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14. ABSTRACT Most Parkinson's disease patients lose their sense of smell, sometimes decades before they develop the symptoms typically associated with the disease, such as tremors and difficulty walking. Olfactory deficits, hyposmia or anosmia, is now recognized as a feature of early Parkinson's disease and an indication that the disease is progressing insidiously. In this FY16 PRP Focused Idea Award application, we will examine the underlying causes for olfactory deficits seen in Parkinson's patients. The focus of our application is directly in line with the Focus Area of ' <i>Identification and evaluation of mechanisms in early Parkinson's disease involving olfactory, microbiome, gastrointestinal, and/or autonomic nervous systems</i> '. In this grant, we will examine how a protein known as alpha-synuclein, that forms abnormal deposits that are a signature of Parkinson's disease, impacts nerve connections or synapses in the olfactory system. We suggest that in Parkinson's disease, due to the presence of these abnormal alpha-synuclein deposits, both the structure and function of olfactory nerve connections is not proper. We will examine this question through our complementary expertise in the olfactory system and alpha-synuclein biology, using high resolution microscopic methods on mouse models that express mutant forms of alpha-synuclein that are linked to the disease. When we have successfully finished this study, we anticipate having a detailed picture of the synaptic abnormalities in the olfactory system in an animal model of early Parkinson's disease. This will allow us to treat the loss of smell in early Parkinson's disease patients in 5-10 years. But more importantly, as the nose and the olfactory system are thought to be a conduit through which alpha-synuclein deposits spreads, eventually reaching the mid-brain, a greater understanding of this pathway will permit us to slow the progression or even prevent Parkinson's disease. This is the ultimate goal of all Parkinson's disease research.					
15. SUBJECT TERMS Hyposmia, Olfactory, Alpha-synuclein, Adult Neurogenesis, Parkinson's disease, Transgenic, Confocal, Electron Microscopy					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 10	19a. NAME OF RESPONSIBLE PERSON USAMRMC
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INTRODUCTION:

Early in the course of Parkinson's disease (PD), most patients (> 90%) become hyposmic and are unable to detect or discriminate odors. Because these olfactory deficits typically occur prior to the onset of motor symptoms in PD, olfactory acuity tests are now being used as a biomarker to diagnose prodromal and early PD patients. Hyposmia in patients correlates with alpha-synuclein pathology in the olfactory bulb (OB) and anterior olfactory nucleus (AON). It is postulated that the restricted pattern of α -synuclein pathology in the olfactory system in early PD eventually spreads to the nigrostriatal system as the disease progresses and motor symptoms ensue. However, to date there have been no studies that have shed sufficient light on the mechanisms via which alpha-synuclein perturbs olfactory function. We hypothesize a primary effect on the synaptic circuitry of the olfactory system as an underlying mechanism. However, given the heterogeneity of olfactory circuits and their dependence upon adult neurogenesis, the specific effect(s) of alpha-synuclein and its precocious effect on olfactory function remains speculative. Therefore, the goal of our grant is to understand why the majority of newly diagnosed PD are hyposmic, by evaluating the role of synaptic dysfunction as a result of alpha-synuclein pathology in situ in the olfactory circuitry.

KEYWORDS:

Hyposmia, Olfactory, Alpha-synuclein, Adult Neurogenesis, Parkinson's disease, Transgenic, Confocal, Electron Microscopy

ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: To characterize the synaptic perturbations in the glomerular and external plexiform layer in α -synuclein transgenic mice.

Specific Aim 2: To examine the migration of newly generated neuroblasts into the OB and their integration into olfactory circuitry on α -synuclein overexpression.

Specific Aim 3: To monitor synaptic vesicle trafficking in OSNs and granule cells in α -synuclein transgenic mice.

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements.

As noted in an earlier report, preliminary data suggested that the α -synuclein transgenic mice (A30P) may have a decrease in synapses in the glomerulus, which is the location of the first afferent synapse in the olfactory system. We have carried out detailed morphometric analysis of electron micrographs of the olfactory bulb glomerulus from aged wild type and α -synuclein transgenic mice and have quantified synapse numbers as shown (**Figure 1**). Indeed, as shown in the attached graph, the number of excitatory afferent synapses from olfactory axon terminals in transgenics ($2.17/27\mu\text{m}^2 \pm 0.19$) is significantly lower than in controls ($3.778/27\mu\text{m}^2 \pm 0.302$) ($p < .001$)(**Figure 1**). This result is exciting because at the level of the light microscope, olfactory

marker protein (OMP) and vGlut2 which is specific to olfactory receptor axon terminals only lightly suggested that the glomeruli may be smaller in the

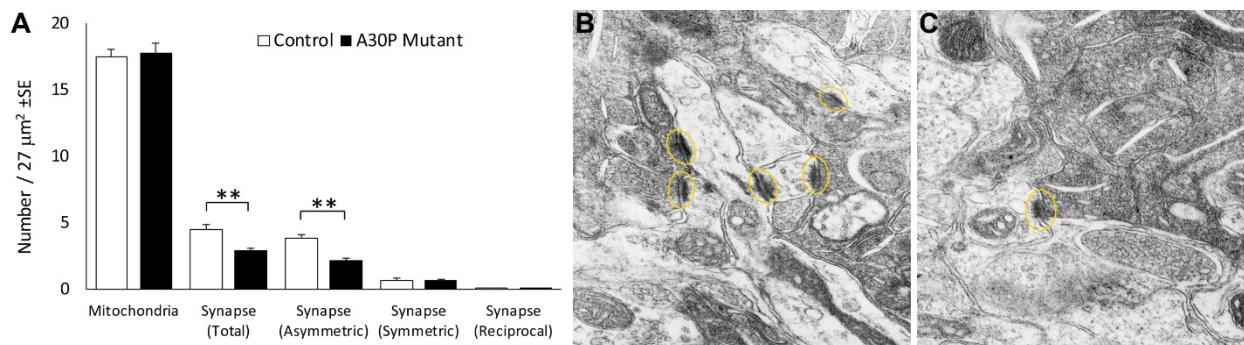


Figure 1. Electron Micrograph quantifications of mitochondria and synapses in the OB glomerulus from control (wildtype) and A30P Alpha-Synuclein transgenics (A30P Mutant). A. Quantifications; ** $p < 0.001$. B-C. Electron micrographs from control (B) and Alpha-Synuclein transgenic (C). Asymmetric synapses have been highlighted with yellow ovals.

transgenic mice (**Figure 2**). The net result of the decreased afferent input from the olfactory sensory neurons suggests that the system as a whole may be less sensitive with a higher threshold for odor detection. The loss of the inhibitory synapses suggests that local synaptic circuits in the glomeruli, which may contribute to odor discrimination, may also be less effective.

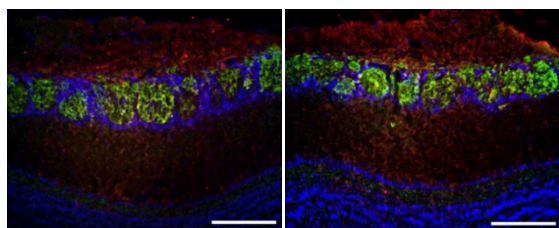


Figure 2. Immunohistochemistry of olfactory marker protein (OMP) (red) and vGlut2 (green) in the glomeruli of wild type controls and the transgenic mutants.

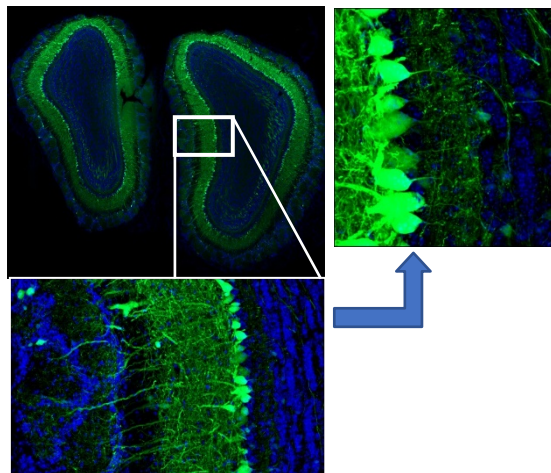
To pursue further the synaptic organization in the transgenic and wildtype controls we quantified the number of synapses in the external plexiform layer of the olfactory bulb where local circuits contribute to activity in the projection neurons, mitral cells and odor discrimination. There was a statistically significant decrease in the density of synapses in the transgenics ($3.493/27\mu\text{m}^2 \pm$) versus wildtype ($4.953/27\mu\text{m}^2 \pm$) ($p < .001$) and a comparable significant decrease in the density of mitochondria at synapses in transgenics ($15.851/27\mu\text{m}^2 \pm$) versus controls ($26.756/27\mu\text{m}^2 \pm$) ($p < .001$). Coupled with the significant decrease in afferent synapses in the glomeruli, these data suggest an overall decrease in synapses in the olfactory bulbs of transgenics and a diminished ability to both detect and discriminate odors.

To pursue this further we carried out a series of behavioral tests in which we compared the ability of the A30P mice to wildtype controls in detecting/finding food rewards hidden in the cage bedding. Consistent with the loss of primary afferent synapses, we found the transgenic mice had a mean time of 78.4 seconds $\pm$ 17.8m seconds to find the food reward while the wildtype controls found it in 25.3 seconds $\pm$ 16.7 (t = 2.84, $p < .006$). These differences were consistent across multiple trials and over the course of 3 weeks.

Given these two sets of observations, we built upon our initial hypotheses to ask if functional activity, assessed with gCAMP6 measuring calcium transients would reflect the loss of synapses and behavioral deficits. The rationale for including gCAMP6 in our experiments as an independent measure of synapse integrity in the olfactory bulbs of the transgenic mice.

This is a work in progress since we need to let the experimental mice age before they can be tested. But we have tested the overall approach and can show the robust expression of gCAMP6 in the olfactory bulb following viral delivery, as shown in **Figure 3**.

Figure 3. gCAMP 6 staining in the olfactory bulb. A low magnification image of a coronal section is shown on the upper left. Lower left is a higher magnification showing staining of mitral cell apical dendrites in the glomerular layer where they receive excitatory afferent synapses. On the upper right a higher magnification is shown to illustrate the strong gCAMP6 labeling in the mitral cell bodies on the left and their exiting axons on the right.



is shown on the upper left. Lower left is a higher magnification showing staining of mitral cell apical dendrites in the glomerular layer where they receive excitatory afferent synapses. On the upper right a higher magnification is shown to illustrate the strong gCAMP6 labeling in the mitral cell bodies on the left and their exiting axons on the right.

Complementing our analyses of primary afferent input to the olfactory bulb glomeruli we have also begun to assess synaptic organization in the first cortical representation of output from the olfactory bulb, piriform cortex, a 3-layer paleo- or allocortex.

We think this assessment is critical because when we assessed the expression of alpha-synuclein we found a particularly high expression in piriform cortex neurons, both in anterior (**Figure 4**) as well as posterior divisions. This is ongoing work but thus far our counts of primary input synapses onto the apical dendrites of piriform cortex pyramidal cells in layer I of piriform are identical for both the transgenic and control ($3.03/27\mu\text{m}^2$ and $3.33/27\mu\text{m}^2$, respectively). The lack of a difference between the two lines of mice is also apparent when the counts are broken down for asymmetric (excitatory) and symmetric (inhibitory). Similarly, we see no difference at this stage in the number of mitochondria associated with synapses in piriform cortex of the transgenics versus controls.

In addition, we have measured features of given classes of synapses, including, number of vesicles, lengths of pre- and postsynaptic specializations, and mitochondria. We found no differences in the ultrastructural properties of synapses in the two lines of mice. When it became apparent from preliminary data from this past year, we moved on to address hypotheses providing more insights into the substrates of olfactory deficits in humans and mouse models. The data suggest that synaptic transmission in those synapses that are present will not differ between the two lines of mice. The study noted above with the gCAMP6 will provide further insight. We also note as shown above in **Figure 3** that while the number of synapses in the glomeruli decreased, the density of mitochondria at synaptic sites did not differ ($17.77/27\mu\text{m}^2$ in transgenics and $17.46/27\mu\text{m}^2$ in controls). Thus, while there is broad speculation in the literature that alpha-synuclein may induce mitochondria dysfunction (e.g. Alpha-synuclein-induced mitochondrial dysfunction is mediated

via a sirtuin 3-dependent pathway. Park JH, et al. Mol Neurodegener 2020. PMID 31931835), this apparently does not translate to fewer mitochondria.

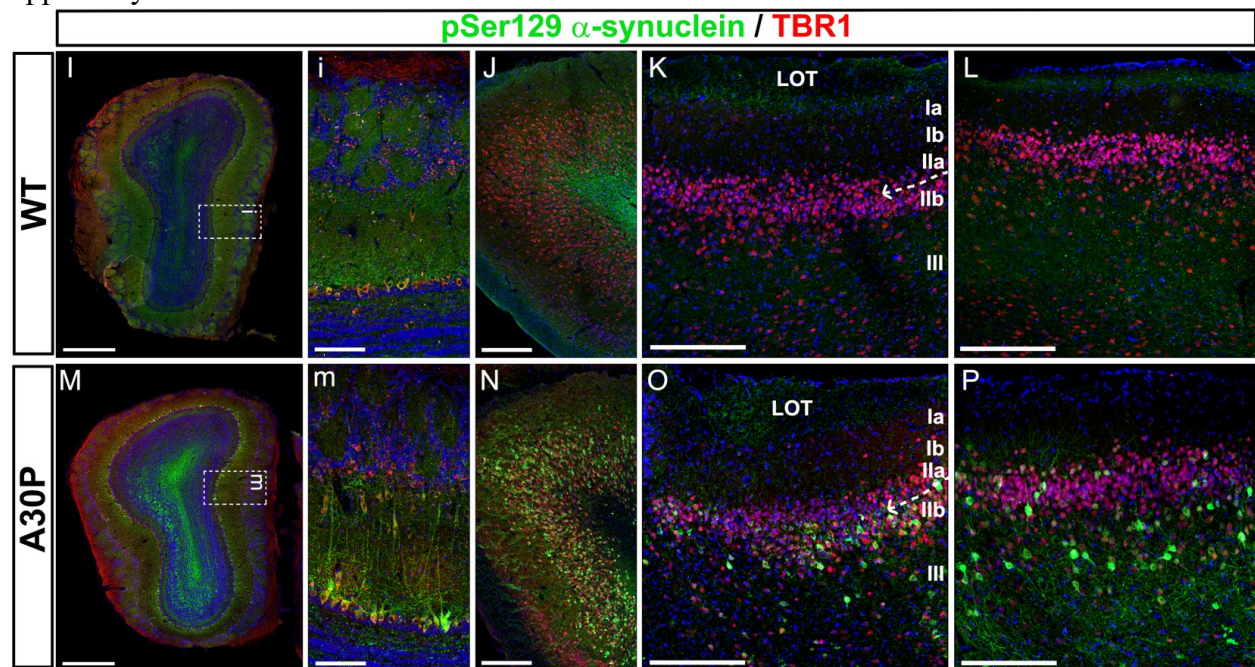


Figure 4. Expression of the phosphorylated alpha-synuclein in the olfactory bulbs at low and high magnification (I & M), in the anterior olfactory nucleus (J & K), in the anterior (K & O) and posterior (L & P) piriform cortex. pSer129 alpha synuclein shown in green and the neuronal marker TBR1 shown in red. Note the heavy alpha-synuclein staining in the transgenic mice in the lower row. LOT – lateral olfactory tract; piriform cortex layers shown in (K).

We have also carried out BrdU experiments to monitor neurogenesis and migration of new neuroblasts into the olfactory bulb. As shown in **Figure 5**, we have confirmed in wildtype control mice and the transgenic alpha-synuclein mutant mice that we can effectively label adult born neurons that originate in the subventricular zone and migrate to the olfactory bulb (**Figure 5**). The subjective interpretation suggests that fewer migrating doublecortin (DCX) labeled neurons are in the olfactory bulb. This appears consistent with the decrease in neurogenesis in the subventricular zone (BrdU labeling) and the subjective decrease in migrating DCX labeled neuroblasts in the rostral migratory stream. To generate objective data that we can test statistically and pursue the hypothesis that a decrease in this process may lead to a perturbation of olfactory bulb circuits in the transgenic mice we must wait for them to age until such time as when they begin to exhibit motor symptoms due to the over-expression of alpha synuclein. Successful breeding of these important mice is poor and motor symptoms are seen at the earliest around 8-12 months postnatal. Thus, this is a very long-term study. The electron microscopy reported above afforded more flexibility because we could use tissue from mice that died prior to sacrifice. The neurogenesis studies, and those with the gCAMP6 reported below, require live animals.

