the next ~6 hours and after this time it decomposes.

BrdU stock: 6 mg/mL in ddH_20, aliquot and store at -20^oC

On the day of interest an intraperitoneal BrdU injection is performed in pregnant mice at a dose of 75μg / g of animal weight in 1xPBS. Mice collected at P6, without perfusion, and the brains were dissected.

2) Immunohistochemistry

After the IHC against the other marker is completed the anti-BrdU staining takes place as follows:

i) Sections are fixed for 10 min in 4% PFA in 1xPBS, RT.
ii) Washes in 1xPBS, 3x5 min
i) Incubation in Denaturation Buffer (2N HCl in 1xPBS, with 0,1% 100xTriton), for 30 min in 37^oC.
NOTE: 37% HCl corresponds to 12N normality.
ii) Incubation in Neutralization buffer (Sodium Tetraborate 0.1M, pH 8.5), 3x8 min, RT.
iii) Washes in 1xPBS and 0.01% Triton.
iv) Incubation in BrdU antibody diluted in 1xPBS, 10% FBS, 0.1% Triton, for 12-18 h, 4^oC.
v) Washes in 1xPBS, 0.01% Triton, 3x10 min, RT.
vi) Incubation in the secondary antibody diluted in 1xPBS, 10% FBS, 0.1% Triton, for 1-2 hours, RT.
vii) Washes in 1xPBS, 0.01% Triton, 3x10 min, RT
viii) Mount sections with MOWIOL and store in 4^oC or -20^oC for longer storage.

2.5. DiI Labeling

For axonal tracing experiments we performed 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbo-cyanine perchlorate (DiI) labeling in embryonic brains. The following set of axons were labeled: the CTAs, TCAs and CST tracks. This dye is lipophilic and incorporates into the cell membrane. Thus, through diffusion we have the ability to label apart from the neuron's cell body, also the neurites.

Embryonic brains were cut using a vibratome and the sections stored at 4ºC (see 2.3.2). Small DiI crystals were inserted into different layers of the cerebral cortex and the thalamus. Then, the fixed slices were incubated at 37ºC in 1xPBS contain NaN_3 from 7 days to up to three weeks to enable the diffusion of the dye.

Subsequently, sections were immersed with the assistance of a painting brush in a solution containing 0.2% (m/v) gelatin (porcine skin gelatin) in dH_2O and 50 mM Tris HCl, pH7.5 and placed on slides prior to mounting with Mowiol and storage at 4ºC.

Images were acquired using either a confocal (TCS SP2, Leica) or epifluorescence microscope (Zeiss).

2.6. Analysis of immunohistochemical experiments and statistical analysis

For the quantification of various cell types in immunohistochemical experiments images were acquired using a confocal microscope (TCS SP2, Leica) and under the same laser and detector settings for all samples. In each analysis we used 2-3 animals and focused in the motor and somatosensory cortex. Images were processed with the Adobe Photoshop CS2, version 9.0 and countings were performed using the ImageJ software, version ImageJA 1.45b.

3. RESULTS

3.1. Colocalization of GFP and TAG-1 in mouse neocortical cells

In order to study the pattern of TAG-1 expression, in stages that the antibody directed against it is not sufficient, the Tag-1$^{loxP\text{-}GFP\text{-}loxP\text{-}DTA}$ (PhD thesis. Vidaki M.) transgenic mouse line was created. Using this mouse model we can gain information about the expression of TAG-1 by following the expression of tsg GFP. We analyzed the colocalization of the tsg GFP and the endogenous TAG1 in a wide range of developmental and postnatal stages: at embryonic days E12.5, E13.5, E15.5, E16.5 (Figure 5) and postnatal days P0, P15 (Figure 6). We observed tsg GFP closely mimics the expression pattern of TAG-1, as visualized using double IHC against GFP and TAG-1 (epitope 4D7). Nevertheless, we need to keep in mind that the overlap of the signal is not always obvious, since GFP protein is located in the cytoplasm, while TAG-1 often seen along the axons.

Moreover, we found that GFP was detected during stages, at which TAG-1 is not detectable with standard IHC procedures, especially at postnatal ages (Figure 5). We performed experiments for the detection of TAG-1 and GFP using IHC in coronal section after the birth of the mouse, at the stages which TAG-1 is not detectable with IHC (Figure 6).

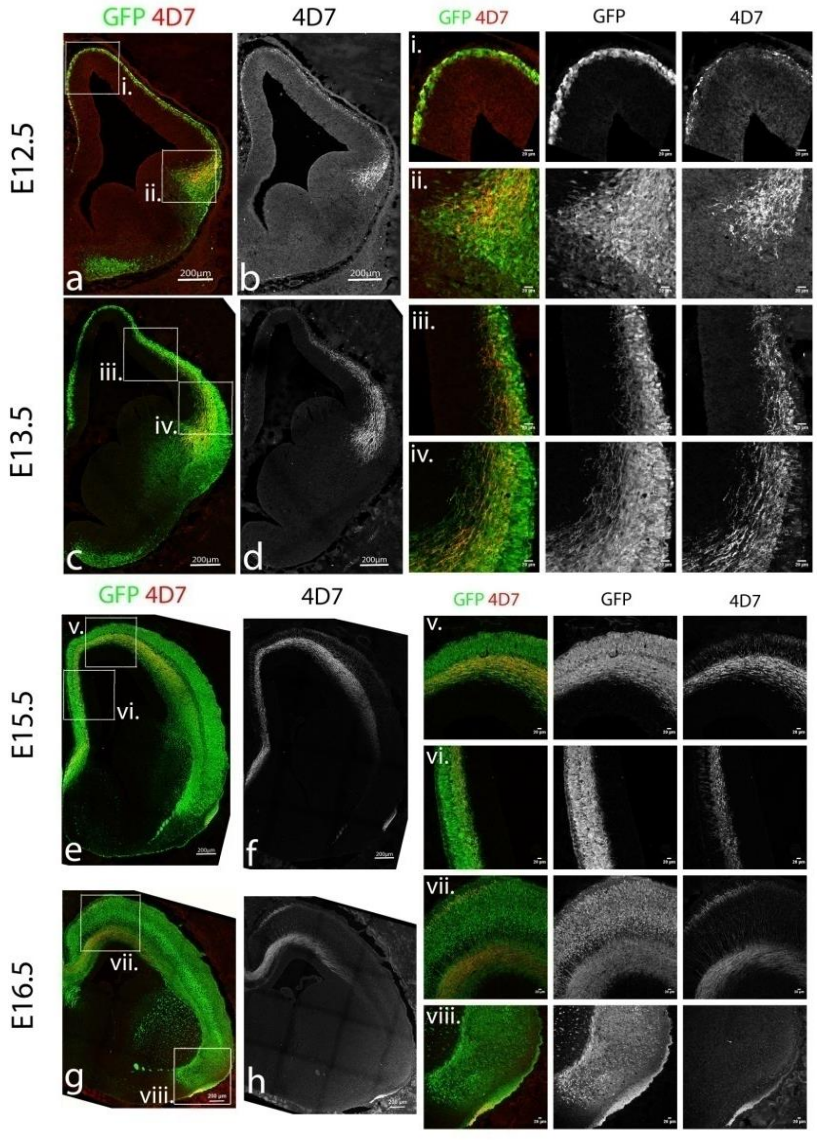

Figure 5 : Collocalization of GFP and TAG-1 in the cortex at embryonic stages. IHC against TAG-1 (4D7, in red) and GFP (in green) in coronal sections of the half hemisphere of the brain at a-b, E12.5; c-d, E13.5; e-f, E15.5 and g-h, E16.5 Zoom in are referred to different brain areas of the cortex (i,ii,iii,iv,v,vi,vii,viii)