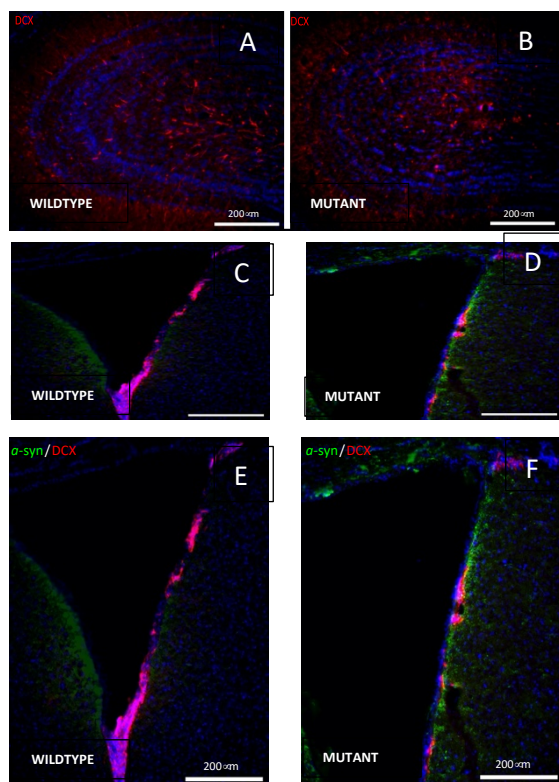


Figure 5. (A) and (B) are example of doublecortin (DCX) (red) labeled neuroblasts in the olfactory bulb. In (A) and (B) are examples of neurogenesis labeling with Ki67 (red) in the subventricular zone. In (D) compared with the control in (C) there is an apparent decrease in DCX labeled neuroblasts in the SVZ. In (E) and (F) the DCX labeled neuroblasts migrating in the rostral migratory stream appear fewer in the mutant compared to control. Alpha-synuclein labeling (green) is show in (E) and (F) and non-specific nuclear labeling (blue) in (A) and (B).

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

We will continue to do immunoelectron microscopy with PhosphoSer129 α -synuclein antibody to determine which specific synapses in the olfactory circuit are impacted by α -synuclein pathology. We also have begun live gCAMP6 imaging in wildtype and α -synuclein transgenic mice. We also plan to complete this study and publish our findings.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our results will help us understand why most newly diagnosed PD patients are hyposmic, i.e. have lost their sense of smell. This proposal is significant and impactful for several reasons. As the olfactory deficits occur prior to the onset of motor symptoms, and the olfactory system is thought to be a conduit through which α -synuclein pathology spreads, eventually reaching the mid-brain, a greater understanding of this pathway will permit us to slow the progression or even prevent

Parkinson's disease. As all available treatments for PD are symptomatic and do not halt or retard the underlying causative neurodegeneration, the translational prospects of our proposal is particularly important.

What was the impact on other disciplines?

We are learning more synapse structure and function in the olfactory circuit and this is important for the fields of synaptic biology and olfaction."

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

CHANGES/PROBLEMS:

Changes in approach and reasons for change: Instead of performing synaptopHluorin imaging we have decided to carry out gCAMP6 imaging, due to unavailability of synaptopHluorin transgenic reporter mice.

Actual or anticipated problems or delays and actions or plans to resolve them: Nothing to Report

Changes that had a significant impact on expenditures: Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Nothing to Report

Significant changes in use or care of human subjects: Not applicable

Significant changes in use or care of vertebrate animals: Nothing to Report

Significant changes in use of biohazards and/or select agents: Nothing to Report

PRODUCTS:

Publications, conference papers, and presentations:

Martin-Lopez E, Meller SJ, Greer CA. Brain Struct Funct. 2018 Dec;223(9):4067-4085. doi: 10.1007/s00429-018-1741-y. Development of piriform cortex interhemispheric connections via the anterior commissure: progressive and regressive strategies.

D.J., Vidyadhara, Lee, J.E., and Chandra, S.S. (2019). Role of the Endolysosomal System in Parkinson's disease. *J. Neurochem.* Invited Review. 150(5):487-506. doi: 10.1111/jnc.14820. PMID: 31287913

Journal publications.

Martin-Lopez E, Meller SJ, Greer CA. Brain Struct Funct. 2018 Dec;223(9):4067-4085. doi: 10.1007/s00429-018-1741-y. Development of piriform cortex interhemispheric connections via the anterior commissure: progressive and regressive strategies.

D.J., Vidyadhara, Lee, J.E., and Chandra, S.S. (2019). Role of the Endolysosomal System in Parkinson's disease. *J. Neurochem.* Invited Review. 150(5):487-506. doi: 10.1111/jnc.14820. PMID: 31287913

Books or other non-periodical, one-time publications. Nothing to Report

Other publications, conference papers, and presentations. Nothing to Report

Website(s) or other Internet site(s): Nothing to Report

Technologies or techniques: Nothing to Report

Inventions, patent applications, and/or licenses: Nothing to Report

Other Products: Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	Sreeganga Chandra
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0001-9035-1733
Nearest person month worked:	.36

Contribution to Project:	Dr. Chandra has obtained IACUC and other regulatory approval. She also has organized the mice breeding for the experiments. She has also supervised the ongoing experiments.
Funding Support:	\$15,390

Name:	Charles Greer
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	.24
Contribution to Project:	Dr. Greer has obtained local IACUC and ACURO approvals. He has also supervised the ongoing experiments.
Funding Support:	4765.53

Name:	Eduardo Martin-Lopez
Project Role:	Post Doc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Martin-Lopez has performed the confocal and electron microscopic experiments.
Funding Support:	\$9016.36

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported

previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

What other organizations were involved as partners?

Nothing to Report

Organization Name:

Location of Organization: *(if foreign location list country)*

Partner's contribution to the project *(identify one or more)*

Financial support;

In-kind support *(e.g., partner makes software, computers, equipment, etc., available to project staff);*

Facilities *(e.g., project staff use the partner's facilities for project activities);*

Collaboration *(e.g., partner's staff work with project staff on the project);*

Personnel exchanges *(e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

Other.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not Applicable

QUAD CHARTS: Attached.

APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***



Development of piriform cortex interhemispheric connections via the anterior commissure: progressive and regressive strategies

Eduardo Martin-Lopez^{1,2} · Sarah J. Meller^{1,2} · Charles A. Greer^{1,2,3}

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Abstract

The anterior commissure (AC) is a phylogenetically conserved inter-hemispheric connection found among vertebrates with bilateral symmetry. The AC connects predominantly olfactory areas but many aspects of its development and structure are unknown. To fill this gap, we investigated the embryonic and postnatal development of the AC by tracing axons with DiI and the piggyback transposon multicolor system. With this strategy, we show that axon growth during establishment of the AC follows a strictly regulated timeline of events that include waiting periods (“regressive strategies”) as well as periods of active axon outgrowth (“progressive strategies”). We also provide evidence that these processes may be regulated in the midline via overexpression of chondroitin sulfate proteoglycans. Additionally, we demonstrate that the ipsi- and contralateral innervation of piriform cortex occurs simultaneously. Morphologically, we found that 20% of axons were myelinated by postnatal day (P) 22, in a process that occurred fundamentally around P14. By immunohistochemistry, we described the presence of glial cells and two new subtypes of neurons: one expressing a calretinin (CR)[−]/MAP2⁺ phenotype, distributed homogeneously inside the AC; and the other expressing a CR⁺/MAP2⁺ phenotype that lies beneath the bed nucleus of the stria terminalis. Our results are consistent with the notion that the AC follows a strictly regulated program during the embryonic and postnatal development similarly to other distal targeting axonal tracts.

Keywords Anterior commissure · Piriform cortex · Axons growth · Embryonic development · Versican · Myelination · PiggyBac

Introduction

The information generated in the telencephalon of bilaterian animals is integrated by connecting the hemispheres through bundles of axons, the brain commissures. In vertebrates several commissures cross the midline along the rostral–caudal axis that connect functionally distinct areas

of the brain, including the pallial and hippocampal (HC) commissures, anterior commissure (AC) and corpus callosum (CC) (Suarez et al. 2014). The AC is the most phylogenetically conserved and the only commissure present in all vertebrates, although the brain areas it connects varies depending on the species. In mice the AC is predominantly paleocortical while in other species (i.e., rats, cats, wallaby and monkeys) it carries neocortical projections mostly from the visual and auditory areas of the ventral temporal lobe (Fitzpatrick and Imig 1980; Gross et al. 1977; Horel and Stelzner 1981; Jouandet 1982; Jouandet and Gazzaniga 1979; Shang et al. 1997). The paleocortical areas projecting through the AC are the olfactory bulb, olfactory cortices (including the anterior olfactory nucleus (AON), piriform cortex (PC) and olfactory tubercle), amygdaloid complex, entorhinal cortex (EC), preoptic area, nuclei of the AC, nuclei of the lateral olfactory tract (nLOT), nuclei of the stria terminalis and nucleus of diagonal band (Brodal 1948; Gurdjian 1925; Haberly and Price 1978a, b; Ramon y Cajal 1901). Fibers from these areas converge in the three main

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00429-018-1741-y>) contains supplementary material, which is available to authorized users.

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tracts: the anterior limb (*pars olfactoria* or *bulbaris*) of the AC (ALAC), the posterior or associational limb (*pars interhemispheric* or *sphenoidal*) of the AC (PLAC), and the portion communicating bilaterally the bed nuclei of the stria terminalis (*pars ad striam terminalem*) (Jouandet and Hartenstein 1983; Lent and Guimaraes 1991; Ramon y Cajal 1901). Within these tracts, bundles of myelinated and unmyelinated axons expressing different neurotransmitters intermingle with neurons and glial cells (Brunjes 2013; Larriva-Sahd et al. 2002; Misaki et al. 2004; Sturrock 1974a, b, 1975, 1977).

Reflecting the complexity of axonal types traveling through it, the embryonic development of the AC is a highly regulated process involving many different families of molecules. Among them are proteoglycans and tenascins (Pires-Neto et al. 1998), ephrins and eph receptor (Henkemeyer et al. 1996; Kudo et al. 2005; Kullander et al. 2001; Robichaux et al. 2016), molecules of the semaphorin-plexin superfamily (Falk et al. 2005; Sahay et al. 2003; Suto et al. 2005), Robo family receptors (Andrews et al. 2006; Calloni et al. 2017), immunoglobulin-like receptors (Abudureyimu et al. 2018), cytoskeleton associated proteins (Deuel et al. 2006; Klingler et al. 2015; Koizumi et al. 2006; Shen et al. 2002), cell adhesion molecules (Lustig et al. 2001; Munakata et al. 2003; Tissir et al. 2005; Zhou et al. 2008), small GTPases (Brouns et al. 2000; Chen et al. 2007; Kassai et al. 2008), FGFs family of growth factors (Tole et al. 2006), Frizzled receptors (Hua et al. 2014; Wang et al. 2002), and autophagy related proteins (Wang et al. 2017; Yamaguchi et al. 2018). Postnatally the development of the AC is simplified to processes of axonal maturation and myelination (Chebat et al. 2006; Collins et al. 2018; Sturrock 1975, 1976).

Although knowledge regarding the molecules involved in the development of the AC is rapidly expanding, less attention has been paid to the dynamics of axonal extension and elongation during AC pathfinding. To date, it seems clear that the onset of AC axogenesis in mice is around embryonic day 13 (E13) (Kallen 1954; Klingler et al. 2015; Silver et al. 1982), although data about how and when they reach and cross the midline (ML) is less clear (Klingler et al. 2015; Schneider et al. 2011; Wahlsten 1981). Since anomalies in the AC development are associated with the appearance of neurological disorders such as schizophrenia, bipolar disorder and autism (Adler et al. 2004; Doderio et al. 2013; Hulshoff Pol et al. 2004; Kikinis et al. 2015; Saxena et al. 2012), precise understanding of mechanisms regulating the establishment of the AC will improve our understanding of disorders of axon pathfinding.

To further our understanding of axon pathfinding we characterized axon elongation during development of the AC, their arborization in the contralateral side, the myelination process at postnatal stages and the phenotype of cells

residing in the AC following maturation. To study embryonic development, we used DiI injections and labeling strategies based on the piggyBac transposon (Martin-Lopez et al. 2017). Interestingly, we found that AC formation follows a sequential process of “pioneer axon” extension which precede the subsequent “follower axons” using both “progressive” (extension and elongation) and “regressive” (pauses and waiting periods) mechanisms. The first appearance of AC axons was at E13.5, simultaneous with the beginning of the PC neuroblast migration through the lateral cortical stream (LCS) (Kallen 1954; Klingler et al. 2015; Silver et al. 1982). For the first time, we suggest a role of chondroitin sulfate proteoglycans (CSPG) in the sorting of AC axons while crossing the ML. Postnatally, we studied the myelination process of the AC using electron microscopy (EM) and staining for the myelin-associated enzyme CNPase. Our CNPase staining showed that myelination of AC progressed rapidly and uniformly after postnatal day 14 (P14), without increasing until P21. The EM analysis revealed that myelination rate of the AC was 20% around weaning day (P22) with an average axon diameter of 0.52 μm . Using specific markers, we characterized two new subpopulations of neurons inside the AC: one expressing the marker MAP2 and distributed throughout the AC, and another expressing both MAP2 and calretinin (CR) that is located exclusively in the ML infiltrating from the bed nucleus of the stria terminalis (BDST). In brief, our observations provide the first evidence of pioneer axons initiating the development of the AC which then proceeds with periods of both progressive axon extension and accompanying periods of quiescence which we characterize as “regression”. Our observations of the timeline of myelination within the AC are largely in agreement with prior reports, while our additional immunohistochemical analyses demonstrate the presence of previously unrecognized populations of neurons, as well as a spatially restricted distribution of CSPGs that may be important determinants of AC boundaries.

Materials and methods

Animals

All experiments were conducted on CD1 embryos and postnatal mice (males and females), obtained from Charles River Laboratories (Wilmington, MA, USA), and housed on a 12-h light cycle with access to standard chow ad libitum at Yale University vivarium.

DiI injections

A stock solution of 1 mg/mL DiI (Molecular Probes) was prepared in dimethylformamide (DMF, Sigma-Aldrich), then

diluted 40:1 in Pluronic F127 (Sigma-Aldrich) and kept in aliquots at -20°C until use. Embryos were obtained from pregnant females euthanized with CO_2 at the embryonic stages of E13, 14, 15, 17 ($N=3$). Tissues were fixed by immersion in 4% PFA-PBS. Postnatal tissues were obtained from pups at the newborn day 0 ($N=3$) and the postnatal day 21 (P21) ($N=3$), euthanized with an overdose of Euthasol (Virbac) before perfusing transcardially with a saline solution followed by 1 mL/g of body weight of ice-cooled 4% PFA-PBS. All tissues were postfixed for 7 days before the DiI injections. The day of injection, borosilicate micropipettes were pulled to obtain tips with an outer diameter of 50 μm and then loaded with the DiI solution. The areas of brain covering both the anterior PC (aPC) and posterior PC (pPC) were injected unilaterally with ~ 50 –300 nL of DiI using a Picospritzer (Parker). Next, all brains were incubated at 37°C in PBS for 1–3 months. Brains were subsequently cryoprotected in 30% sucrose-PBS, embedded in OCT compound (Fisher Scientific), and sectioned in a Reichert Frigocut (E-2800) cryostat to collect 50 μm -thick sections in the horizontal plane (Schambra et al. 1992). Immediately before imaging, sections were rehydrated with PBS and slides mounted with Mowiol 4-88 (Sigma-Aldrich).

In utero injections and electroporations (IUE)

We labeled the PC progenitor cells using the piggyBac transposon system expressing either one single or multiple fluorophores as we previously described (Martin-Lopez et al. 2017). Briefly, pregnant females at gestational day 11 (E11) were deeply anesthetized with 100 mg/kg ketamine (Ketaset, Pfizer) + 10 mg/kg xylazine (AnaSed, Lloyd), an incision was made in the midline of the abdominal wall through the alba line, and embryos were pulled out from the abdomen for IUE. Two sets of animals were injected with 0.2–0.5 μL of plasmids solution at 1 mg/mL: (1) one set was injected with a mix of three plasmids expressing the fluorophores EGFP (pPB-CAG-EGFP), tdTomato (pPB-CAG-tdT) and iRFP670 (pPB-CAG-iRFP670), together with the transposase-expressing plasmid (pCAG-PBase), and used to study the growing and formation of AC (E13–P0); (2) another set was injected with only pPB-CAG-tdT + pCAG-PBase and used for the analysis of ipsi- and contralateral PC projections (E17, P0, P1, P3 and P7) and colocalization with CSPG (E16). Plasmids were injected into the cerebral ventricle of embryos using a Picospritzer, targeting the lateral ganglionic eminence from which PC projection neurons originate. Electroporations were performed by delivering five pulses of 35 V using a pair of gold tweezers (Genepaddles-542, Harvard Apparatus) connected to an ECM 830 electroporator (BTX Harvard Apparatus). Following electroporation embryos were rehydrated with pre-warmed saline solution (Hospira) and introduced back into the abdominal cavity.

The peritoneal membrane and skin were closed using 5/0 PGA absorbable (AD Surgical) and a 5/0 silk braided (Ethicon) sutures, respectively. Postsurgical care was applied for five consecutive days by close monitoring of animals and by injecting 4 mg/kg of the analgesic Meloxicam (Eloxject, Henry Schein).

Tissues that received IUE were studied at embryonic and postnatal stages to track axonal navigation. Embryonic tissues were collected at consecutive stages of E13–E18 ($N=4$ /each) and fixed by immersion in 4% PFA-PBS (see above), whereas postnatal tissues were obtained from P0–P3 ($N=2$), P7 ($N=2$) and P21 ($N=3$) pups. Postnatal tissues were fixed by transcardial perfusion with saline solution followed by 4% PFA-PBS at 1 mL/g of body weight (see above). Both embryonic and postnatal brains were postfixed overnight at 4°C and then cryoprotected in 30%-sucrose-PBS prior to sectioning in a cryostat (Reichert Frigocut). Animals electroporated with the multicolor method were sectioned at 50 μm in the horizontal plane (Schambra et al. 1992), while animals electroporated exclusively with a plasmid expressing just one fluorophore were sectioned at 20 μm the horizontal plane. Sections from multicolor electroporations were rehydrated with PBS and mounted with Mowiol 4-88 before imaging, whereas one fluorophore-expressing tissues were kept at -80°C until use for immunostaining.

Processing of non-electroporated tissues

A group of non-electroporated animals were used for the immunohistochemistry analysis. Tissues from embryos of E12–E18 stages ($N=3$ /each) and postnatal pups of P7 and P21 ($N=3$ /each) were collected and processed equally to the electroporated animals (see above), with the exception that P21 animals were sectioned at 20 μm in the coronal plane.

Electron microscopy

Animals at postnatal day 22 (P22) were euthanized with an overdose of Euthasol (Virbac) and immediately perfused transcardially with 1 mL/g of body weight of saline solution followed by ice-cooled 4% paraformaldehyde (PFA, JT Baker) and 0.2% glutaraldehyde (Electron Microscopy Sciences) in phosphate-buffered saline (PBS). Brains were postfixed overnight at 4°C , and then 50 μm coronal sections were collected with a vibratome (Pelco 101). Lipids were fixed by treating sections with 4% osmium (Electron Microscopy Sciences) for 1 h, dehydrated through graded alcohols, and treated with uranyl acetate (Electron Microscopy Sciences) and lead citrate (Electron Microscopy Sciences) to increase the contrast of tissue ultrastructure. Finally, sections were polymerized in EPON 812 (Electron Microscopy Sciences) and the AC was microdissected for sectioning at 0.07 μm with an ultramicrotome (UltraCut-E). Sections were

mounted on formvar-coated slot grids (Electron Microscopy Sciences) before imaging.

Immunohistochemistry

Immunohistochemically characterization of the AC was made in the following groups: postnatal tissues at P21; embryonic tissues from E12 to E18; and embryonic tissues at E16 from animals electroporated only with the tdTomato-expressing plasmid to detect CSPG. Sections were thawed in a slide warmer at 60 °C, and then rehydrated on PBS. All tissues were treated for antigen unmasking by incubating the slides in 0.01 M citrate buffer [for CNPase staining, this buffer was supplemented with 0.05% Tween 20 (Sigma-Aldrich)], pH 6.0 for 35 min at 80 °C. After cooling down to room temperature (RT), sections were washed with PBS and, exclusively on sections to be stained with the versican antibody, tissues were digested with 0.1 U/mL of protease-free chondroitinase ABC (from *Proteus vulgaris*, Sigma-Aldrich) in 50 mM Tris pH 8.0 (American Bioanalytical), 60 mM sodium acetate (JT Baker) and 0.02% bovine serum albumin (BSA, Sigma-Aldrich), for 2 h at 37 °C. Enzyme was heat inactivated at 70 °C for 5 min., and sections were washed with PBS containing 0.1% Triton X100 (Sigma-Aldrich) (PBST). Nonspecific antibody binding was blocked by incubating the slides with PBST supplemented with 5% normal goat serum (NGS, Accurate Chemicals) + 0.1% bovine serum albumin (BSA, Sigma-Aldrich) at RT for 1 h. Next, sections were incubated with specific primary antibodies (Table 1) overnight in blocking solution at 4 °C, washed three times with PBST, and incubated with specific secondary antibodies (Table 1) diluted in PBST containing 1 µg/mL of DAPI (Invitrogen) and 5 µM DRAQ5 (BD Pharmingen)

for 2 h at RT. Finally, sections were washed with PBST and mounted with Mowiol 4-88.

Imaging and statistical analysis

Ultrastructural micrographs were taken in a JEOL Electron Microscope (JEM-1200 EX II) in the ALAC, at the level at which a small tail of axons from the PLAC were seen. To quantify the percentage of myelinated axons, the area of the AC was subdivided in six symmetric fields (Fig. 7a), and random images were taken from each of these fields at 10,000× primary magnification. Between 2000 and 3000 axons were counted per animal ($N=3$) for statistical analysis. To calculate the diameter of axons, we measured the area of at least 40 axons on each of the random images taken per animal, and the diameters were calculated on the assumption of an axon being circular in transverse section. The number and diameters of myelinated vs. unmyelinated axons were compared using a Student's *t* test (Statistica 7.0).

To automatically quantify CNPase staining, we first isolated the AC on the ML region on a 16-bit maximum projection of a 20 µm image with a freehand drawing tool. The area of this drawn ROI was measured to later determine the density of stained objects. To reduce the noise in the image and correct for uneven illumination, a difference of Gaussians was applied by subtracting a Gaussian Blur of the image with sigma 25 from a Gaussian Blur of the image with sigma 1. To then segment the CNPase stained pixels from the rest of the image, we used the Fiji automatic Otsu threshold function; Otsu's method identifies a threshold pixel value that minimizes the interclass pixel intensity variance between the two-pixel classes ('on' or 'off') within the image and created a binary image by replacing all values above this globally

Table 1 Primary and secondary antibodies

Antigen	Primary AB	Source (catalog no.)	Dilution	Secondary Ab	Source	Dilution
Brevican	Mouse IgG1	BD Biosciences (610894)	1:200	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
Calretinin	Mouse IgG1	EMD Millipore (MAB1568)	1:400	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
CNPase	Mouse IgG1	Biolegend (836404)	1:1000	Goat anti-Ms IgG1 Alexa 488	Invitrogen	1:1000
CS56	Mouse IgM	Sigma-Aldrich (C8035)	1:200	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000
Doublecortin	Rabbit Poly	Cell Signaling (4604)	1:500	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000
GAD65/67	Mouse IgG2a	EMD Millipore (MAB5406)	1:500	Goat anti-Ms IgG2a Alexa546	Invitrogen	1:1000
GFAP	Rabbit Poly	Dako (Z0334)	1:500	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
Iba1	Rabbit IgG	Wako (016-2000001)	1:200	Goat anti-Rb IgG Alexa 546	Invitrogen	1:1000
Map2	Chicken IgG	EMD Millipore (AB5543)	1:500	Goat anti-Chk IgG Alexa 568	Invitrogen	1:1000
NeuN	Mouse IgG1	EMD Millipore (MAB377)	1:500	Goat anti-Ms IgG1 Alexa 546	Invitrogen	1:1000
Neurocan	Mouse IgG	Chemicon (MAB5212)	1:200	Goat anti-Ms IgG Alexa 546	Invitrogen	1:1000
PSA-NCAM	Mouse IgM	EMD Millipore (MAB5324)	1:250	Goat anti-Ms IgM Alexa 555	Invitrogen	1:1000
Reelin	Mouse IgG	EMD Millipore (AB78540)	1:500	Goat anti-Ms IgG Alexa 546	Invitrogen	1:1000
S100β	Mouse IgG1	Sigma-Aldrich (S2532)	1:100	Goat anti-Ms IgG1 Alexa 546	Invitrogen	1:1000
Versican	Rabbit Poly	EMD-Millipore (AB1033)	1:250	Goat anti-Rb IgG Alexa 488	Invitrogen	1:1000

determined threshold with 1 s and setting all other values to 0 s. After making a mask of this image, quantification of the area of CNPase-stained pixels was completed using the Fiji Analyze Particles function.

Quantification of DAPI-stained nuclei was performed as above but with additional steps using Fiji (ImageJ) software with the MorphoLibJ integrated library and plugin (Legland et al. 2016). Following segmentation with an Otsu threshold, we applied a closing morphological filter of a 0.8-pixel radius disk to close the staining of nuclei. Finally, the stained nuclei were isolated by applying a gray scale attribute opening filter of area minimum of 150 pixels and connectivity of 8 before quantifying nuclei number with the Analyze Particles function. Comparisons of stained CNPase area fractions and number of DAPI nuclei between P0 and P21 postnatal ages was made by applying a one-way ANOVA followed by a post-hoc Tukey test (Statistica 7.0).

For DiI staining, sections were imaged using an Olympus BX51 epifluorescence microscope coupled to a digital camera Olympus HKH027241. Immunostained sections and tissue from electroporated animals were imaged using a Leica TCS-SL confocal equipped with the following laser lines: 488 nm used to capture EGFP and Alexa 488 signals; 543 nm used to capture tdTomato and Alexa 546 signals; and 633 nm used to detect iRFP670 and Draq5 signals (both emitting in far-red but shown in blue). Since AC axons elongated from neurons migrate ventrally from the LGE during

the development, we manually photomerged images taken in different consecutive sections, along the dorsal–ventral axis, to illustrate the embryonic development in both the ipsi- and contralateral sides (Fig. 2).

Results

Developmental dynamics of the anterior commissure studied with DiI and IUE

Our initial approach was injecting DiI along the anterior–posterior axis of PC at different embryonic and postnatal stages to study the development of the AC (Fig. 1). At E13 the dye was covering the extension of the PC but no axon was distinguishable (Fig. 1a, a'). The first commissural growth cones were observed at E14 forming small bundles of pioneer axons extending from the PC and approaching the ML (Fig. 1b, b', arrowhead); they reached the ML at E15 (Fig. 1c, c', arrowhead). The first evidence of AC axons crossing the ML was observed at E17 (Fig. 1d), forming a delineated and engrossed bundle of axons at the ML at P0 (Fig. 1e). At P21, the AC displayed a mature morphology with the presence of both ALAC and PLAC (Fig. 1f). Although we could trace a rough map of AC axons growth development (Fig. 1g), the contralateral innervation and a clear visualization of axonal navigation through the brain

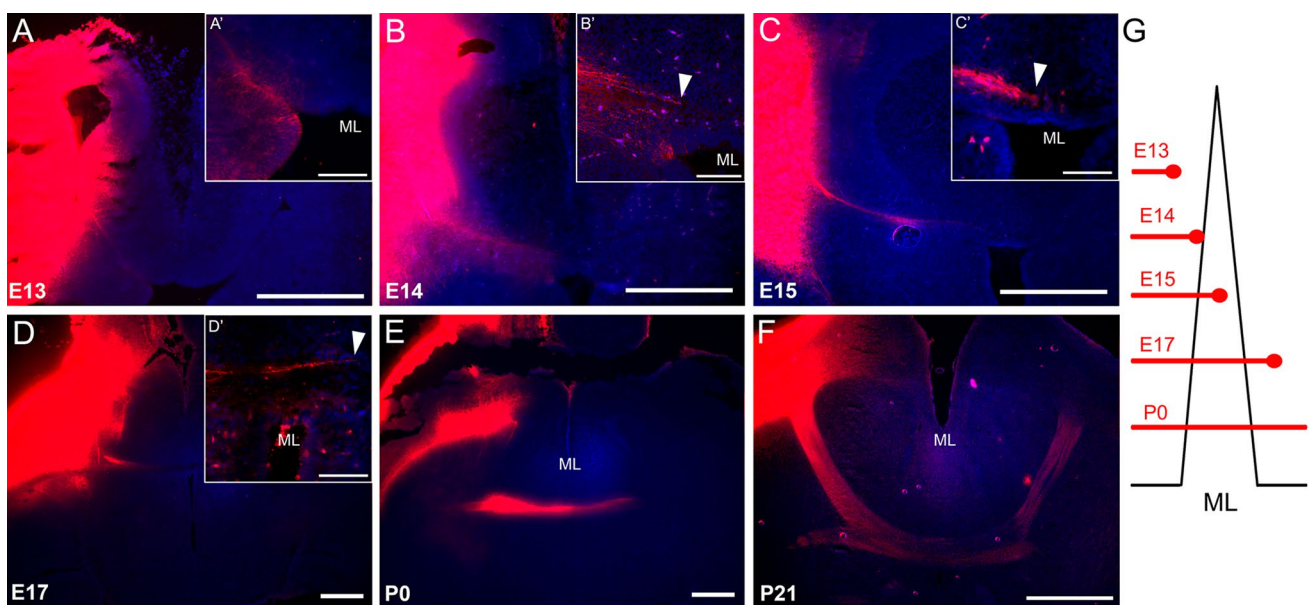


Fig. 1 Development of the anterior commissure. DiI was injected into fixed brains along the anterior–posterior axis of PC to label both anterior (ALAC) and posterior (PLAC) limbs. Images are shown in the horizontal plane as described in the “atlas of the prenatal mouse brain” (Schambra et al. 1992). **a** Section at E13 showing the first growth cones. **b** Section at E14 showing pioneer axons approaching

the ML (arrowhead in b'). **c** First evidence of AC axons crossing the ML at E15. **d** AC axons crossing the ML at E16 and general thickening of the tract. **e** Thickness of AC is seen equally in both sides of the ML at P0. **f** Aspect of a mature AC at P21 showing both the ALAC and PLAC. Nuclei counterstained with DAPI (blue). Scale bars = 1 mm in f; 500 μ m in a–e; 100 μ m in insets (a'–d')

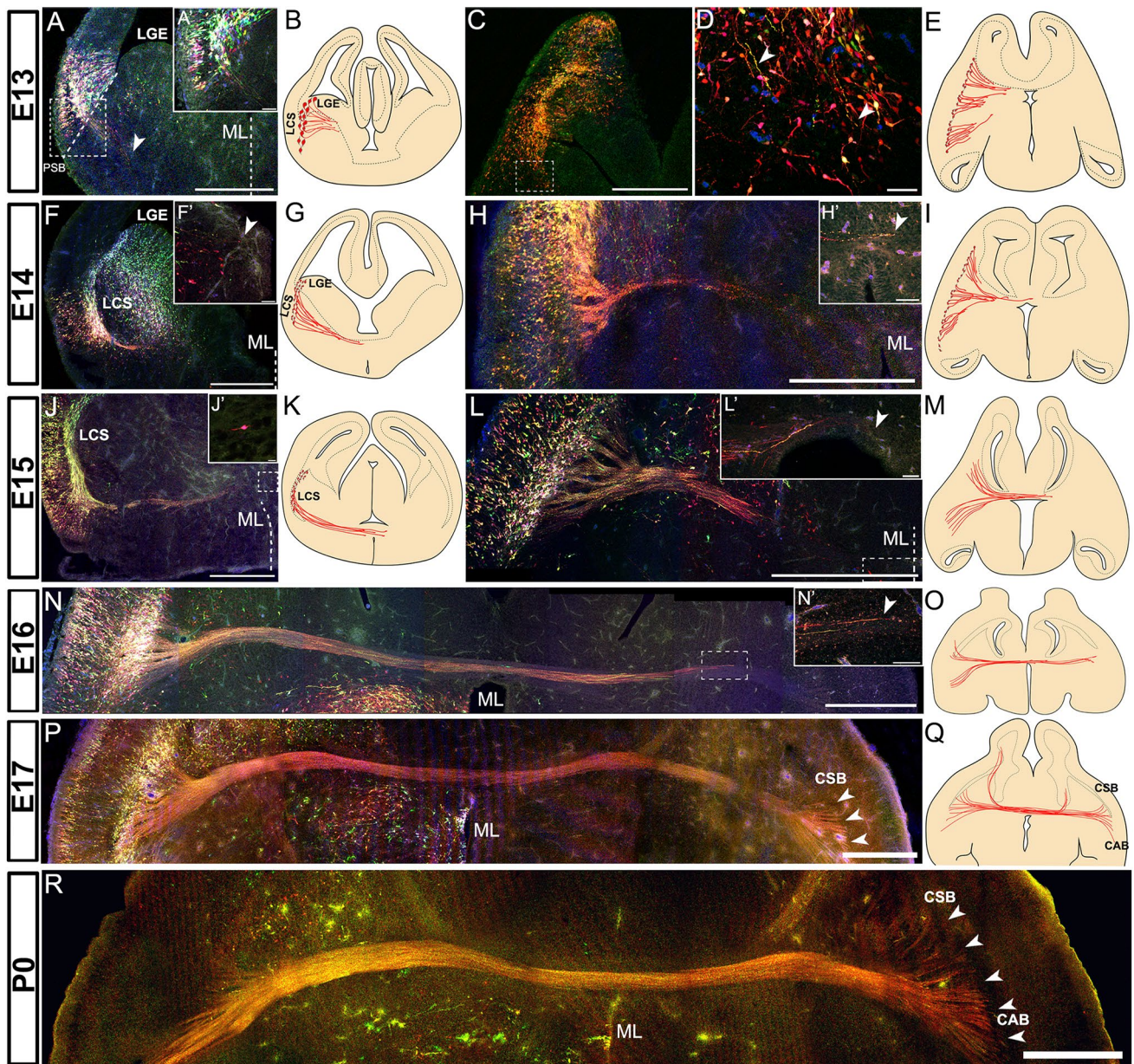


Fig. 2 Timeline of anterior commissure development as demonstrated by labeling piriform cortex precursor cells with the piggyBac transposon system (Martin-Lopez et al. 2017). **a, b** Coronal section and diagram at E13 illustrating the first commissural axons (“pioneer axons”) extending from “PC commissure pioneer neurons”. AC axons elongate while PC precursor cells migrate ventrally through the LCS. **c–e** Horizontal sections and diagram showing pioneer axons navigating towards the ML at E13. **f, g** Coronal section and diagram at E14 illustrating the AC pioneer axons approaching the ML and PC neuroblasts migrating through the LCS. **h, i** Horizontal section and diagram showing the first AC pioneer axons reaching the ML at E14 (arrowhead in **h'**). **j, k** Horizontal section and diagram at E15 showing the thickening of the AC in the ipsilateral parenchyma. Followed axons coalesce upon the pioneer to form a thicker AC bundle. **l, m** Horizontal section and diagram illustrating pioneer axons crossing

but staying near the ML at E15 (arrowhead). Pioneer axons around the ML represent the first “regressive event” during the AC development. **n, o** Horizontal section and diagram of the AC at E16 showing the axonal fasciculation to form a tract elongating through the contralateral parenchyma. No evidence of pioneer axons is observed arriving to the contralateral PC (arrowhead). **p, q** Horizontal section and diagram at E17 showing the AC axons reaching the contralateral inner border of the PC formed by the CAB/CSB. Axons defasciculate and spread out along the anterior–posterior axis (arrowheads) at E17. **r** Horizontal section at P0 showing a similar morphology observed at E17, with no axons innervating the contralateral PC. E17–P0 represent the second “regressive event” of the AC development. CAB cortico-amygdaloid border, CSB cortico-striatal border, LCS lateral corticostriatal stream, ML midline. Scale bars = 500 μ m in **a, c, e, f, h, j, l, n, p** and **r**; 50 μ m in **a', d, f', h', l', n'**; 5 μ m in **j'**

parenchyma was not possible due to technical issues DiI diffusion through axons.