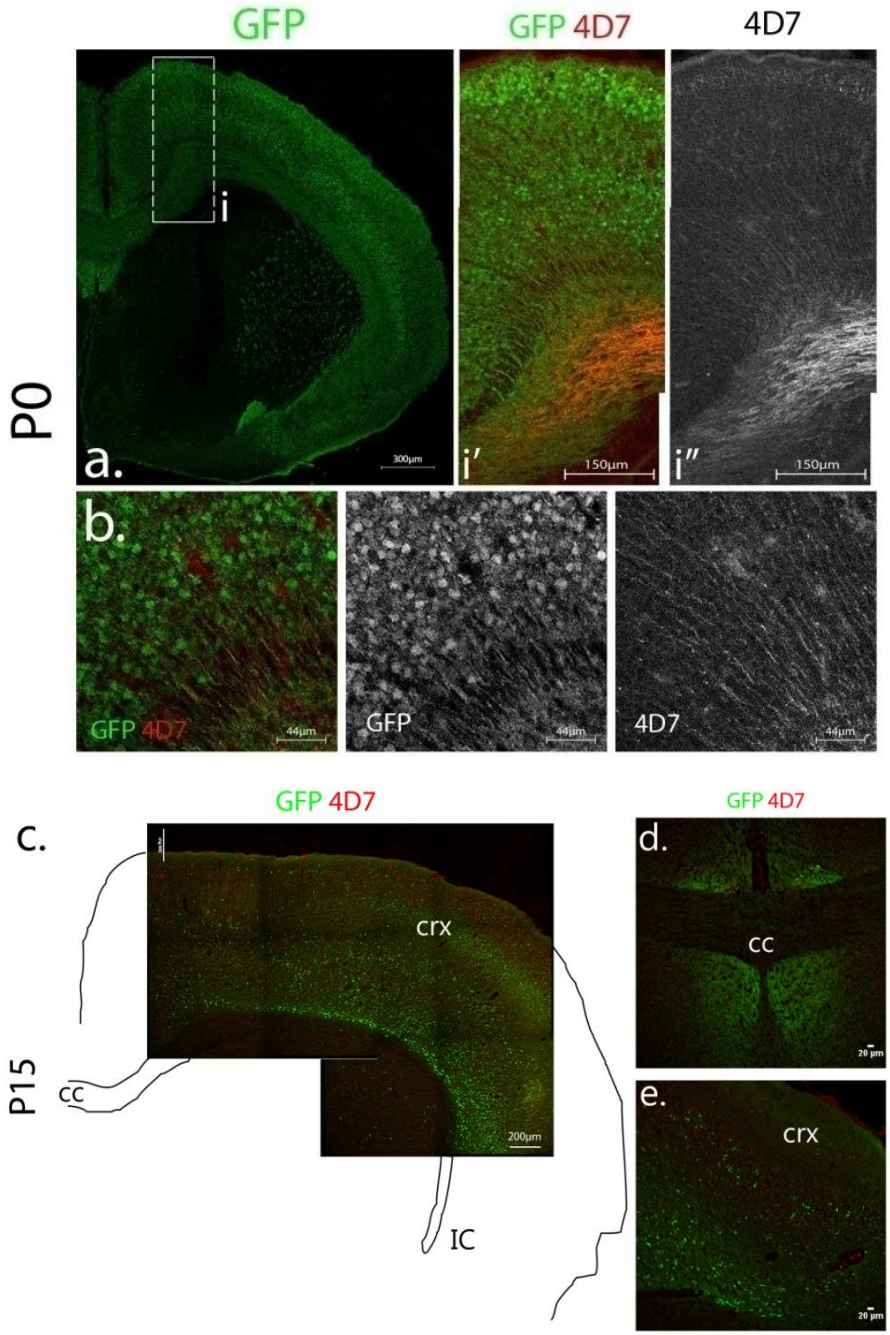

Figure 6 : Collocalization of GFP and TAG-1 in the cortex after birth. a) IHC against GFP (in green) in coronal sections at P0. i') zoom in and IHC against GFP(in green) and TAG-1 (in red-4D7), i'') zoom in with TAG-1 in gray scale. b) IHC against GFP, TAG-1 in the cortex. c) IHC against GFP, TAG-1 in P15 showing d) the corpus callosum and e) area in the cortex.

3.2. Profiling of GFP+ cells

Additionally, we performed double IHC against tsg GFP combined with neuronal-type-specific markers in order to conclude on the identity of the TAG-1/GFP+ neurons of the cortex. Tbr1 is a transcription factor widely used as a marker for the subplate at early stages and late on for layer V, VI. Double IHC against the tsg GFP and Tbr1 in *Tag-1*::GFP animals revealed that the profile of GFP+ cells corresponds partially to Tbr1+, as seen in the cortex at P6 (Figure 7). In layers V and VI almost all GFP+ cells are also Tbr1+, as seen in Figure 7b, where in layer V the 98% of GFP+ cells were also Tbr1+ and in layer VI the 93%.

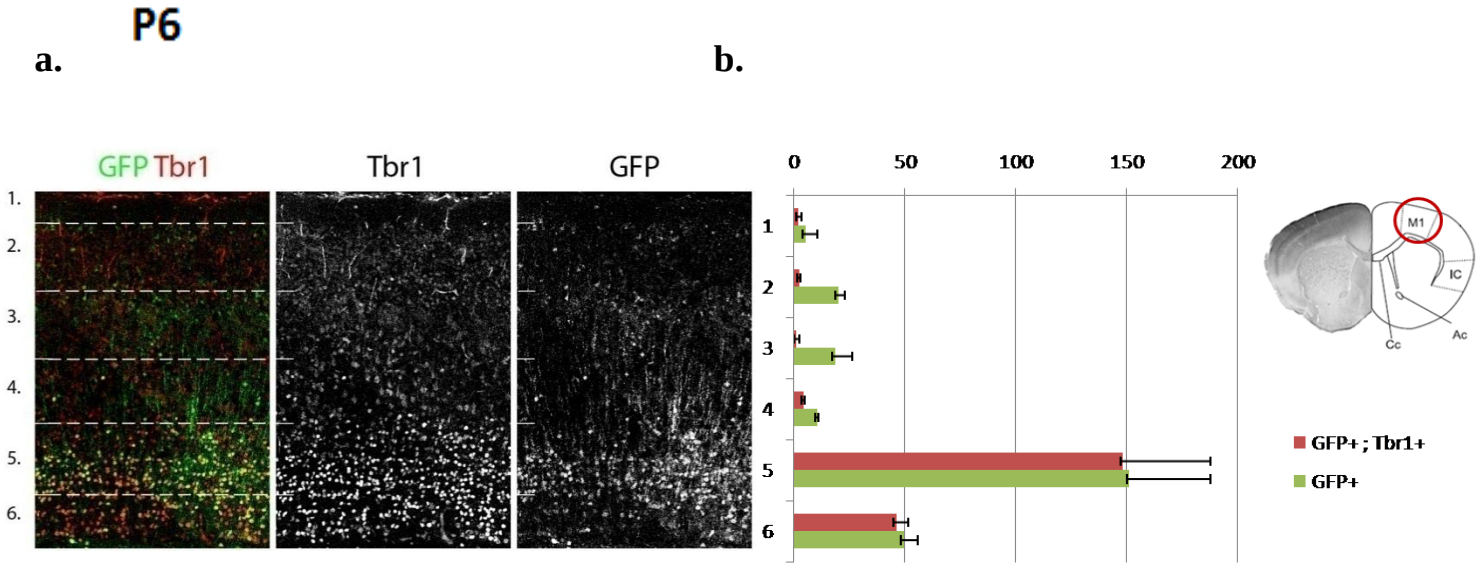

Figure 7 : (a) Immunohistochemistry against Tbr1 (in red) and GFP (in green) in coronal sections, in motor cortex area at P6. (b) Counts of cells in different bins, GFP+ ; Tbr1+ double positive cells compare with total GFP+ cell counts.

A previous study mentions that Reelin(Rln)+ cells, which constitute another subpopulation in the cortex located in the Marzinal Zone (MZ), are also TAG-1+ (Morante-Oria, et al., 2003). Thus, we wondered whether if Rln+ cells are also GFP+ (Figure 8). In *Tag-1*::GFP mice, almost all Rln+ cells are also GFP+.

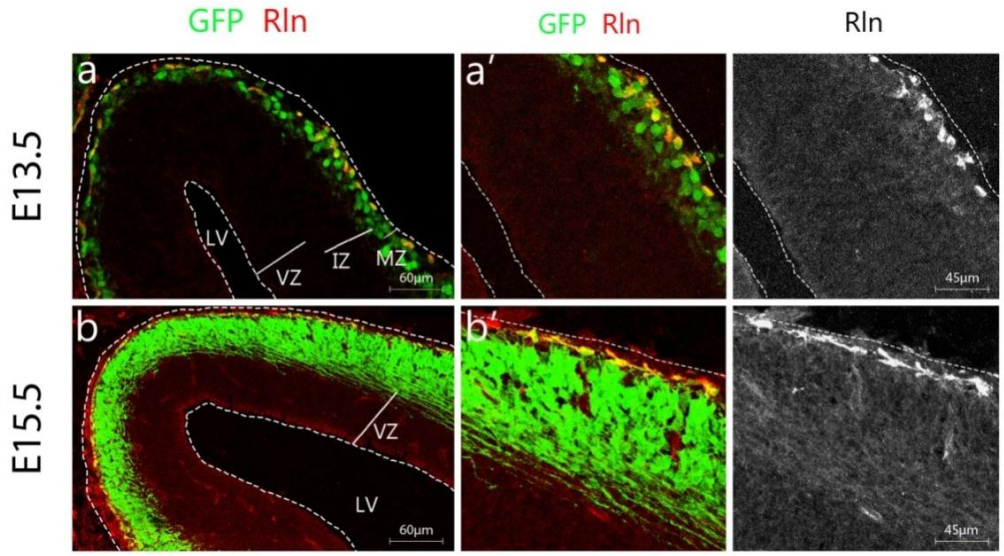

Figure 8 : Immunohistochemistry against Reelin (in red) and GFP (in green) in *Tag-1* :: GFP coronal sections of a) E13.5 and b) E15.5 developmental stages brain. Abbreviations, LV, Lateral Ventricle; VZ, Ventricle Zone; IZ, Intermediate Zone; MZ, Marginal Zone.

Cell birthdating experiments using BrdU pulses were performed for the analysis of the day of generation of the GFP+ cells which correlates also with their positioning in the neocortex. We confirmed a simultaneous birthdate of GFP+ and TAG-1+ neurons, with the majority located at layer V and VI, when taking in account previous information that has been published about the birthdate of TAG-1+ neurons in E11.5, E12.5 and E13.5 (Wolfer et al., 1994)(Figure 9).

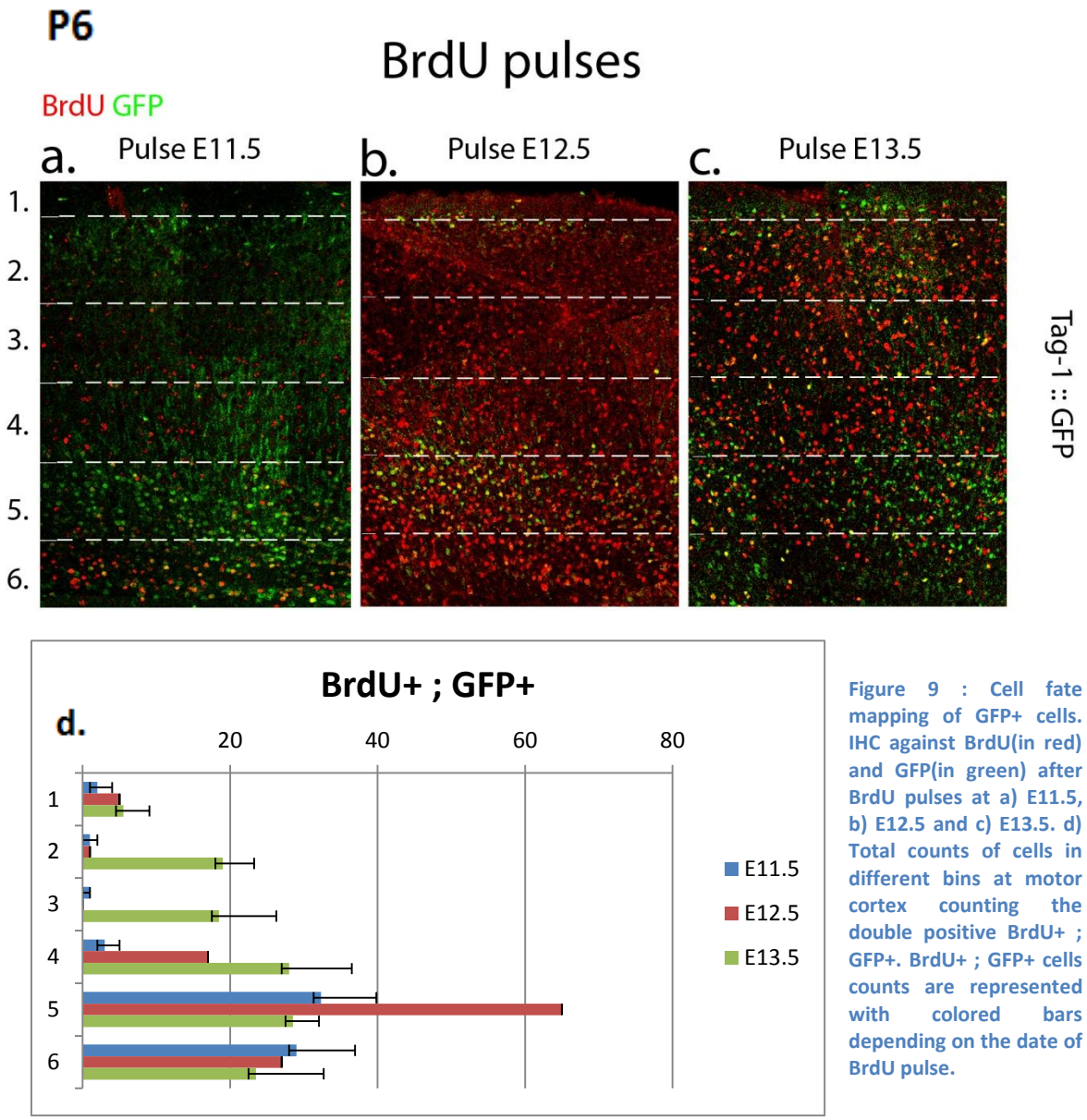

Figure 9 : Cell fate mapping of GFP+ cells. IHC against BrdU(in red) and GFP(in green) after BrdU pulses at a) E11.5, b) E12.5 and c) E13.5. d) Total counts of cells in different bins at motor cortex counting the double positive BrdU+ ; GFP+. BrdU+ ; GFP+ cells counts are represented with colored bars depending on the date of BrdU pulse.