To properly track AC axon navigation and improve the visualization of growth cones, we expressed different fluorescent reporter proteins in PC neuroblasts of the dorsal LGE at E11, which is the area from which the PC projection neurons originate and migrate ventrally through the lateral cortical stream (LCS) (Garcia-Moreno et al. 2008; Huilgol and Tole 2016; Martin-Lopez et al. 2017), using a multicolor piggyBac-transposon system (Martin-Lopez et al. 2017). This method was shown to specifically label AC axon outgrowth and decussation during embryonic development (E13–17), as well as their projections and arborizations to the contralateral PC at postnatal stages (Figs. 2, 3).

In contrast with the observed after DiI injections, we first showed that PC neuroblasts begin extending commissural axons at E13, while these cells were still proximal to the LGE (Fig. 2a–e). These cells continue migrating ventrally through the LCS until E15, where they were no longer visible next to the LGE (Fig. 2j, k). However, after E15 many other PC neuroblasts were still being produced in the LGE and migrating through the LCS (Martin-Lopez et al. 2017), suggesting that commissural PC neurons are produced at restricted times during the development. Similar behavior has been reported for other commissural tracts such as the CC (Lent et al. 1990). At E13 the AC axons were defasciculated and they seemed to establish the pathway for later axons, and therefore, we identified them as the group of “AC pioneer axons” and the neuroblasts extending them as “AC pioneer neurons” (Pires-Neto and Lent 1991; Silver et al. 1982). As described, the elongation of AC axons occurred while the AC pioneer neurons continued migrating to areas nearby the prospective PC (Fig. 2a, b, f, g, j, k); we concluded that the emission and extension of AC axons is a dynamic and intrinsic process occurring independently of the PC neuronal activity. Although the onset of AC axons at E13 supported previous descriptions in mice (Kallen 1954; Klingler et al. 2015; Wahlsten 1981), we did not find any approaching or crossing the ML as previously reported (Schneider et al. 2011).

AC pioneer axons continued to elongate towards the ML at E14 and some had reached the ML region, but without crossing (Fig. 2f–i, arrowheads). Interestingly, we observed that commissural axons initially clustered in small fascicles prior to converging into a primary fascicle that is the nascent AC (Fig. 2h). Up to this point, the AC pioneer axons adopted a “progressive strategy” of growth based on a process of extension and elongation (Lent and Guimaraes 1990, 1991). This strategy was also observed at E15 by the arrival of more axons (“follower axons”) following the path established by pioneer axons towards the ML (Fig. 2j, k), producing a thickening of the AC. However, all pioneer axons arriving to the ML paused their growth prior to invading the contralateral

parenchyma (Fig. 2l, m), and remained proximal to the ML in the first evidence of a “regressive strategy” of growing not previously reported for the AC.

By E16, commissural axons restarted a progressive strategy, fasciculated into a single tract and massively crossed the ML to the contralateral parenchyma (Fig. 2n, o). The pioneer axons were still visible and continued to lead the navigation process (Fig. 2n', arrowhead). One day later, at E17, AC axons reached the internal areas of the contralateral olfactory cortex, where they were positioned in the deeper mass of cells comprising the cortical plate along the cortico-striatal border (CSB) in the anterior regions, or the cortico-amygdaloid border (CAB) in the posterior regions (Fig. 2p, q). At this location, axons began to defasciculate and spread out along the anterior–posterior axis to cover the extension of the contralateral PC (Fig. 2p, q, arrowheads). At this age, we recognized a second “regressive event” where AC axons stopped their growth and remained stationary in the CSB/CAB without invading the contralateral PC until P1 (Fig. 2r, arrowheads). This second regressive event resembled similar events that occur during the development of other systems containing distal targeting neurons such as the CC (Lent et al. 1990; Wise and Jones 1976), the commissure of the superior colliculus (Chebat et al. 2006) or the OB (Treloar et al. 1999). At P0, AC axons continued to spread out along the anterior–posterior axis without invading the layers of the contralateral PC (Fig. 2r). Our data contrast with the previously reported in hamsters, in which the AC followed exclusively progressive strategies (Lent and Guimaraes 1990, 1991).

Ipsi- and contralateral arborization of anterior commissure axons

We next investigated the AC axonal innervation and arborization of contralateral PC following the second waiting period established at E17–P1. Our analysis focused on the following events: (1) the ipsilateral innervation of aPC from the pPC; (2) the contralateral innervation of PC from AC axons; (3) innervation of the contralateral olfactory bulb (OB) from the aPC.

To evaluate whether the ipsi- and contralateral cortico-cortical innervation of PC were established simultaneously, we first addressed innervation of the aPC from neurons labeled in the ipsilateral pPC. During the second regressive event at E17–P0, there was an absence of parallel axons along the layers of the aPC, indicating that axons from the pPC had not yet reached the anterior part (Fig. 3a, b) and confirming that cortico-cortical innervation in both hemispheres might occur simultaneously. Later at P1–P3, numerous centrifugal projections were seen arriving in the aPC and running parallel to the surface of layers I and III, and some crossing layer II of the aPC (Fig. 3c, d). Finally, at P7

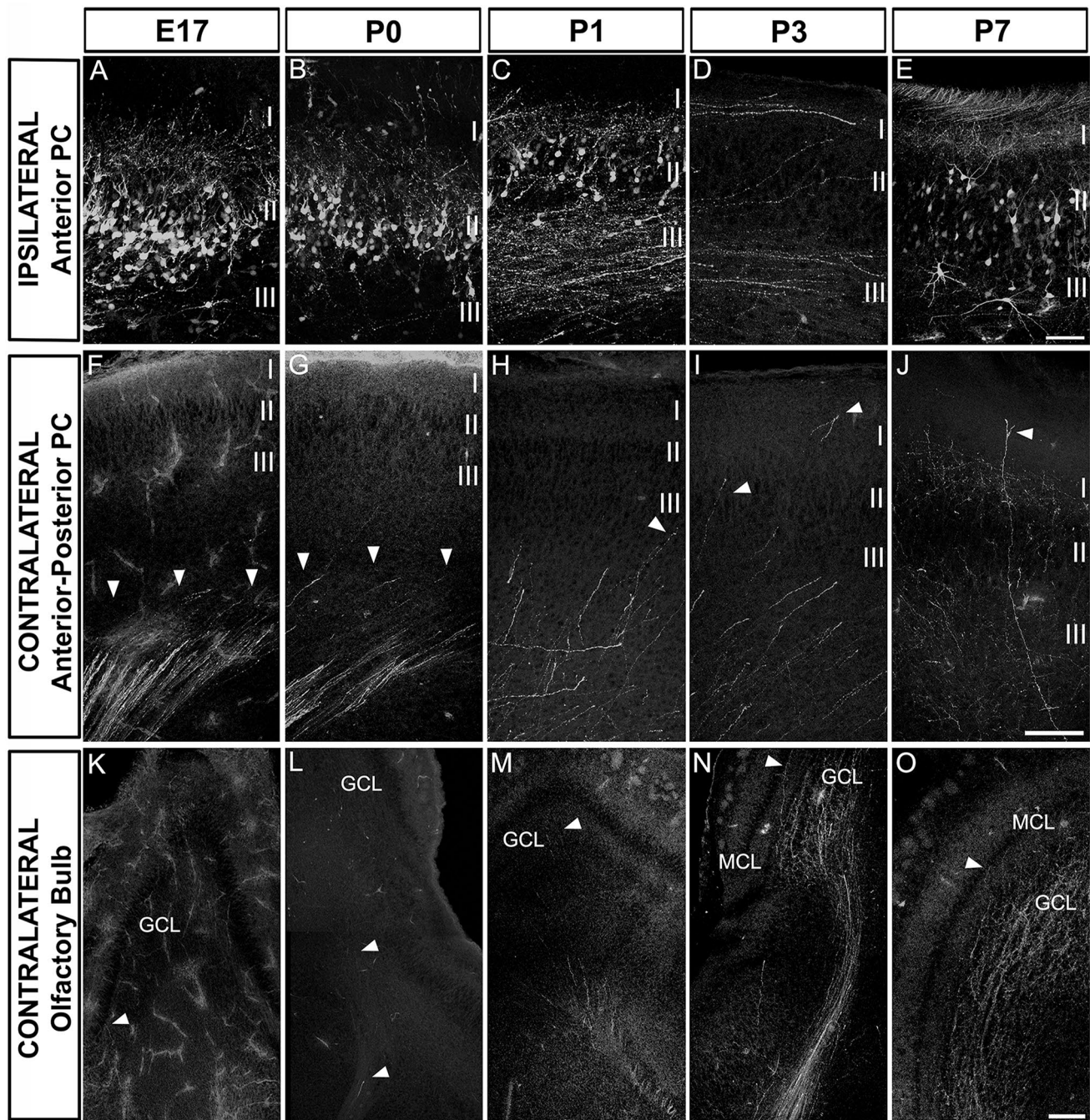


Fig. 3 Ipsi- and contralateral cortico-cortical innervation of piriform cortex during embryonic and neonatal development. Animals received unilateral IUE with tdTomato-expressing piggyBac transposon at E11. **a–e** Ipsilateral cortico-cortical projections of aPC with axons from the pPC. **a, b** No axons are seen approaching the aPC coming from the pPC. **c–e** At P1–P7 we observe the first evidence of ipsilateral innervation in aPC, represented by the arrival of parallel fibers crossing layer II and invading layers I and III. **f–j** Contralateral innervation of PC. AC axons approach to the CSB/CAB at E17, where they stall (regressive event) without innervating the PC (**f, g**, arrowheads). At P1, some axons begin to innervate the contralateral layer III of PC (**h**, arrowhead) and slowly arborize until P3 (**i**). At P7, the arborization is more elaborated and complex in layers I and III

(**j**), with some axons crossing layer II. Some growth cones are still visible at P7 suggesting that maturation continue (**j**, arrowhead). **k–o** Contralateral innervation of the OB from ALAC. Growth cones reach the OB peduncle at E17–P0 (**k, l**, arrowheads). From P1, axons penetrate in the GCL (**m**, arrowhead), to arborize in the GCL and MCL at P3–P7 (**n, o**, arrowheads). In all images, layer II of the PC is defined as the region of highly packed cell bodies shown by darker spherical nuclei. Layer I and III are the areas above and below layer II, respectively. ALAC anterior limb of the anterior commissure, aPC anterior piriform cortex, CAB/CSB cortico-amygdaloid border/cortico-striatal border, GCL granule cell layer, MCL mitral cell layer, OB olfactory bulb, pPC posterior piriform cortex. Scale bars=100 μ m in **f–o**; 50 μ m in **a–e**

we observed a more elaborated innervation of aPC predominantly in layers I and III (Fig. 3e).

Simultaneously, in the contralateral PC the AC growth cones that remained beneath the CSB/CAB during the second regressive event (Fig. 3f, g, arrowheads), initiated the contralateral innervation of PC from the deep layer III at P1 (Fig. 3h, arrowhead). At P3, more axons arrived in layer III and a few of them penetrated and crossed layer II to reach layer I (Fig. 3i). Finally, commissural axons arborize densely the contralateral PC at P7 (Fig. 3j), arborizing predominantly layers I and III. Since layer Ib has been reported to receive associational fibers from PC (Haberly and Price 1978a), we presumed that fibers observed in layer II were in transit to layer I. Note that many growth cones were still visible at P7 predominantly in layers I and III of PC (Fig. 3j, arrowhead), indicating the arrival of axons and suggesting that the maturation of connections continued.

Additionally, we also assessed the contralateral innervation of the OB of axons coming from the aPC traveling through the ALAC. The first growth cones arrived in the OB peduncle at E17–P0 (Fig. 3k, l, arrowheads) to reach the inner granule cell layer (GCL) of the OB at P1; a few axons were seen innervating the superficial GCL (Fig. 3m, arrowhead). From P3 to P7, the contralateral innervation spread out in the OB and predominantly within the GCL, although some axons appeared to arborize in the mitral cell layer (MCL) (Fig. 3n, o, arrowheads).

In summary, we established a precise timeline of the mouse AC development that occurs as follows (Fig. 4): AC axon outgrowth began at E13, with pioneer axons reaching the ML using a progressive strategy at E14. At this time pioneer axons paused outgrowth and adopted a regressive strategy, while the AC follower axons, which began outgrowing at E14, continued growing towards the ML until E15; ultimately both pioneer and follower axons fasciculated and extended through the contralateral parenchyma at E16–17 in a progressive manner. From E17 to P1, AC axons

reached the internal border of the contralateral PC and once again adopted a regressive strategy (or a second pause), subsequently invading the contralateral layers of PC. Finally, commissural axons innervated and arborized the contralateral PC during the first postnatal week of the development in the third progressive growth event.

Influence of chondroitin sulfate proteoglycans in sorting the anterior commissure axons

Although many different families of molecules have been reported as acting as chemoattractant or chemorepellent signals inside the extracellular matrix (ECM) during the axonal guidance process (Dickson 2002; Kaprielian et al. 2000; Lent et al. 2005), the full orchestra of molecules has not yet been fully delineated. We describe here for the first time a distribution of chondroitin sulfate proteoglycans (CSPG) relative to the AC and propose them as candidates to influence the development of the AC. The proteoglycans (PGs) are an abundant family of molecules in the embryonic ECM (Iozzo 1998; Maeda 2015; Maeda et al. 2011; Margolis and Margolis 1997), and may be involved in the formation of brain commissures (Conway et al. 2011; Feraud-Espinosa et al. 1996; Inatani et al. 2003; Klambt and Goodman 1991; Pires-Neto et al. 1998). Here, we used the monoclonal antibody CS56 to recognize both chondroitin-4-sulfate and chondroitin-6-sulfate, and versican to recognize a specific family of PGs part of the lectican family, in the brain at different embryonic and neonatal stages.