3.3. Directed cell death of TAG-1+ neocortical pyramidal neurons

For the ablation of TAG-1+ corticofugal axons, the tsg line*Tag1*$^{\text{loxP-GFP-loxP-DTA}}$ was crossed with the *Emx1*$^{\text{Cre}}$, giving rise to the *Emx1*$^{\text{Cre}}$;*Tag1*$^{\text{DTA}}$. This results in removal of the GFP and the expression of DTA only where the *Emx1* gene is expressed.

Emx1 is a homeobox gene which starts to be expressed from E9.5 and on until the adult life (Chan et al., 2001). During the initial stages of neurogenesis Emx1 is expressed in almost all mitotic cells and in all cortical neurons regardless of the stage of differentiation which they are (Cecchi & Boncinelli, 2000). Moreover, it is expressed in the dorsal chamber (dorsal thalamus) or along the path of thalamocortical axons (Bishop et al., 2003).

After crossing the two tsg lines, Cre-recombinase expression in the neocortex area leads to the ablation of loxP sites and and the eGFP-coding sequence that is found between them while the DTA-coding sequence comes closer to the Tag-1 promoter and is expressed. Since we have proven the expression of GFP by TAG-1+ cells, we can assume that the toxicity of DTA leads to the death of TAG-1+ cells, which is expected to happen early in the development, after the E11.5, when TAG-1 expression starts (Figure 10).

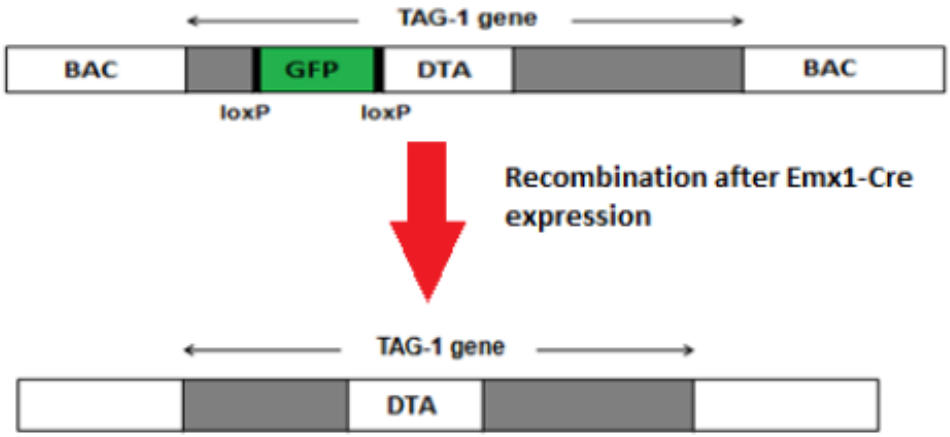

Figure 10 : Schematic representation of the transgene and the recombination after crossing with the Cre line, leading to the expression of the DTA toxin.

3.3.1. Analysis of Cortical Layering after the ablation of TAG-1+ cortical neurons

For checking which layers and subpopulations of the cortex are affected by the elimination of TAG-1+ neurons, experiments for the analysis of cortical layers are performed.

Macroscopically, *Emx1*::Cre;*Tag-1*::DTA mice have a significantly smaller cerebral cortex compared to control animals. Cortical layering is found unaffected in double transgenic animals using IHC against different layer markers as Tbr1 (layers V, VI) and Ctip2 (layer V) at P6 (Figure 11a, b). Nevertheless, when analyzing the cortical marker Satb2 (layers II/III & IV), double transgenic animals did not show a similar distribution compared with the control mice (Figure 11c).

Counting the total cell number in all the animals we observe a reduction of 36,4% in Satb2+, 31,2% in Tbr1+ and 11,6% in Ctip2+ cells in the total length of the cortex (Figure 11d).

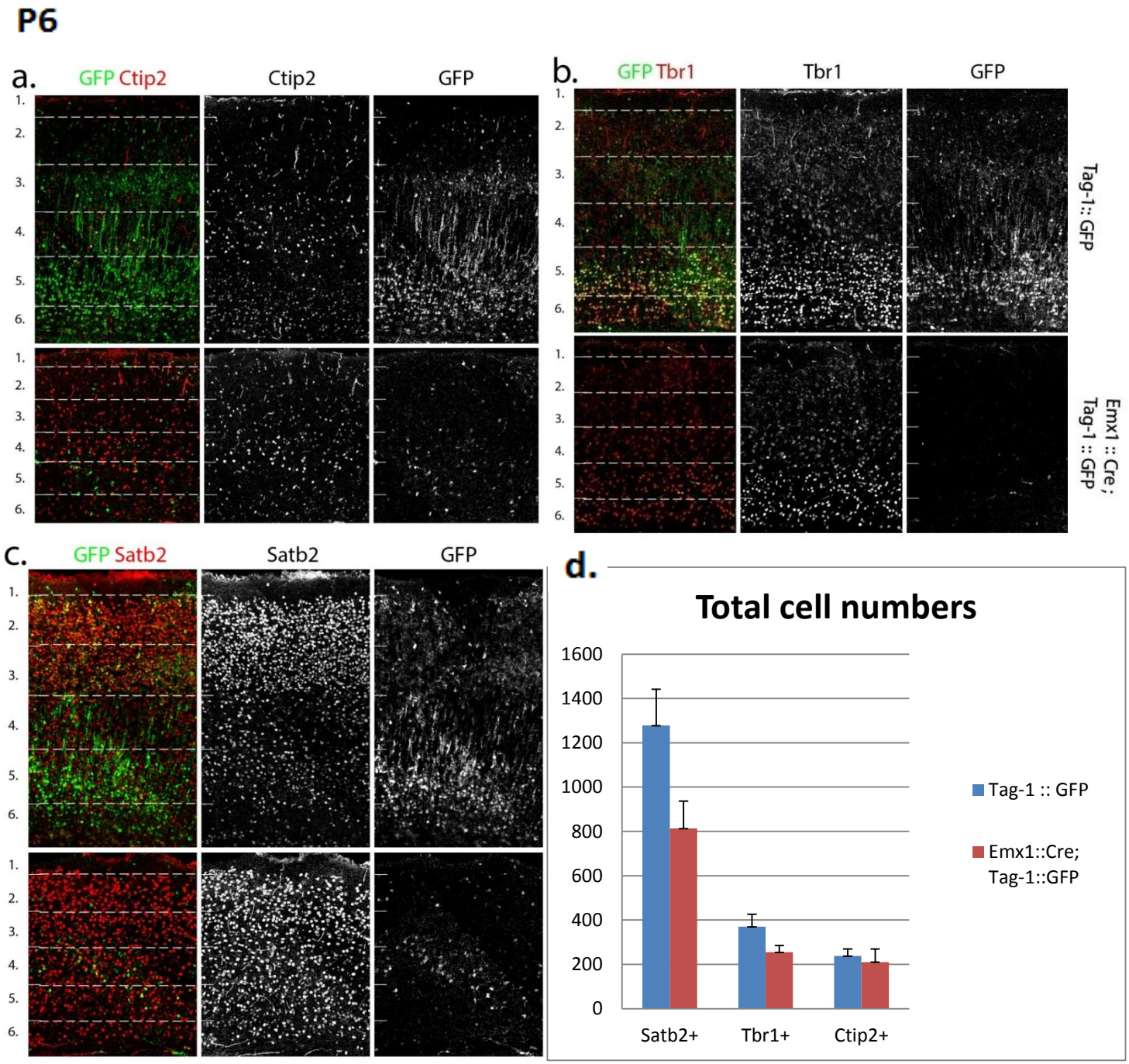

Figure 11 : Cortical layering partially unaffected in Emx1::Cre;Tag-1::GFP mice. IHC in Tag-1::GFP control mice and Emx1::Cre;Tag-1::GFP against a) Ctip2, b) Tbr1 and c) Satb2 (in red) and GFP (in green) in coronal sections, at the motor cortex at P6. d) Total cell counts for each layer marker in these animal models