The expression of CS56 was initially detected as sparsely distributed within the brain parenchyma around the ML at E13 (Fig. 5a). When the initial pioneer axons approached the ML at E14, the staining for CS56 formed a CSPG tunnel around the nascent AC, possibly forming a molecular barrier to keep the axons enclosed in a bundle (Fig. 5b). The tunnel of CS56 was more obvious at E15 (Fig. 5c). Interestingly, at E16 expression of CS56 flipped from forming a tunnel to

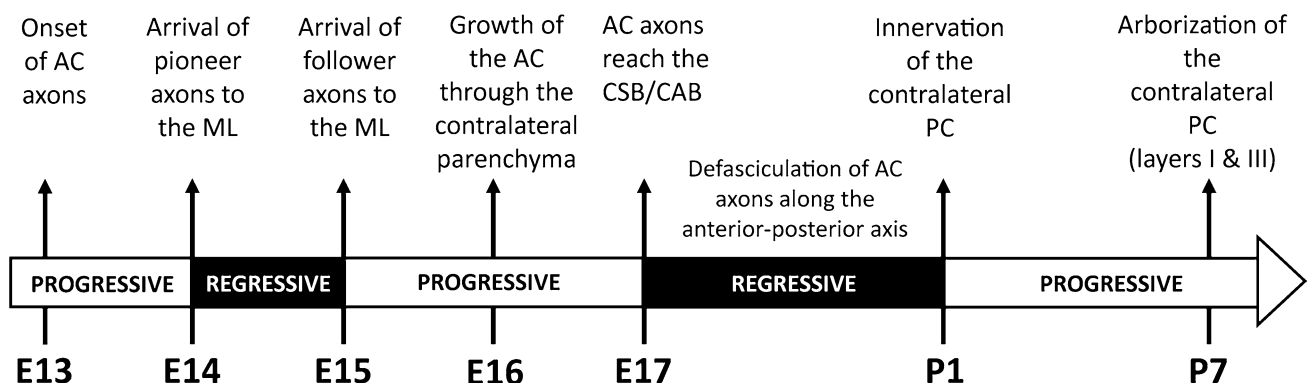


Fig. 4 Diagram illustrating the progressive (growth and elongation) and regressive (pauses and waiting) periods during the timeline of AC embryonic and postnatal development

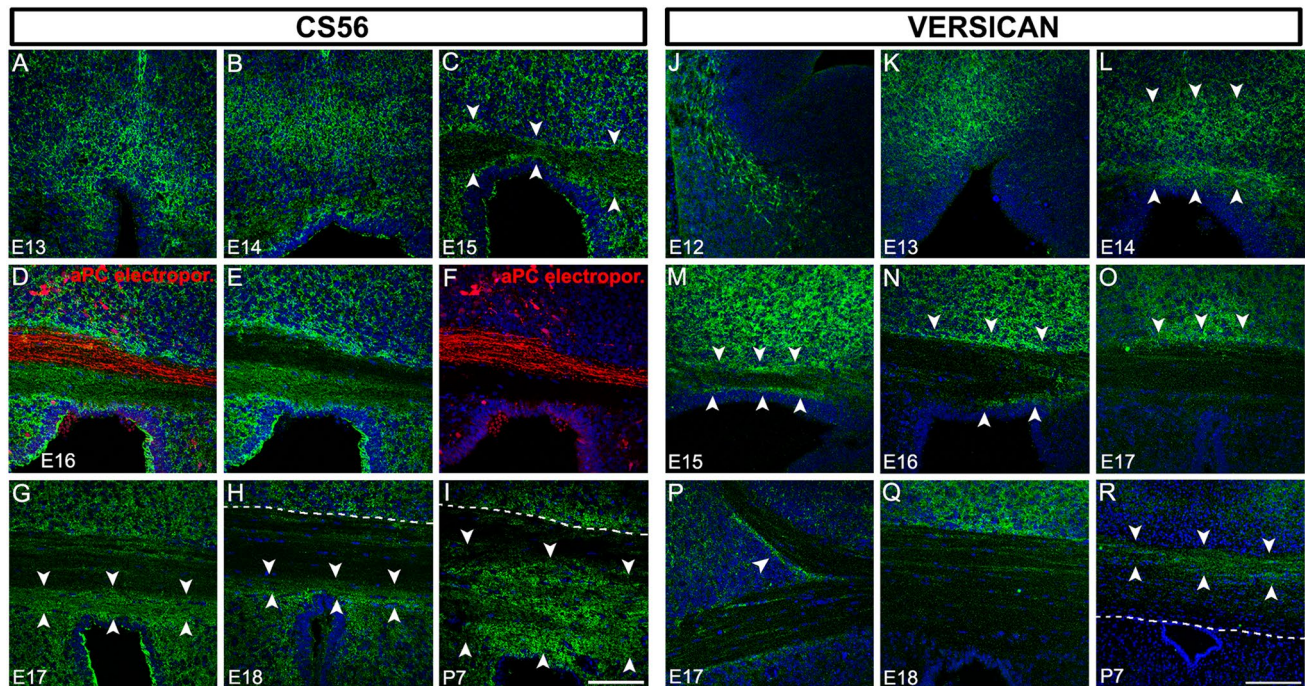


Fig. 5 Expression of chondroitin sulfate proteoglycans during the development of anterior commissure. Images show horizontal planes (Schambra et al. 1992) of the ML region, illustrating the anterior pole (rostral) in the upper part of the images, and the posterior pole (caudal) at the bottom. **a–i** CSPG distribution in the ML during the AC development detected with antibody against CS56. **a** Intense staining is observed in the brain parenchyma around the ML region at E13. **b, c** From E14 to E15, CS56 staining forms a tunnel surrounding the area through which the AC axons navigate (arrowheads). **d–f** At E16, CS56 expression reaches the region from which axons extend from the PLAC. AC axons coming from the aPC that form the ALAC were specifically labeled using regionalized IUE in the anterior LGE (red); these axons are seen navigating exclusively through the area absent of CSPG (green). This CS56 expression pattern is maintained until the end of embryonic (E17–E18) (**g, h**) and postnatal (P7) (**i**) devel-

opment. **j–r** Expression of the versican CSPG around the AC region during embryonic–postnatal development. **j** Expression of versican by neuroblasts migrating from the LGE at E12. **k** Expression of versican in the brain parenchyma around the ML at E13; **l–n** Formation of a versican tunnel around the AC at E14–E16 and absence of expression inside the AC. **o** At E17, versican is only expressed in the rostral part of the AC (arrowheads) and the caudal edge of the ALAC in the area where coalesce with axons from the PLAC (**p**, arrowheads). **q** At E18, versican expression forms longitudinal stripes within the lumen of AC in the ALAC area (**q**). **r** Overexpression of versican in the ALAC region at P7 (arrowheads). Nuclei counterstained with Draq5 (blue). ALAC anterior limb of anterior commissure, CSPG chondroitin sulfate proteoglycans, LGE lateral ganglionic eminence, ML midline, PLAC posterior limb of anterior commissure. Scale bars = 100 μm

being within the lumen of the AC in the caudal half of the AC at the ML, which is the area through which the axons from the PLAC travel (Fig. 5d–f). To confirm that CS56 from E16 onwards might have a role in the pathfinding of AC axons within the ML, we performed IUE to specifically labeling the anterior cells that populated the aPC and extend axons through the ALAC. Indeed, the axons coming from the aPC and traveling through the ALAC avoided the area of CS56 overexpression at the ML (Fig. 5d–f). These data strongly suggested that CSPG could be involved in the sorting of AC axons coming from the anterior and posterior limbs and thus serve as both permissive and repulsive signals. This expression pattern was sustained during embryonic development until postnatal day 7 (Fig. 5g–i).

We next sought to identify at least one type of CSPG detected by the CS56 antibody. Immunohistochemistry against distinct CSPGs (Table 1) showed that only versican

was expressed around the ML of the AC (Fig. 5j–r), while brevican and neurocan were absent (data not shown). Versican is a CSPG expressed during brain development and can function as either an attractive or inhibitory signal for axonal outgrowth (Schmalfeldt et al. 2000; Snyder et al. 2015; Wu et al. 2004). We initially detected versican in the neuroblasts migrating from the LGE at E12 (Fig. 5j) and around the ML at E13, predominantly in anterior areas (Fig. 5k). From E14 to E16, versican formed a tunnel around the AC with no expression detected within the AC (Fig. 5l–m), which is an expression pattern similar to that previously reported during the development of the internal capsule (Popp et al. 2003). The tunnel expression of versican was lost from E17–18, as it was restricted to the brain parenchyma surrounding the anterior side of the AC (Fig. 5o, q). Conversely, in the area where the ALAC and PLAC converge at the ML, versican was overexpressed in the posterior edge of the ALAC and

the anterior edge of the PLAC (Fig. 5p), supporting a role for versican in helping axons maintain a correct trajectory. Interestingly, at P7 versican was also expressed inside the AC at the ML, but was restricted to the ALAC (Fig. 5r). As at P7 AC axons have already crossed the ML, the expression of versican in the lumen of the ALAC suggests a role in the stabilization of ALAC axons.

Postnatal maturation of the AC studied by axonal myelination

We next evaluated the maturation of the AC while it forms a functional axon tract. Here, we focused in analyzing the myelination within the AC from P0 to P21 by detecting the expression of CNPase, an enzyme involved in the maturation of oligodendrocytes during myelination (Raasakka and Kursula 2014). Our data demonstrate that the first oligodendrocytes expressing CNPase were found around the AC at P0 (Fig. 6a) and continue invading the AC at P7 (Fig. 6b). The first evidence of myelination was observed at P14 and continues until P21 (Fig. 6c, d). The fraction of AC covered

by the CNPase staining increased dramatically from P7 to P14 (Fig. 6e), indicating that most of the myelination process occurred near P14; these support the recently reported *rapid myelination period* occurring in the AC between P13 and 15 (Collins et al. 2018). The AC thickness also increased predominantly during the peak of myelination at P14, coinciding with an increase in the number of cells per unit area (Fig. 6f).

Finally, we studied the ultrastructure of myelin in the ALAC at P22 as representative of a mature AC. First, we established the density of AC axons to be 517 axons/100 μm^2 . We then determined that the percentage of myelinated axons was approximately 20% (Fig. 7b, c). This number coincided with the myelination rate measured with EM in older animals at P32–35 (Sturrock 1975, 1976), although the diameter of axons, 0.52 μm for myelinated 0.25 μm for unmyelinated (Fig. 7d) was similar to animals of the same age (Sturrock 1975, 1976). The external architecture of the AC revealed a boundary of parenchyma composed predominantly of unmyelinated neuronal fibers (Fig. 7e), although there was no clear evidence of a

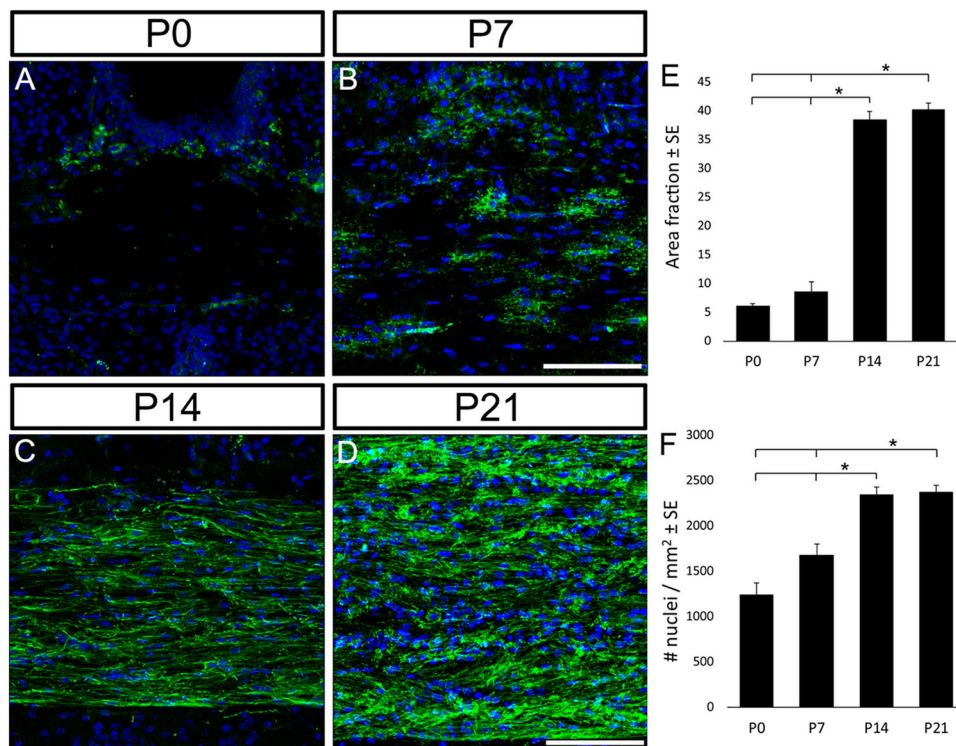


Fig. 6 Temporal dynamics of anterior commissure myelination. **a–d** Expression of myelin-associated enzyme CNPase (green) in the ML of AC at the postnatal stages P0–P21. Nuclei counterstained with Draq5 (blue). The first cells expressing CNPase were observed at P0 (**a**) around the ML. At P7 (**b**) there is an invasion of CNPase cells in the lumen of the AC, but no evidence of myelination was observed. At P14 (**c**) there is an abrupt increase in CNPase staining showing myelinated fibers throughout the lumen of the AC (*rapid myelina-*

tion process) that stabilize until P21 (**d**). These processes are shown graphically after performing quantification of the CNPase stained area (**e**). Maturation of AC showed by an increase of the number of cells (Draq5 nuclei) in the AC during postnatal development. Statistical significance $*p < 0.01$. AC anterior commissure, CNPase 2',3'-cyclic-nucleotide 3'-phosphodiesterase, ML midline. Scale bar = 100 μm

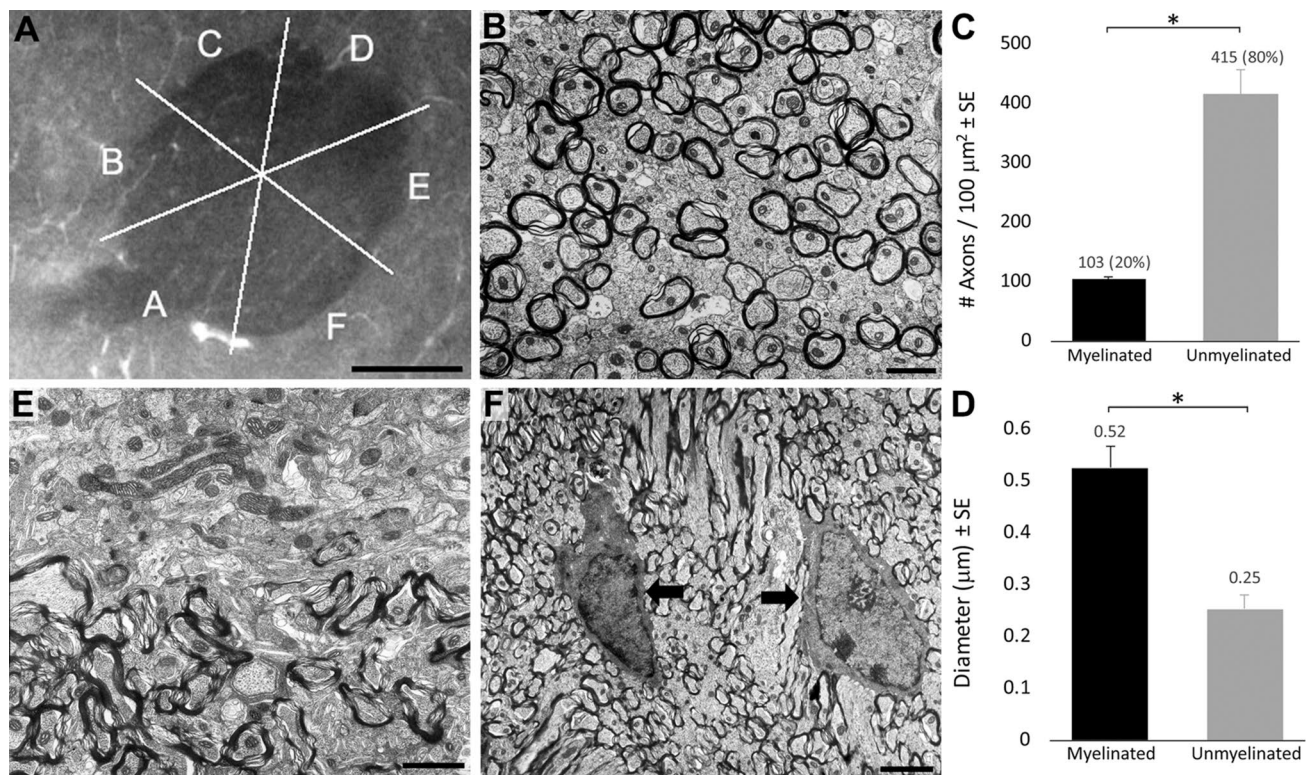


Fig. 7 Characterization of the anterior commissure ultrastructural profiles and myelin at P22. **a** Brightfield image of the ALAC in the coronal plane showing the subdivisions made for myelin quantifications (areas A–F); dark staining is the product of pre-treatment with osmium before processing for electron microscopy (EM). **b** Representative cross-sectional EM image of AC axons showing myelinated axons surrounded by myelin sheaths. **c** Comparison of myelinated vs. unmyelinated axons at P21 in the ALAC represented as no. of

axons/100 μm^2 ($t = -7.62$; $*p < 0.01$). **d** Comparison in the diameter between myelinated and unmyelinated axons ($t = 5.43$; $*p < 0.01$). **e** External boundary between the lumen of the AC occupied by myelinated/unmyelinated axons and the surrounding tissue (absence of myelinated axons). **f** Representative image of two oligodendrocytes found inside the ALAC, characterized by a dark cytoplasm and irregular nuclei with clumped chromatin. Scale bars = 100 μm in **a**; 2 μm in **f**; 1 μm in **b** and **e**

specialized anatomical structure that might restrict the AC axons. We observed the somata of oligodendrocytes with processes engulfing axons distributed throughout the AC (Fig. 7f, arrows). These cells were identified due to their characteristic dark cytoplasm with long cisternae of rough endoplasmic reticulum, and irregular nuclei with clumped chromatin both beneath and within the nucleoplasm (Sturrock 1974a).

Characterization of molecular profiles in the anterior commissure

We further characterized the anatomy of the mature AC by detecting different subpopulations of neurons and glial cells by immunohistochemistry (Table 1). Among the neurons, we found at least five subpopulations of cells, including: (1) some scattered NeuN⁺ neurons (Fig. 8a); (2) a few Reelin (Reln)-expressing neurons exclusively located in the PLAC (Fig. 8b). In contrast to reports in rats, we did not find Reln cells in the ML (Misaki et al. 2004); (3) neurons

expressing the postmitotic neuronal marker MAP2, which represented approximately the 35% of the total and displayed small and fusiform cell bodies (Fig. 8c). These cells distributed homogeneously through the lumen of the AC and were generally aligned to the longest cross-sectional axis of the commissure. Densely packed neuronal processes expressing calretinin (CR) in the nucleus accumbens surrounded the ALAC (Fig. 8c); (4) a group of neurons found beneath the bed nucleus of the stria terminalis, lateral division, posterior part (BSTLP), which expressed a CR⁺/MAP2⁺ phenotype. This phenotype was shared by the BSTLP neurons and, because they displayed a single process pointing ventrally from the BSTLP, it is suggestive of a migratory behavior of cells infiltrating from the BSTLP (Fig. 8d–f). To date, the presence and function of these last two type of cells in the AC is unknown; (5) a sparse group of neurons expressing glutamic acid decarboxylase 65/67 (GAD65/67) and displaying the typical morphology of inhibitory interneurons (Fig. 8g, arrowheads). Interestingly, we found in the core of the ALAC the expression of the immature neural

markers doublecortin (DCX) and polysialylated neural cell adhesion molecule (PSA-NCAM), typical of neuroblasts and suggesting the presence of an immature profile within this region (Fig. 8h, i, dotted lines). The marker DCX was first detected at E14 (data not shown) and increased in expression throughout embryonic development until eventually declining after birth (Supplementary Fig. 1A–C).