3.3.2. Analysis of ablated neuronal populations

We used BrdU pulses at E11.5, E12.5, E13.5 and E15.5, to determine the identity of the neurons that were ablated after the recombination. We observe reduction of cells located in layers V, VI, which are born mainly at E11.5 (Figure 12a,b,e) and E12.5 (Figure 12c,d,f).

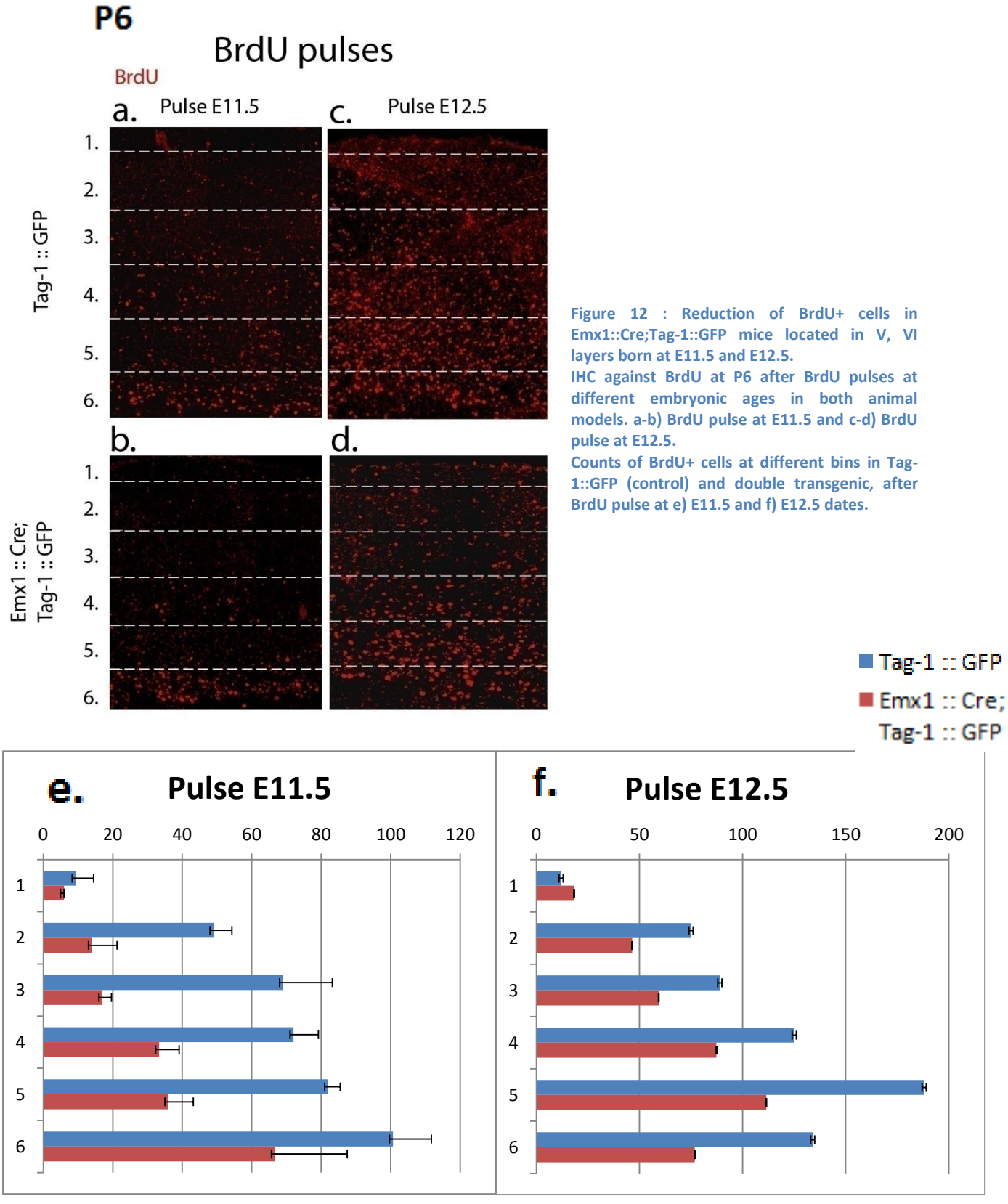

Figure 12 : Reduction of BrdU+ cells in Emx1::Cre;Tag-1::GFP mice located in V, VI layers born at E11.5 and E12.5.
IHC against BrdU at P6 after BrdU pulses at different embryonic ages in both animal models. a-b) BrdU pulse at E11.5 and c-d) BrdU pulse at E12.5.
Counts of BrdU+ cells at different bins in Tag-1::GFP (control) and double transgenic, after BrdU pulse at e) E11.5 and f) E12.5 dates.

3.4. Analysis of axonal tracts

In the *Emx1*::Cre;*Tag-1*::DTA mice we have observed reduction of corticofugal axons, abnormal corpus callosum (cc) and anterior commissure (ac), all of them basic tracts for the communication between various brain areas. This implied a major communication problem in this mouse model, but more elaborate investigation of the structures should be performed.

The anterior commissure is a basic structure for the communication of the two temporal lobes and seems to be a key player in the olfactory system. By applying double IHC against two different antibodies against TAG-1 (epitopes TG3 and 4D7) and GFP in the control mouse, we confirmed the expression of the transgene in this structure (Figure 13). IHC against GFP revealed severe reduction of the structure (Figure 14).

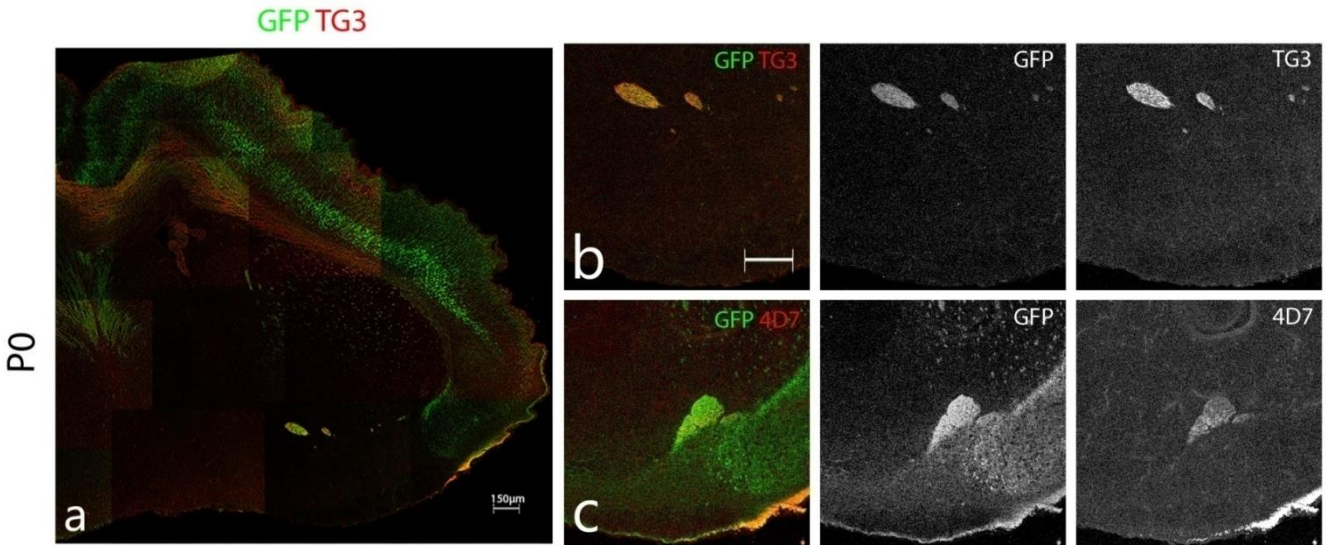

Figure 13 : Collocalization of GFP and TAG-1 in the Anterior Commissure. IHC against TAG-1 (in red) and GFP (in green) in coronal sections at P0 *Tag-1* :: GFP mouse brain. a) Whole hemisphere IHC against GFP and TG3 for TAG-1. Zoom in the area of the Anterior commissure with IHC against GFP and b) TG3 and c) 4D7 for TAG-1.

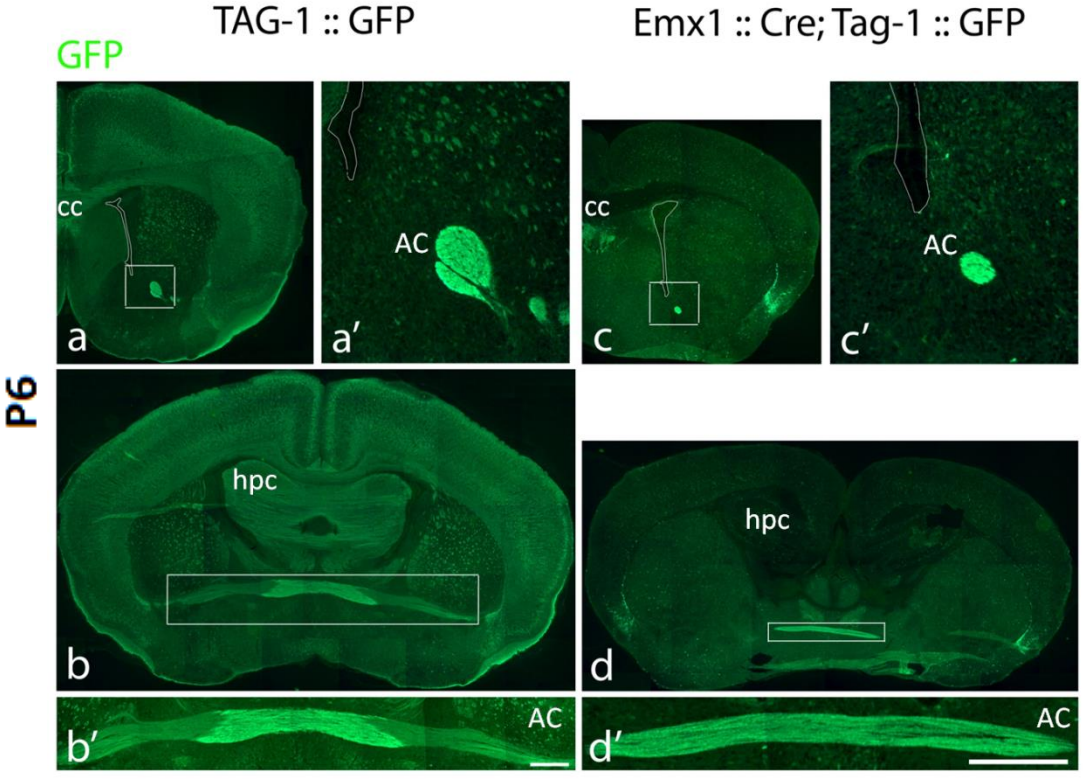

Figure 14 : Anterior Commissure reduction in double transgenic.
IHC against GFP in coronal brain sections of
a-b) control animal;
c-d) double transgenic animal
a-c) Rostral part and
b-d) causal part of the commissure.

3.4.1 DiI labeling of corticothalamic and subcerebral axons

Since a large percentage of corticofugal axons is removed in the double tsg, we found important to study the pattern of CTAs growth, as well as that of the TCAs in the context of the "handshake hypothesis". Additionally, we were also interested in potential defects in other axonal tracts arising from the cortex, collectively known as the corticospinal(CSP) tracts.

First, DiI crystals were placed in layer VI area of E16.5 neocortex and successfully labeled CT axons in both animal models. Thus, we confirm the existence of CTAs in both genotypes with no significant evidence of a severe defect (Figure 15).