The analysis of glial cells showed abundant astroglial lineages with immature cells expressing S100 β and mature cells expressing GFAP (Raponi et al. 2007) in the AC (Fig. 8j, k), as well as subpopulations of microglial cells detected by IBA1 expression (Fig. 8l). Whereas the microglial marker IBA1 was only detected postnatally, the astroglial markers GFAP and S100 β showed progressively increased expression in the AC from E18 to postnatal stages (Supplementary Fig. 1D–F).

Secondary to the discovery of two new neuronal subpopulations not previously reported (CR[−]/MAP2⁺ and CR⁺/MAP2⁺), we investigated their appearance during development. Interestingly, the first evidence of both cell types occurred postnatally at P7, at which time the expression of both cell types increased abruptly to reach maturity 1 week later at P14. No evidence of later maturation stages was found when we analyzed the tissue at P21 (Supplementary Fig. 2).

Discussion

The AC represents the most important tract to establish interhemispheric connections in the paleocortex and predominantly connects olfactory areas of the brain (Brodal 1948; Haberly and Price 1978a, b; Ramon y Cajal 1901). Because of the variety of areas connected via the AC, this tract is formed by a complex mixture of myelinated and unmyelinated axons of diverse origin (Brunjes 2013; Collins et al. 2018; Sturrock 1975), that follow a strictly regulated program during the development. To augment understanding of AC development, in this work, we describe a detailed program of progressive and regressive strategies utilized by pioneer and follower axons during the elongation process from the PC. We also found that some families of CSPG may be involved in regulating the sorting of axons coming from the anterior and posterior limbs of AC in the ML. Postnatally, we show that AC myelination occurs predominately after the second week of life in a rapid myelination period, consistent with recently reported data (Collins et al. 2018), to finally reach a 20% level of myelination at P22. Additionally, we report two new subpopulations of neurons detected by CR and MAP2, one homogeneously distributed throughout the lumen of the commissure and displaying small-enlarged cytoplasm (CR[−]/MAP2⁺), and the other infiltrating from the BSTLP (CR⁺/MAP2⁺) with unknown functions.

During the last two decades there has been increasing evidence that failures in the development of the AC may mediate serious neurologic disorders (Adler et al. 2004; Dodero et al. 2013; Hulshoff Pol et al. 2004; Kikinis et al. 2015; Saxena et al. 2012). Despite this evidence, the knowledge we have regarding the mechanisms of AC development are still very limited. Accordingly, we performed a thorough and detailed analysis of AC axonal wiring during the embryonic and postnatal development by applying both classical (DiI injections) and more innovative (piggyBac transposon system) techniques. The quality of DiI labeling to track developmental axons has been previously reported to poorly discriminate individual fibers (Lent and Guimaraes 1991). In our work, we employed DiI injections to roughly approach the AC development but could not distinguish the precise sequence of events for axonal extension and elongation we sought using only DiI. To effectively illustrate axon outgrowth in the AC we thus used a piggyBac technique to label progenitor cells, which we and others have previously employed to study cell lineages to good effect (Figueres-Onate et al. 2016; Garcia-Marques and Lopez-Mascaraque 2013; Garcia-Moreno et al. 2014; Martin-Lopez et al. 2013, 2017). We were thereby able to precisely track axon growth through the AC and could describe the “progressive” (growth) and “regressive” (pauses, waiting periods and fiber pruning) strategies observed in other brain tracts, including the CC, the commissure of the superior colliculus or the afferent innervation of the OB (Chebat et al. 2006; Lent et al. 1990; Treloar et al. 1999; Wise and Jones 1976). However, our current results contradict what was previously reported in hamsters, whose AC develop using only progressive events (Lent and Guimaraes 1990, 1991). Our interpretation of these discrepancies is based on the method used to label the AC axons using DiI, as they may not be effective in revealing the regressive events during development of axonal tracts. Therefore, we demonstrate that the piggyBac-transposon multicolor method is a powerful technique for tracking axons during the development of the AC pathfinding.

Consequently, we could establish a detailed timeline of developmental events in the AC of mouse (Fig. 4). These events include the arrival of the first axons in the ML around E14, 1 day later than the previously reported at E13.5 (Kallen 1954; Silver et al. 1982), although we agree that ML is initially crossed at E14.5 (Wahlsten 1981). However, a more recent work reported AC axons reaching and even crossing the ML at E13.5 (Schneider et al. 2011). Discrepancies could be due to differences in the mouse strain or targeting of distinct neurons subpopulations, such as subplate neurons projecting through the internal capsule showing that behavior (De Carlos and O’Leary 1992). In our opinion, and considering that the first subpopulations of PC cells start migrating through the LCS at E11 (Pax6 cells from pallial

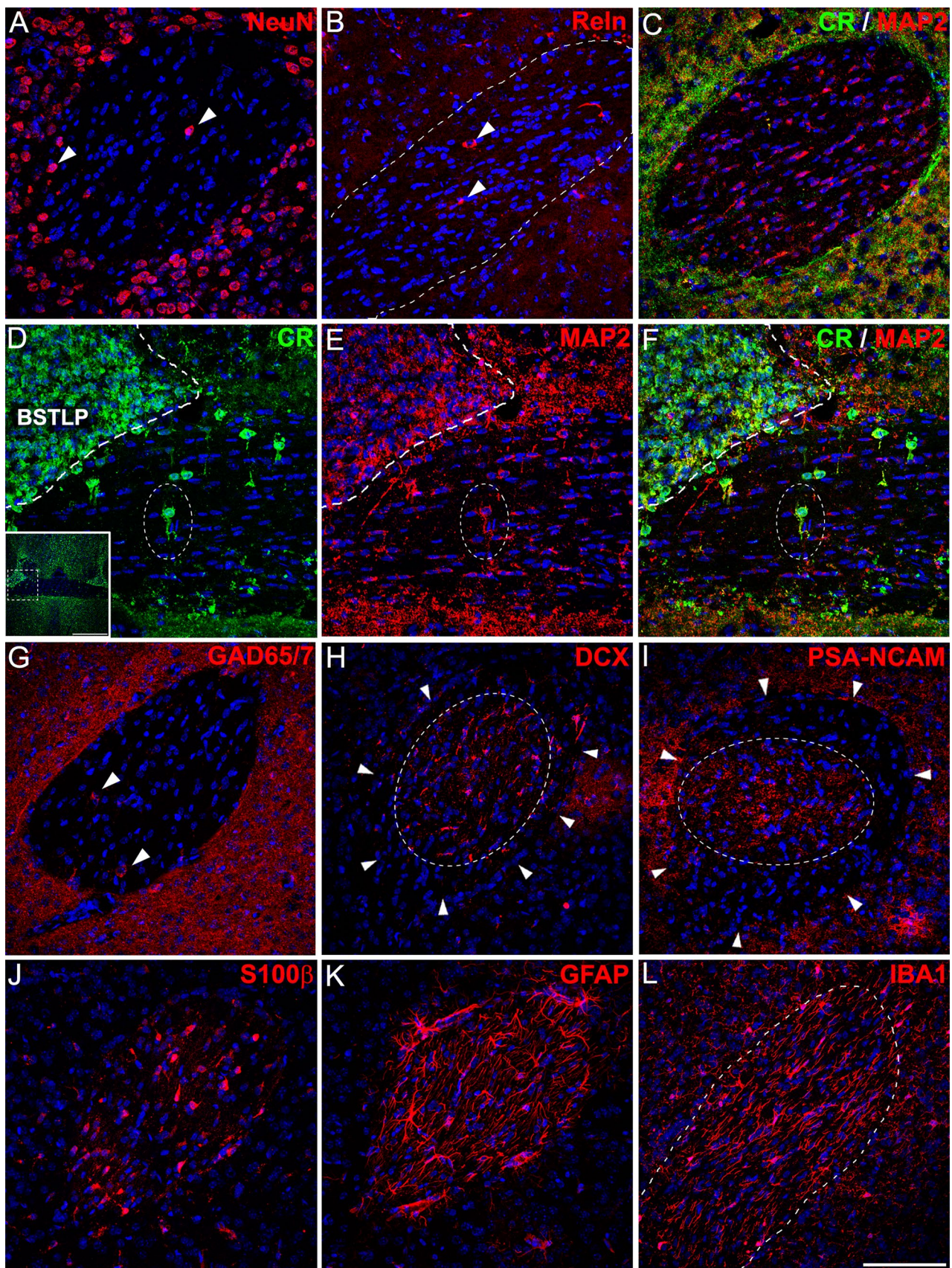


Fig. 8 Characterization of neuron and glial cells in the anterior commissure by immunohistochemistry at P21. All images show cross-sectional planes of the AC with nuclei counterstained with Draq5 (blue). **a** NeuN neurons (red) located within the lumen of the AC (arrowheads). **b** Scattered Reln neurons (red) located exclusively in the PLAC (arrowhead). **c** Immunoreactivity to CR (green) and MAP2 (red) showing a population of MAP2⁺ neurons with small and enlarged cell bodies within the lumen of the ALAC and a strong staining for CR surrounding the AC. **d–f** AC section at the level of axons crossing the ML. A subpopulation of neurons expressing the CR⁺/MAP2⁺ phenotype depicted in the area underneath the BSTLP. A ventral cell process emerges from these cells, suggesting migration from the BSTLP. **g** Expression of GAD65/67 (red) positive inhibitory neurons inside (arrowheads) and around the AC. **h** DCX staining (red) reveals the presence of immature neurons in the core of the AC (dotted line). The external edge of the AC is shown by arrowheads. **i** Labeling for PSA-NCAM (red) in the core of the AC (dotted line vs. arrowheads). **j** Subpopulations of astroglial cells expressing S100 β (red) inside the AC. **k** GFAP staining (red) showing astrocytes inside the AC. **l** Iba1 positive microglial cells (red) intermingled with other cells in the AC. **AC** anterior commissure, **BSTLP** bed nucleus of the stria terminalis, lateral division, posterior part, **CR** calretinin, **MAP2** microtubule associated protein-2, **ML** midline. Scale bars = 500 μ m in inset in **d**; 100 μ m in all other images

origin) and E13 (Dlx2 cells from subpallial origin) (Carney et al. 2006), the suggestion that commissural axons reached (and even crossed) the midline at E13 seems unlikely. Our data, on the other hand, supported the results obtained from magnetic resonance (Zhang et al. 2005) and AC labeling using L1CAM (Klingler et al. 2015).

Besides our interest in establishing the timeline of AC development, we wanted to further investigate the influence CSPG may have on guiding axons through the AC to incorporate this family of molecules to the other types influencing the development of brain commissures (Chedotal 2014; Dickson 2002; Kaprielian et al. 2000; Nawabi and Castellani 2011; Suarez et al. 2014). The CSPG are a family of glycoproteins that have been reported to form boundary tunnels around the AC and act as guidance cues (Fernaund-Espinosa et al. 1996; Inatani et al. 2003; Pires-Neto et al. 1998), although the roles of specific CSPG remain enigmatic. Despite the difficulties in understanding the function of PGs in vivo, we know they might act by generating instructive “sugar codes” to instruct axonal guidance (Holt and Dickson 2005). Here, we speculated that some CSPG such as versican, may be playing a similar role in sorting axons traveling between the ALAC and PLAC. We first mapped the expression of all CSPGs by analyzing staining with anti-CS56, an antibody that recognizes all types of CSPGs. Our data showed that CS56 was overexpressed in the PLAC at the level of the ML, an expression pattern that strongly implied that CSPG may have both permissive and repulsive behaviors depending on the type of axon. This dual behavior of CSPG has been reported before and is related to the intrinsic properties of neurons (Kantor et al. 2004; Shen 2014). It is, therefore, reasonable to speculate with

the existence of differing intrinsic mechanisms expressed by ALAC and PLAC axons. Among the CSPG investigated, only versican was observed in and around the AC during development. The expression pattern was found to be similar compared with that previously described around the internal capsule (Popp et al. 2003), which makes it reasonable to speculate that this CSPG could have similar activities in the axonal guidance in the AC.

Our next step was analyzing the postnatal maturation of the AC by describing the innervation and arborization of the contralateral PC and the myelination of AC axons. The arborization process in the contralateral PC was found to be similar to the arborization of callosum fibers, which exhibit a waiting period in the deeper layers before arborizing the contralateral cortex while cortical neurons mature (Lent et al. 1990; Wise and Jones 1976). This process contradicts what was observed in hamsters (Lent and Guimaraes 1990). On the other hand, the myelination process studied by the CNPase staining agreed with the recently described studies made with other myelin markers and EM (Collins et al. 2018). In contrast, the myelination rate we found of 20% (103 ± 5 axons/100 μ m²) was higher than the reported recently (Collins et al. 2018) and more similar to the previously described for more mature animals at P35 (Sturrock 1975, 1976) and P240 (Sturrock 1974b). Indeed, our recent data in PC describe a mature architecture and organization of PC by the weaning day at P21 (Martin-Lopez et al. 2017; Sarma et al. 2011) suggesting that the AC axons are similarly mature by this age. The consistency in our data may be accounted for in part by the large number of axons counted per animal, > 2000 (Sturrock 1975), and differences in the maturation rates might be related to the use of different mouse strains (Wahlsten 1981). However, we did not rule out the possibility of a later maturation of the tract as immature markers like DCX and PSA-NCAM were still detected in the core of the AC at this age.

Finally, characterization of cell populations in the AC revealed two previously unrecognized subpopulations of neurons: one expressing MAP2 (CR⁻/MAP2⁺), and the other expressing both MAP2 and CR (CR⁺/MAP2⁺) located in the ML region ventral to the BSTLP (Fig. 8). The CR⁻/MAP2⁺ were homogeneously distributed within the AC and may represent a subpopulation of neurons that could be involved in olfactory circuits and derived from the adjacent septal nuclei (Sturrock 1977). However, the absence of staining of the lateral dendrites with MAP2 also suggested they could belong to the population of AC interfascicular neurons (Larriva-Sahd et al. 2002). The CR⁺/MAP2⁺ phenotype exhibited a larger cytoplasm and extended a single cell process pointing ventrally in a behavior suggesting cell migration infiltrating from the adjacent BSTLP. This behavior has been previously described by a population of neurons infiltrating the AC from the amygdaloid complex in the caudal parts of the

AC (Gurdjian 1925). Regardless of their origin, the function of both populations of neurons remains a mystery.

Conclusions

In summary, our findings demonstrate: (1) the development of AC in mouse follows dynamics of *progressive* (extension and elongation) and *regressive* (waiting periods and pauses) strategies during development, in contrast to that described for other rodents; (2) the ipsilateral and contralateral innervation of the PC occurs later in the development than the projections to the OB; (3) the sorting of anterior and posterior axons of the AC at the level of the ML may be regulated by the expression of selective chondroitin sulfate proteoglycans; (4) the myelination of the AC occurs postnatally in a *rapid period* involving an increase in the number of cells after P14; (5) the myelination rate of CD1 mouse at P22 show a maturation degree homologous to older animals from other strains; (6) we report two new subpopulations of neurons in the AC: one with small and enlarged cell bodies expressing CR⁻/MAP2⁺ phenotype and distributed uniformly throughout the AC; and one other expressing the CR⁺/MAP2⁺ phenotype as neurons in the BSTLP that could be infiltrating from that nucleus; (7) these both subpopulation of neurons are generated after postnatal day 7.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal procedures were approved by Yale University Animal Care and Use Committee.

Informed consent This manuscript has been approved by all co-authors and agreed with the submission of the manuscript to Brain Structure and Function.

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REVIEW

Role of the endolysosomal system in Parkinson's disease

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Abstract

Parkinson's disease (PD) is one of the most common neurodegenerative disorders, affecting 1–1.5% of the total population. While progress has been made in understanding the neurodegenerative mechanisms that lead to cell death in late stages of PD, mechanisms for early, causal pathogenic events are still elusive. Recent developments in PD genetics increasingly point at endolysosomal (E-L) system dysfunction as the early pathomechanism and key pathway affected in PD. Clathrin-mediated synaptic endocytosis, an integral part of the neuronal E-L system, is probably the main early target as evident in auxilin, RME-8, and synaptotagmin-1 mutations that cause PD. Autophagy, another important pathway in the E-L system, is crucial in maintaining proteostasis and a healthy mitochondrial pool, especially in neurons considering their inability to divide and requirement to function an entire life-time. *PINK1* and *Parkin* mutations severely

perturb autophagy of dysfunctional mitochondria (mitophagy), both in the cell body and synaptic terminals of dopaminergic neurons, leading to PD. Endolysosomal sorting and trafficking is also crucial, which is complex in multi-compartmentalized neurons. *VPS35* and *VPS13C* mutations noted in PD target these mechanisms. Mutations in *GBA* comprise the most common risk factor for PD and initiate pathology by compromising lysosomal function. This is also the case for *ATP13A2* mutations. Interestingly, α -synuclein and LRRK2, key proteins involved in PD, function in different steps of the E-L pathway and target their components to induce disease pathogenesis. In this review, we discuss these E-L system genes that are linked to PD and how their dysfunction results in PD pathogenesis.

Keywords: Autophagy, Clathrin-mediated endocytosis, LRRK2, Lysosomes, Retromer complex, α -Synuclein.

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Parkinson's disease (PD) is the second most common neurodegenerative disorder, clinically identified by motor symptoms, with an increasing recognition of non-motor manifestations (Hoehn and Yahr 1967; Chaudhuri and Schapira 2009). Loss of dopaminergic neurons of the substantia nigra pars compacta (SNpc) (40–50%) and the presence of α -synuclein pathology in surviving neurons are the neuropathological hallmarks of PD. At the onset of motor symptoms, dopamine levels have decreased 60–70% in the dorsal striatum, where most SNpc dopaminergic neurons form synaptic connections (Fearnley and Lees 1991; Ma *et al.* 1997; Dickson *et al.* 2009; Kordower *et al.* 2013). Under physiological conditions, α -synuclein is predominantly present in

Abbreviations used: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATP13A2, ATPase cation transporting 13A2; CCVs, clathrin coated vesicles; CME, clathrin-mediated endocytosis; DCVs, dense core vesicles; E-L, endolysosomal; ESCRT, endosomal sorting complex required for transport; GAK, cyclin G associated kinase; GBA, Glucosidase Beta Acid; Gcase1, glucocerebrosidase 1; GD, Gaucher disease; GlcCer, glucosylceramide; GTPase, guanosine triphosphatase; GWAS, genome wide association study; Hsc70, heat shock cognate 70; HSP40, heat shock protein 40; HSPGs, heparan sulfate proteoglycans; ILVs, interluminal vesicles; iPSCs, induced pluripotent stem cells; KO, knockout; LAMP2a, lysosome-associated membrane glycoprotein 2; L-dopa, Levodopa; LRRK2, leucine-rich repeat kinase 2; M6P, mannose-6-phosphate; MAPK/ERK, mitogen-activated protein kinase/ extracellular signal-regulated kinase; MSNs, medium spiny neurons; MVBs, multivesicular bodies; PARK, Parkinson's disease gene; PD, Parkinson's disease; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PINK1, PTEN Induced putative Kinase 1; PIPs, phosphoinositide phosphates; PRKN, parkin gene; RME-8, receptor mediated endocytosis 8; siRNA, small interfering ribonucleic acid; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; SNCA, alpha-synuclein gene; SNpc, substantia nigra pars compacta; SVs, synaptic vesicles; SYNJ1/ SynJ1, synaptotagmin-1; TGN, trans-golgi network; USP19, ubiquitin-specific protease 19; VPS, vacuolar protein sorting.

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pre-synaptic terminals, aiding neurotransmitter release, whereas in PD, α -synuclein tends to aggregate and form Lewy bodies (the defining pathology of PD) (Spillantini *et al.* 1997; Spillantini *et al.* 1998). Aggregation of α -synuclein, perturbed proteostasis, mitochondrial dysfunction, oxidative stress, and impaired calcium homeostasis are the major cell-autonomous mechanisms of PD pathogenesis. Non-cell-autonomous mechanisms include cell-to-cell transmission of pathological α -synuclein and neuroinflammation (Jyothi *et al.* 2015; Poewe *et al.* 2017). So far, incomplete knowledge on causal mechanisms has limited treatment strategies to symptomatic relief. Recent studies targeting the endolysosomal (E-L) system reveal that its dysfunction can lead to the mentioned disease mechanisms, suggesting the E-L system as a key pathway in PD pathogenesis. In this review, we discuss important E-L system components, their roles in neuronal survival, and how their dysfunction causes PD.

Endolysosomal system in neurons: Custom-made for neuronal function

The E-L system comprises of complex, highly dynamic membrane-enclosed tubular-vesicular structures with overlapping properties and functions (Klumperman and Raposo 2014). They facilitate nutritional intake from a cell's microenvironment through endocytosis, neutralize pathogenic materials via phagocytosis, promote cellular proteostasis through autophagy, and maintain overall cellular homeostasis through endosomal sorting and trafficking of various proteins/enzymes across organelles (Repnik *et al.* 2013; Klumperman and Raposo 2014). E-L system functions vary greatly depending on cell type and functional state. Neurons are unique in their highly polarized intracellular compartmentalization of cell body/soma and processes including axons, axon terminals, and dendrites. This organization enables them to form complicated networks that mediate specialized functions (Fig. 1). This complexity comes at a price: burdensome demands on the E-L system, which have been increasingly implicated in neurological diseases including PD (Kett and Dauer 2016; Wang *et al.* 2018).

In neurons, synthesis of E-L components occurs primarily in the cell body/soma (Fig. 1a). These components are trafficked through the secretory pathway to their subcellular location. A prime example of this is lysosomal hydrolases trafficking. Lysosomal hydrolases are produced in the endoplasmic reticulum, then undergo glycosylation and other post-translational modifications at the Golgi apparatus [e.g. addition of mannose-6-phosphate (M6P) groups] (Nixon and Cataldo 1995). This modification directs hydrolases to lysosomes. More specifically, hydrolases form complexes with transmembrane M6P receptors, which are sequestered into clathrin-coated vesicle (CCVs) budding from the trans-Golgi networks (TGN) to form endosomes/early endosomes (Nixon and Cataldo 1995) (Fig. 1a). Inside early endosomes,

M6P receptors detach from hydrolases and are recycled back to the TGN for their next round of transport (Saftig and Klumperman 2009), while hydrolases are delivered to lysosomes (discussed below).

Simultaneously, early endosomes are also derived from the cell surface through the endocytic pathway, an integral part of the E-L system. Many of the nutrients required for neuronal function and surface receptors designated for recycling are internalized through clathrin-mediated endocytosis (CME) at the plasma membrane and delivered to early endosomes (Fig. 1a). Early endosomes derived from both the TGN and the plasma membrane mature into late endosomes, where Rab5 and Rab7 play a prominent role (Saftig and Klumperman 2009). This process occurs with the help of the endosomal sorting complex required for transport (Fig. 1a), which facilitates ubiquitin-dependent sorting of cargo/hydrolases from the outer membrane of early endosomes into interluminal vesicles (ILVs) (Wollert *et al.* 2009). Early endosomes derived from CME, if not sorted, follow a recycling pathway to the plasma membrane (Fig. 1a) (Saftig and Klumperman 2009). ILVs are enveloped inside multivesicular bodies (MVBs), which mature into late endosomes. These late endosomes deliver their luminal contents either by forming tubular extensions or by fusing with pre-existing lysosomes to form endolysosomes (Luzio *et al.* 2007). These may be considered the final compartment of the endocytic pathway, where hydrolases degrade endocytic cargo and the membranes of ILVs for reuse (Saftig and Klumperman 2009; Klumperman and Raposo 2014). In addition, there is retrograde transport of receptors (such as M6P, LAMP2a, etc) from endosomes to the TGN for reuse through the retromer complex, comprised mainly of VPS35, VPS26 and VPS29 (Saftig and Klumperman 2009) (Fig. 1a). Proteins that are meant for secretion follow a secretory pathway from the TGN (Saftig and Klumperman 2009; Klumperman and Raposo 2014). Selective capture and elimination of a cell's damaged organelles and misfolded proteins happens through a process called macroautophagy (termed hereafter as autophagy) (Ohsumi 2014), an integral part of the E-L system. Double membrane vesicles called autophagosomes encapsulate unwanted/toxic cytosolic constituents including aggregated proteins and deliver them to the lysosome for degradation (Fig. 1a) (Mizushima 2007; Kawamata *et al.* 2008). Non-selective autophagy also occurs during starvation, through which cells can recycle proteins and reuse amino acids for the synthesis of basic proteins essential for cell survival (Ohsumi 2014). Therefore, autophagy plays a pivotal role in house-keeping and maintenance of neuronal homeostasis.

Customization of the E-L system for neurons mainly happens in axons (Fig. 1b) and synapses (Fig. 1c). At pre-synaptic terminals, vesicle precursors are delivered from the cell body and undergo vesicle cycling to form functional synaptic vesicles (SVs). However, delivery of new vesicle precursors cannot match the number of SVs undergoing

exocytosis to achieve neurotransmission at an active synapse (Saheki and De Camilli 2012). This deficit is solved by highly responsive CME, recapturing fused vesicular membranes from the pre-synaptic membrane to form CCVs, which are in turn converted back to SVs through clathrin uncoating (Fig. 1c) (Heuser and Reese 1973; Morgan *et al.* 2000; Saheki and De Camilli 2012). Less understood clathrin-independent bulk endocytosis (Holt *et al.* 2003; Wu and Wu 2007) and the “kiss and run” mechanism (Fesce *et al.* 1994) are also parts of the synaptic endocytic repertoire. In addition, there are dense-core vesicles derived from the TGN that are transported down the axons. Unlike SVs, dense core vesicles do not participate in SV recycling and are primarily used to secrete long-acting neuropeptides (Nurrish 2014). While these processes are mostly confined to the pre-synaptic compartment, the E-L system's postsynaptic compartment contributes to membrane trafficking pathways primarily aimed at receptor, transporter, and ion-channel recycling (Rosendale *et al.* 2017).

Although this complex E-L system allows a neuron to function far away from its site of protein synthesis, the neuron is beholden to anterograde transport of proteins and membranes synthesized and modified in the soma for maintenance of axons and the synapses (Maday *et al.* 2014). Furthermore, constant synaptic activity, especially in continuously firing neurons like SNpc dopaminergic neurons may result in considerable build-up of damaged organelles, membranes, and misfolded proteins. These need to undergo encapsulation by an autophagosome or endosome (Fig. 1C) and retrograde transport to the soma for lysosomal degradation (Fig. 1B) (Yang *et al.* 2013). Here, long-distance axonal microtubule networks provide significant support to the neuronal E-L system (Yang *et al.* 2013). These spatial challenges to address a unique structural geometry, high metabolic demand, enhanced responsiveness to chemical/electrical stimulation, and the requirement for longevity because of their postmitotic nature, place a tremendous burden on the E-L system. Nigrostriatal dopaminergic neurons have an additional burden in terms of long and highly branched axons, larger surface area, selective transporters for external toxins, large fluctuations in free Ca^{2+} , less buffering capacity, and higher oxidative stress, reducing their threshold for degeneration (Vidyadhara *et al.* 2016; Surmeier *et al.* 2017; Vidyadhara *et al.* 2017; Vidyadhara *et al.* 2019). These vulnerabilities are further exacerbated with aging. Faced with environmental and genetic risk factors, disease pathogenesis abounds.

E-L system dysfunction: the holy grail of PD pathophysiology?

In 1997, identification of dominant mutations in the α -synuclein gene (*SNCA*, *PARK1*) which encodes α -synuclein as a cause of familial PD (Polymeropoulos *et al.* 1997),

brought a conceptual shift toward the importance of genetics in disease pathogenesis. Subsequent findings revealed various other genetic mutations, with at least 10-15% of PD patients having familial PD of an absolute genetic origin. Leucine-rich repeat kinase 2 (*LRRK2*, *PARK8*) and Parkin (*PRKN*, *PARK2*) mutations are the most common genetic variants of dominant and recessively inherited PD respectively (Deng *et al.* 2018). Mutations and gene multiplications of *SNCA* (*PARK1* and *PARK4*) (Singleton *et al.* 2003; Chartier-Harlin *et al.* 2004) and Glucosidase Beta Acid (*GBA*) are also highly prevalent, whereas PD pathology was also noted with abnormal forms of Parkinsonism associated with deglycase or DJ1 (*PARK7*), and PTEN induced putative kinase 1 (*PINK1*, *PARK6*) (Deng *et al.* 2018). There is also compelling evidence for a genetic component to sporadic PD. In fact, a rigid distinction between the genetic and environmental basis of PD pathogenesis may not exist considering that cases are phenotypically indistinguishable (except for the onset) (Baba *et al.* 2006), implying genetic and sporadic cases share common underlying mechanisms. Even after considerable studies on these familial mutations and their interaction with environment, the core pathomechanisms of PD are still unclear, limiting treatment strategies. Over the past 10 years, advancements in high-throughput sequencing, the application of genome wide association studies (GWAS), and meta-analysis, have identified new genetic mutations (refer to Deng *et al.* 2018 for a list of all PARK genes and risk alleles). Intriguingly, most of these identified genes have links to the E-L system (refer Table 1). Mechanistic studies also suggest that these proteins are the downstream targets of previously identified broad spectrum dysfunction in α -synuclein and *LRRK2* mutations. It is now becoming clear that most PD-linked genes (Table 1) and known pathomechanisms are, in one way or another, associated with the E-L system, strongly suggesting this pathway as the key master regulator of PD pathogenesis. Recent studies suggest that neurodegeneration is initiated at the dopaminergic pre-synaptic terminals of striatum, years before the clinical diagnosis (Imbriani *et al.* 2018). Evidence that most of the E-L system genes associated with PD function at this terminal further establishes their fundamental role in PD pathogenesis. Below, we discuss our understanding of the functions and dysfunctions of these PD-linked E-L system genes. We also discuss how other key PD-associated proteins target E-L pathways.

Clathrin-mediated synaptic vesicle endocytosis: A prime target?

SV endocytosis is predominantly mediated through CME (Fig. 1c). Following exocytosis, the SV membrane is endocytosed primarily through CME, which involves formation of a clathrin coat, composed of an outer clathrin layer, and an inner layer of adaptors (Saheki and De Camilli 2012). The most common adaptors, such as AP-2 and AP180, bind

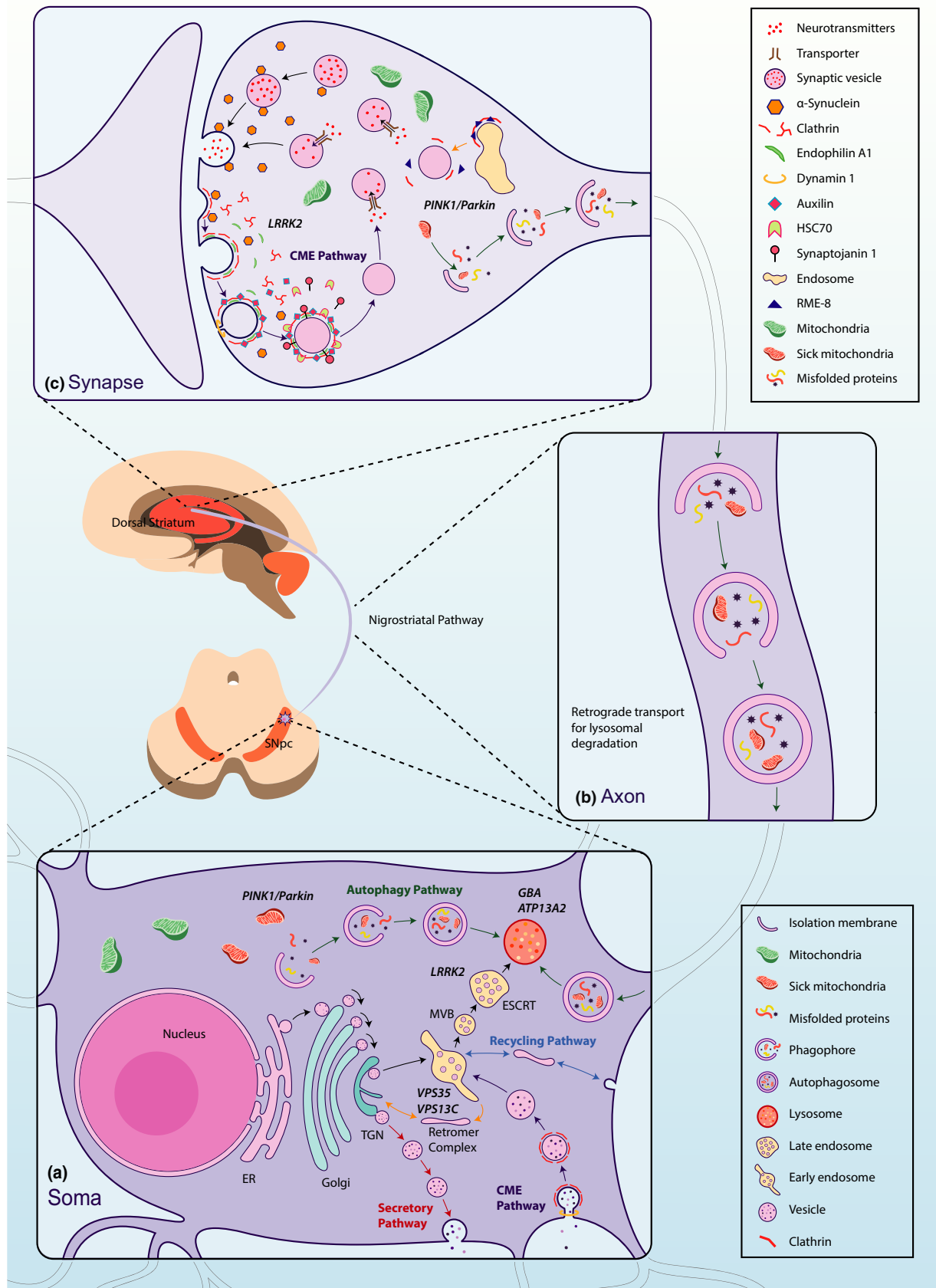


Fig. 1 E-L system in the compartments of a nigrostriatal dopaminergic neuron, with the normal functional locations of E-L system proteins linked to PD. (a) Soma: E-L system proteins that form structural and functional components are synthesized in the neuronal soma. Following production in the endoplasmic reticulum (ER) and post-translational modifications in the Golgi apparatus, proteins (e.g. lysosomal hydrolases) are packed into vesicles and delivered to the trans-Golgi network (TGN). In the TGN, based on the nature of post-translational modification, the vesicles are diverted either toward lysosomes (black arrows) or to the secretory pathway (red arrows). Vesicles that are destined for lysosomes bud off from the TGN to form early endosomes. Simultaneously, early endosomes carrying nutrients and surface receptors for recycling are also derived from the plasma membrane through clathrin mediated endocytosis (CME, purple arrows). These early endosomes, with the help of the endosomal sorting complex required for transport (ESCRT), pass through highly dynamic tubulovesicular structures like multivesicular bodies (MVB) and/or late endosomes before fusing with lysosomes. Leucine-rich repeat kinase 2 (LRRK2) plays a significant role in this sorting, whereas Glucocerebrosidase 1 (GBA/Gcase 1) and ATPase cation transporting 13A2 (ATP13A2) are crucial for normal lysosomal function. Early endosomes from the cell surface that are not sorted follow the recycling pathway back to the plasma membrane (blue arrows). Retrograde transport of receptors that are used for sorting from early endosomes to TGN occurs through the retromer complex where Vacuolar Protein Sorting 35 (VPS35) and VPS13C take part (yellow arrows). An expanding membrane sac called a phagophore sequesters malfunctioning organelles (e.g. sick mitochondria) and misfolded proteins to form a double-membraned autophagosomes. These later