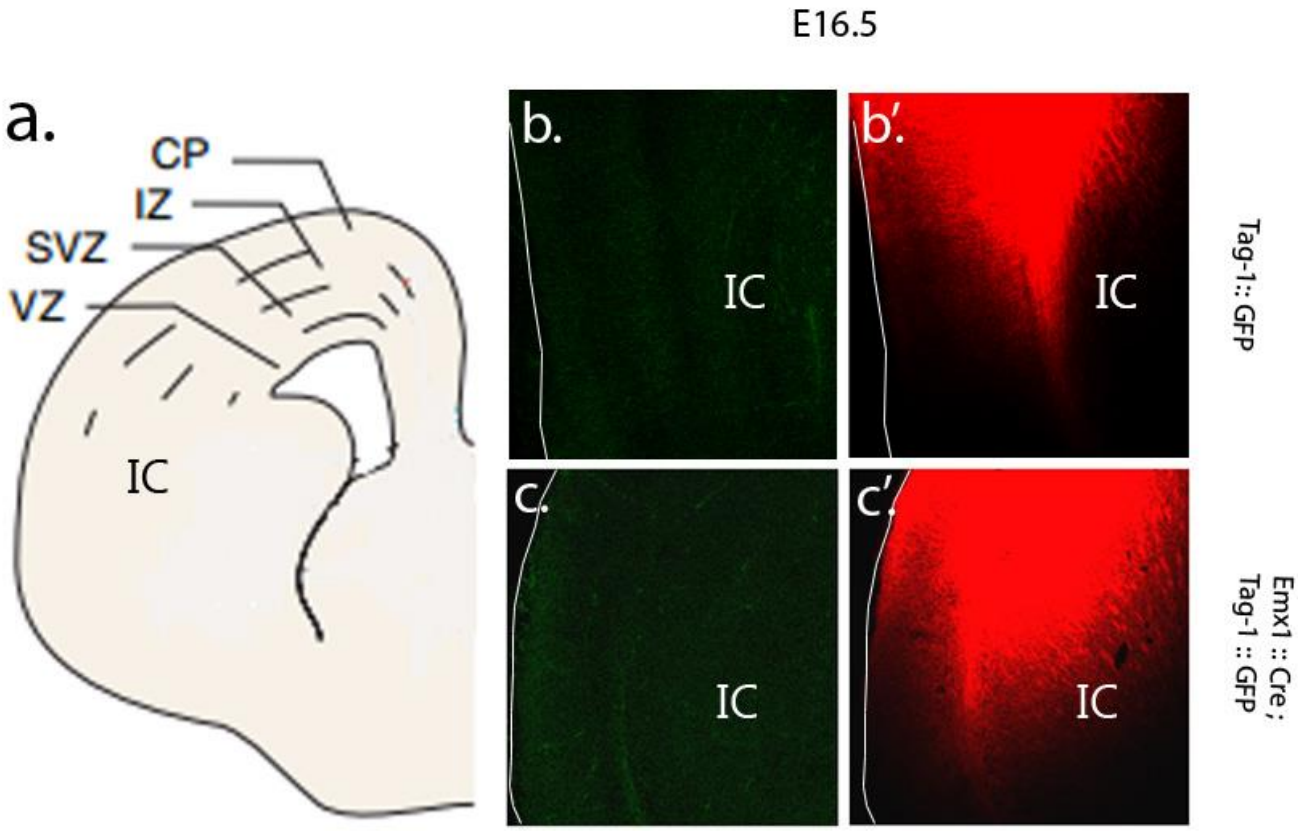

Figure 15 : DiI labeling of CTAs. a) Schematic representation of a mouse brain at E16.5. DiI labeling against CTAs at E16.5 coronal section b) in the control model and c) in double transgenic. Abbreviations, IC, Internal Capsule.

Afterwards we aim to label the CS tract. DiI tracing, starting from the layer V at E17.5, lead to the marking of both CTAs and CSPs. We confirmed the normal growth of the CSP axons in double tsg animals (Figure 16c,c') and also the existence and the right turn of the course of the eliminated CTAs to reach the thalamus (Figure 16b,b').

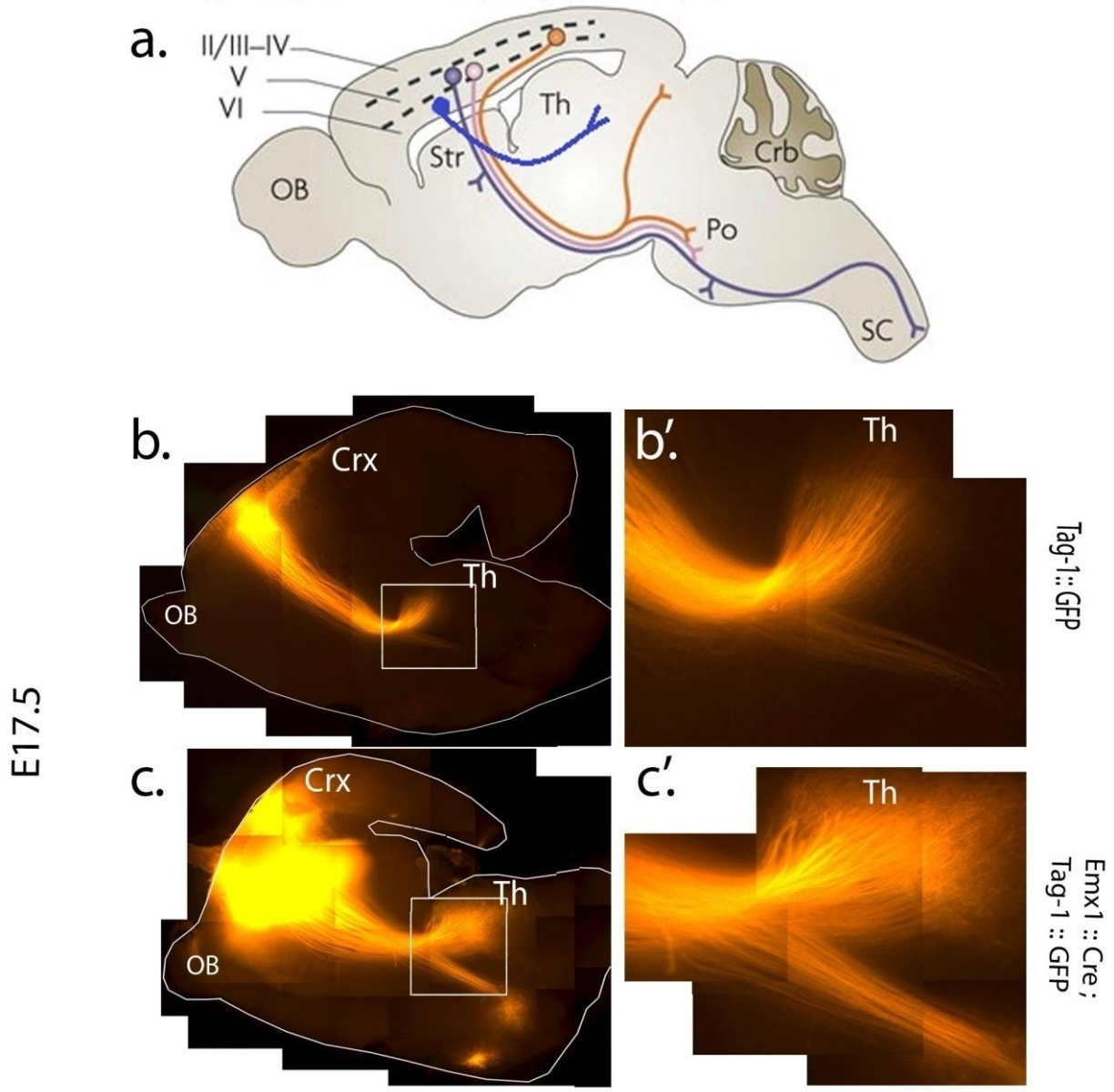

Figure 16 : DiI labeling of CTAs and CSP axos. a) Schematic representation of a sagittal section with all the axonal tracts that start from the cortex, CTAs (in blue) and subcerebral (in purple,pink, orange). DiI labeling at E17.5 sagittal sections b) in control and c) in double transgenic animals. b'-c' represent zoom in pictures at the separation point in which CTAs and CSP changing direction. Abbreviations: Crx, Cerebral Cortex; Th, Thalamus; OB, olfactory bulbs; Str, Striatum; Crb, Cerebellum; Po, Pons; SC, Spinal Cord.

Summary of Results

Tag-1 :: GFP animal model

- GFP mimics TAG-1 expression
- Almost all GFP+ cells are Tbr1+ cells in layer V and VI
- Almost all GFP+ cells are Rln+ cells in Marginal Zone
- GFP+ cells and TAG-1+ cells ar born simultaneously

Emx1 :: Cre; *Tag-1* :: GFP animal model

- Small cortex size
- Mild difference in the distribution of cortical layer II/III and IV
- Reduction of cell numbers in cortical layer V and VI
- Abnormal formation of the corpus callosum, reduction of the anterior commissure and smaller hippocampal formation
- Reduction of corticofugal thickness
- Normal corticospinal tract

4. DISCUSSION

TAG-1, a membrane protein belonging to the immunoglobulin superfamily, is detected in corticofugal axons during embryonic development, but after birth only in juxtaparanodes of myelinated fibers. In juxtaparanodes, TAG-1 forms a complex with the glial-TAG-1 promoting myelination of the fiber, but also the clustering of the potassium channels, which are important for the repolarization of the membrane after action potential propagation (Traka et al., 2003). Even though it has been shown to play a role in axonal outgrowth and fasciculation, it has been investigated so far whether the TAG-1+ axons acts as a scaffold for other axonal sets. Additionally, the inability to detect it after birth led us to create a reporter mouse line, expressing the GFP under its promoter.

The transgenic mouse line *Tag1*$^{\text{loxP-GFP-loxP-DTA}}$ (PhD thesis, Vidaki M.), also carries upstream and downstream of GFP, two loxP sites, as well as the DTA-coding sequence downstream (Diptheria Toxin A subunit). Upon crossing with a line carrying the Cre recombinase in pyramidal neocortical neurons (*Emx1 ::* Cre), the domain between the loxP sites is removed, the DTA-coding sequence comes closer to the *Tag-1* promoter and starts being expressed, leading cells to apoptosis. Thus, a mouse is created with ablated TAG-1+ cells in the neocortex.

The *Emx1* gene is expressed. *Emx1* gene is expressed early in the development, from E9.5 and later on, in almost all mitotic cells in the neocortex, until the adulthood (Chan et al., 2001). Previous members of the lab applied IHC against the activated (cleaved) form of Caspase3 form of Caspase3, as a marker of cell death; double transgenic animals were compared with controls, revealing that the pattern of the cell death was restricted in E11.5-E13.5, when TAG-1+ neurons are born (MSc thesis, Theodosiou M.).

The colocalization of the transgenic GFP and the endogenous TAG-1 in embryonic and postnatal brain in *Tag-1*::GFP animals was tested and confirmed in a range of ages. Transgenic GFP mimics the expression pattern of TAG-1, as seen by performing double IHC against GFP and TAG-1 (Figure 5, 6). Additionally, aiming to identify which subpopulation express the tsg GFP in *Tag-1*::GFP animals, IHC against tsg GFP and Tbr1, as a marker for layer V and VI, was performed. Knowing that most of TAG-1+ neurons are localized in layer V and VI, we expected the colocalization of GFP and Tbr1 in these layers. The IHC against GFP and Tbr1 revealed that the profile of GFP+ cells corresponds partially to Tbr1+, as it was tested in P6 (Figure 7a). Especially in layer V and VI, almost all GFP+ cells were also Tbr1+ (Figure 7b).

Using BrdU pulse experiments, we confirmed a simultaneous birthdate of GFP+ and TAG-1+ neurons, taking in account previous published information on the birthdate of TAG-1+ neurons (Wolfer, et al., 1994). In double tsg animals, however, we notice that we have a severe reduction in TAG-1+ (GFP + cells), which takes place between E11.5 and E13.5, corresponding to the period that TAG-1+ neurons are born (Figure 9).

These results make more apparent the similar expression pattern of the transgene and TAG-1.

Rln+ cells, another subpopulation in the cortex located in the MZ, are also TAG-1+ (Morante-Oria, et al., 2003). Rln is crucial in regulating the attachment of migrating neurons to the scaffold created by radial glia (Marin & Rubenstein, 2003). Radial glial cells are used as a scaffold for newly formed neurons to migrate towards the cortex and reach their final position (Lizarraga et al., 2010). It has been shown that in the absence of Rln+ cells, radial glial cells extend their projections in multiple orientations and neurons migrate abnormally (Nomura et al, 2008). In *Tag-1*::GFP mice, almost all Rln+ cells are also GFP+, which points to the importance of further analysis of this subset of cells (Figure 8).

Macroscopically, the *Emx1*::Cre;*Tag-1*::DTA mice have a significantly smaller cerebral cortex compared to control animals. Cortical layering is found unaffected in double transgenic animals using IHC against different layer markers as Tbr1 (layers II/III & VI) and Ctip2 (layer V) (Figure 11a, b). Nevertheless, when analyzing the cortical marker Satb2 (layers II/III & IV) double transgenic animals did not show a similar distribution compared with the control mice (Figure c). In *Emx1*::Cre;*Tag-1*::DTA mice we observe an expansion of Satb2+ cells in all layers, compared with the control where we observe a restricted distribution between layers II-III and IV. Previous studies showed that after the ablation of a layer-specific cortical subtype, other subtypes expand to "fill the gap" which is generated in the knock-out mice (Lodato et al., 2011; McKenna et al., 2011). Thus, we can propose that Satb2+ neurons expand to fill the gap created in layer V and VI by the reduction of TAG-1+ cells.

Several structures were found to be affected in newborn double transgenic animals. Postnatally, smaller hippocampal formations (hpc), abnormal corpuc callosum (cc), as well as a reduction of the anterior commissure (ac) are observed (Figure 14). Several neurodevelopmental disorders have the same characteristics, such as corpus callosum dysgenesis and the L1 syndrome, which makes this animal model a potential tool for studying these conditions (Engle et al., 2010).

Corpus Callosum reduction is often present in knockout mice for different cortical neuronal subtypes (Alcamo et al., 2008; Rouaux & Arlotta, 2010; Lodato et al., 2011). So far, we do not know the cause for the observed atrophy in the cc in the double transgenic mice. The affected pattern of Satb2+ cells which regulate callosal projection may partially explain this phenotype.

The anterior commissure is a central in the olfactory system of the mouse. Nevertheless, its specific role and how its elimination or total ablation affects directly the brain is unclear. Previous experiments performed by our lab, revealed either ablation of the whole structure, as seen by morphological staining, or severe reduction compared with the control in IHC experiments (Figure 14).

The "handshake hypothesis" between CTAs and TCAs constitutes a well-known hypothesis, on which several contradicting pieces of evidence exist. Aiming to test the "handshake hypothesis" in the double transgenic mouse, DiI experiments performed

from other members of the lab, revealed a normal crossing of CTAs and TCAs through the pallium-subpallium boundary at different embryonic days. Furthermore, IHC against TAG-1 and L1 confirms the presence of both axonal tracts, but the corticothalamic tract thickness is found reduced in the double transgenic animals at E15.5. These experiments confirm the existence of both tracts, but do not answer whether the tracts go through the described stages during development. Also, DiI staining revealed that CTAs and subcerebral axons seem to find their way to their target, and separate from each other to do so with no misguidance (Figure 16). As far as the subcerebral axons are concerned, it is known that they do not take part in this set, and reach the pons and spinal cord independently. Thus, we assumed that they would not be affected in the double transgenic.

In summary, in this study we focused on two transgenic mouse models, one used as a reporter line for the detection of TAG-1 through GFP expression and the second being *Emx1*::Cre;*Tag-1*::DTA, in which we ablate TAG-1+ neurons, which correspond partially to the neurons giving rise to the corticofugal axons. Through these series of experiments we tried to characterize the impact of the ablation of a large amount of TAG-1 corticofugal axons on the cortex growth and the cortex-thalamus connection.

Further experiments are needed for the characterization of double transgenic animals, which are focused on specific developmental stages of CT, TC and CSP tracts observation in more detail. Detailed analysis of the anterior commissure, corpus callosum and hippocampal development must be done, which are impaired in this animal model. Until now behavioral phenotype is unknown in these animals; taking on account the disruption of axonal tracts and other structures, behavioral tests must be performed.

By analyzing the phenotype of double transgenic *Emx1*::Cre;*Tag-1*::DTA mice, the function of TAG-1+ neurons and their involvement in the developing neocortex will be elucidated. Moreover, we will acquire important information about the corticofugal tracts, among which the corticothalamic and subcerebral tracts. The characterization of these two mouse models, especially that of the double transgenic, aims ultimately to use it as a potential model for neurodevelopmental disorders.

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