fuse with lysosomes and release contents for degradation and recycling of components; the complete process is called autophagy (green arrows). PTEN Induced Putative Kinase 1 (PINK1) and Parkin are important in identifying sick mitochondria and diverting them to a specialized form of autophagy called mitophagy. (b) Axon: Axons form a crucial bridge for retrograde transport of endosomes/autophagosomes from pre-synaptic terminals to soma for lysosomal degradation. A gradual development of phagophore to full-fledged autophagosome happens within the axon (green arrows) as the encapsulated cargo arrives towards the proximal end. (c) Synapse: Neurotransmitter release in the pre-synaptic terminal is regulated principally by the synaptic vesicle (SV) cycle, an integral part of the neuronal E-L system. Neurotransmitters are loaded into these SVs through specialized transporters. Upon arrival of an action potential in the synaptic terminal, these SVs fuse with the plasma membrane at active sites and release neurotransmitters via exocytosis (black arrows). Empty SVs are then recycled through CME (Purple arrows). α -Synuclein and endophilin A play roles here in membrane bending and SV curvature formation. Clathrin forms a layer on these invaginations with the help of adaptors, and in conjunction with dynamin leads to membrane fission forming clathrin coated vesicles (CCV). Clathrin uncoating is a prerequisite for SV recycling, which is performed by coordinated action of auxilin, Hsc70, endophilin A1, and synaptojanin 1. Phosphorylation by LRRK2 is necessary for activation of these proteins. Endosomes derived from the plasma membrane also form SVs in the nerve terminal where RME-8 plays a role in clathrin sorting (yellow arrow). Synaptic autophagy and mitophagy are also crucial in maintaining the health of the pre-synaptic terminal (green arrows), where PINK1/Parkin are crucial.

Table 1 PD genes linked to E-L system with their PARK nomenclature, proteins they encode, and their primary function and original reference of their discovery.

PD genes that are linked to E-L system				
PARK	Gene	Protein	Function	Reference
PARK1	<i>SNCA</i>	α -Synuclein	Synaptic vesicle cycling	Polymeropoulos <i>et al.</i> (1997)
PARK2	<i>PRKN</i>	Parkin	E3 Ubiquitin ligase	Mori <i>et al.</i> (1998) Kitada <i>et al.</i> , (1998)
PARK4	<i>SNCA</i>	α -Synuclein	Synaptic vesicle cycling	Singleton <i>et al.</i> , (2003) Chartier-Harlin <i>et al.</i> , (2004)
PARK6	<i>PINK1</i>	PINK1	Serine/Threonine kinase	Valente <i>et al.</i> , (2004)
PARK8	<i>LRRK2</i>	LRRK2	Kinase/GTPase	Paisán-Ruiz <i>et al.</i> , (2004); Zimprich <i>et al.</i> , (2004)
PARK9	<i>ATP13A2</i>	ATPase type 13A2	ATPase	Ramirez <i>et al.</i> , (2006); Di Fonzo <i>et al.</i> , (2007)
PARK16	<i>RAB29</i>	Ras-Related Protein Rab-7L1	Rab GTPase	Satake <i>et al.</i> (2009)
PARK17	<i>VPS35</i>	VPS35	Retromer	Wider <i>et al.</i> , (2008) Vilarinho-Güell <i>et al.</i> , (2011)
PARK19	<i>DNAJC6</i>	Auxilin	Co-chaperone	Edvardson <i>et al.</i> , (2012) Köroğlu <i>et al.</i> , 2013
PARK20	<i>SYNJ1</i>	Synaptojanin-1	Polyinositol phosphatase	Krebs <i>et al.</i> , (2013) Quadri <i>et al.</i> , (2013)
PARK21	<i>DNAJC13</i>	RME-8	Co-chaperone	Vilarinho-Güell <i>et al.</i> , (2014)
PARK23	<i>VPS13C</i>	VPS13C	Retromer, Lipid transporter	Lesange <i>et al.</i> , (2014), Schormair <i>et al.</i> , (2018)
Risk Genes	<i>GBA</i>	Glucocerebrosidase 1	Glucosidase	Sidransky <i>et al.</i> , (2009)
	<i>GAK</i>	Cyclin-G Associated Kinase	Co-chaperone	Nalls <i>et al.</i> , (2014) Chang <i>et al.</i> , (2017)
	<i>SH3GL2</i>	Endophilin A1	Membrane bending protein	Nalls <i>et al.</i> , (2014) Chang <i>et al.</i> , (2017)

to endocytic motifs on the SV membrane, as well as phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] present in the plasma membrane. Endophilin A1 and α -synuclein drive membrane bending and curvature formation of the developing clathrin coated pit (Bai *et al.* 2010; Westphal and

Chandra 2013). Following this, the clathrin coated pit in conjunction with dynamin and other accessory proteins undergoes membrane fission to form a CCV (Saheki and De Camilli 2012). Regeneration of SVs from CCVs and recycling of clathrin and related coat components for further

endocytosis requires un-coating of clathrin from CCVs (Fig. 1c). The clathrin uncoating step is carried out by the coordinated action of the PI phosphatase synaptojanin-1 and the co-chaperone auxilin with Hsc70. Endophilin A1 and cyclin G Associated Kinase (GAK) also regulate clathrin uncoating to facilitate CME at the pre-synaptic terminal (Fig. 1c) (Saheki and De Camilli 2012). Mutations of these E-L system genes in familial PD suggest synaptic endocytosis dysfunction as a prime trigger for PD pathogenesis.

The DNAJ family of heat shock proteins in the pre-synaptic terminal and beyond

Auxilin

DNAJC6 gene encodes auxilin, the major pre-synaptic endocytic protein linked to PD. Auxilin is a brain specific co-chaperone belonging to the DNAJC family (Table 1), a subclass of heat shock proteins (HSP40). Auxilin plays an important role in clathrin un-coating, recruiting ATP-activated Hsc70 to CCVs, which in turn, disassembles clathrin from vesicles facilitating their recycling (Lemmon 2001; Gorenberg and Chandra 2017) (Fig. 1c). Loss-of-function autosomal recessive mutations of auxilin (*PARK19*) have been shown to cause juvenile/early onset PD, first observed in two teenage brothers of a Palestinian family (Edvardson *et al.* 2012). The study noted abnormal mRNA splicing and a decrease in auxilin mRNA levels. Other variants of auxilin mutations were later noted in a Brazilian family with similar mis-splicing of mRNA, whereas mutations affecting the J-domain (p.Q791*, p.Q846* and p.R927G) were observed in a few other consanguineous families (Koroglu *et al.* 2013; Olgiati *et al.* 2016). Along with typical PD symptoms, patients with nonsense mutations have cognitive impairment, one of the cardinal non-motor symptoms of PD. Epilepsy and mental retardation have also been noted for some PD patients with auxilin mutations (Koroglu *et al.* 2013). These human genetic studies suggest widespread neuronal dysfunction besides nigrostriatal dopaminergic neurodegeneration. Hence, future studies on post-mortem brains documenting PD-typical Lewy body pathology and dopaminergic neuronal loss are necessary to concretely categorize the pathology as PD or parkinsonism.

One of the few preclinical studies characterizing *DNAJC6* reveals that auxilin down-regulation leads to progressive dopaminergic neuronal loss and motor disabilities in *Drosophila*. Furthermore, loss of auxilin exacerbates α -synuclein over-expression and paraquat (an environmental toxin)-mediated dopaminergic loss, thus aggravating previously established phenotypes in fly models of PD (Song *et al.* 2017). Interestingly, the brains (deep cerebellar nuclei) of adult homozygous auxilin knockout (KO) mice revealed an increased number of CCVs and empty cages at the synapse. Similar defects were noted in primary cortical and hippocampal neurons accompanied by defective SV endocytosis

(Yim *et al.* 2010). The impairment was not immediately detrimental to the neurons, which may explain the post-developmental onset of PD symptoms and slow disease progression. Evaluating whether similar disruptions occur in nigrostriatal dopaminergic neurons and how it mediates pathogenesis will prove crucial in understanding the role of auxilin and the E-L system in PD. An initial step toward this suggests that LRRK2 phosphorylates auxilin, attenuating auxilin's normal function in human iPSC-derived dopaminergic neurons. The study further revealed that phosphorylation of auxilin by over-expressing LRRK2 leads to accumulation of oxidized dopamine and α -synuclein over-expression, pathological hallmarks of PD (Nguyen and Krainc 2018). Whether loss-of-function mutations in auxilin independently exert pathology through the same mechanisms is unknown. However, this newfound link between LRRK2 and auxilin points to the crucial and widespread involvement of E-L system in PD pathogenesis.

GAK

GAK is another DNAJC family protein (*DNAJC26/Auxilin 2*), homologous (~50%) to auxilin but expressed throughout the body (Lemmon 2001). In a meta-analysis of PD GWAS across 13,708 patients and 95,282 controls (Nalls *et al.* 2014), GAK was identified as a risk gene for PD which was confirmed in another recent largescale study (Chang *et al.* 2017) (Table 1). However, GAK's role in PD is still contentious because of presence of mutations in *TMEM175* in a nearby independent locus, which shares the same promoter as GAK. What adds credence is that, like auxilin, GAK functions in clathrin un-coating by recruiting Hsc70 to CCVs (Lemmon 2001) (Fig. 1C). Results show that GAK over-expression can partially compensate for loss of auxilin, and is also noted to up-regulate naturally in auxilin-KO mice (Yim *et al.* 2010). Another similarity between auxilin and GAK has been noted in their interaction with LRRK2 (Jinn *et al.* 2017; Nguyen and Krainc 2018). Brain-specific loss of GAK and ubiquitous KO of GAK are both lethal (postnatal and embryonic respectively) (Lee *et al.* 2008a). The inability of auxilin to compensate for GAK in these mice suggests another unique role for GAK in neurons, perhaps a significant one in dopaminergic neurons. Further *TMEM175* deficiency is noted to cause PD-like α -synuclein aggregation, along with impairment of lysosomal and mitochondrial function (Jinn *et al.* 2017). Clarity on the role of GAK will prove to be crucial not only in establishing its involvement in PD but also in consolidating CME as the prime target in PD pathogenesis.

RME-8

Receptor mediated endocytosis 8 (RME-8) or *DNAJC13* is another DNAJC family co-chaperone and principally functions in regulating retromer and clathrin un-coating dynamics on endosomes (Gorenberg and Chandra 2017) (Fig. 1C).

DNAJC13 mutations (p.N855S) in autosomal-dominant PD (*PARK21*) noted first in a Dutch-German-Russian family of Mennonite origin, with multiple incidences (Table 1) (Vilarino-Guell *et al.* 2014; Gustavsson *et al.* 2015). Post-mortem brains of PD patients with *DNAJC13* mutations revealed strong immunoreactivity for Lewy bodies in the substantia nigra, along with brain areas implicated in non-motor symptoms of PD. Nigral Lewy bodies were also found to be co-localized with RME-8 (Vilarino-Guell *et al.* 2014). Unlike other DNAJC proteins implicated in PD, *in vitro* studies have revealed that the *DNAJC13* mutation confers toxic gain-of-function, leading to E-L cargo trafficking deficits (Norris *et al.* 2017). The *DNAJC13* mutation is also predicted to affect overall endosomal trafficking, even in the soma and postsynaptic compartment (Vilarino-Guell *et al.* 2014), unlike the synapse-specific dysfunction predicted for auxilin. Recently, the link between *DNAJC13* and PD has been muddled by an independent analysis of the same Mennonite family which did not find *DNAJC13* mutations. Instead, all the affected individuals were identified to carry mutations in *TMEM230* (Deng *et al.* 2016), which also functions in E-L system, perhaps at synapses. Hence, further studies are warranted to confirm the genetic link between *DNAJC13* and PD. Still, evaluating the RME-8/*DNAJC13* mediated E-L system dysfunction might provide valuable insights in understanding PD.

Synaptojanin-1 and endophilin A: Partners in crime?

Two other major synaptic endocytic proteins besides the DNAJ family that are implicated in PD are synaptojanin-1 (SYNJ1) and endophilin A. SYNJ1 is a Sac domain-containing protein with two different phosphatase domains; 5-phosphatase and Sac1, which target different phosphoinositide phosphates (PIPs) (McPherson *et al.* 1996). 5-phosphatase regulates SV endocytosis by dephosphorylating PI(4,5)P₂ to facilitate uncoating of CCVs in cooperation with Hsc70 and auxilin (Fig. 1c), whereas Sac1 domain has gained importance because of its link to PD (*PARK20*, Table 1) (Cremona *et al.* 1999; Mani *et al.* 2007; Saheki and De Camilli 2012). The p.R258Q missense mutations in the Sac domain were identified independently in two consanguineous families of Italian and Iranian origin. Homozygous missense mutations of *SYNJ1* Sac domain are noted to cause autosomal recessive PD with extrapyramidal features identical to patients with auxilin mutations (Krebs *et al.* 2013; Quadri *et al.* 2013; Olgiati *et al.* 2014). Interestingly, they responded poorly to L-dopa treatment, indicating the need for preclinical studies to understand the exact pathomechanisms. Recent observations by Cao *et al.*, suggest that the Sac domain plays a previously unappreciated role in clathrin uncoating. Mice with *SynJ1* Sac mutations (knock-in) developed PD-like characteristics and featured synaptic endocytosis defects, including accumulating of CCVs. Furthermore, elevated

auxilin and Parkin levels were noted in these mice, suggesting the profound effect of Sac mutations on other proteins linked to PD (Cao *et al.* 2017). Studies on fibroblasts from patients with *SYNJ1* mutations suggested defective endocytic trafficking (Quadri *et al.* 2013). Other studies have shed light on the involvement of SynJ1 in regulating synaptic autophagy. Mutations in the Sac1 domain of *SynJ1* in both *Drosophila* neurons and *PARK20* patient-derived iPSCs severely affected synaptic autophagosome formation but not endocytosis (Vanhouwaert *et al.* 2017). This discrepancy and the precise relationship between SV endocytosis and synaptic autophagy remains to be clarified. However, the *Drosophila* model revealed loss of dopaminergic neurons, providing evidence for the contribution of synaptic autophagy defects in pathogenesis of PD (Vanhouwaert *et al.* 2017). Considering its dual enzymatic activity, SynJ1 is also believed to play a role in fast endophilin-mediated, clathrin-independent endocytosis which is an alternative to CME (Watanabe *et al.* 2018) and several other processes. It remains to be determined if SYNJ1 exert its effects on PD pathogenesis through any of the alternative endocytic pathways.

SYNJ1 recruitment to endocytic membrane is facilitated by endophilin A (Fig. 1c), a protein drawing attention in PD pathogenesis, despite its locus variation only being identified as a risk factor (Table 1) (Chang *et al.* 2017). This is because of endophilin A's close association with SYNJ1 (Song and Zinsmaier 2003; Cao *et al.* 2017) and its link to most important proteins involved in PD. One of our earlier studies indicated that over-expression of α -synuclein decreases endophilin A levels, while deletion of synucleins had the opposite effect (Westphal and Chandra 2013). As noted in *SynJ1* knock-ins, endophilin KO also leads to synaptic vesicle endocytosis defects, accumulation of CCVs, elevation of Parkin levels, and other parkinsonian features (Milosevic *et al.* 2011; Cao *et al.* 2014). Gain-of-function mutations in LRRK2 hyper-phosphorylate endophilin A, the fly homolog, leading to synaptic endocytosis dysfunction (Matta *et al.* 2012). LRRK2-mediated phosphorylation of endophilin A is also noted to regulate synaptic autophagy (Soukup *et al.* 2016). Owing to the relatively higher frequency of LRRK2 mutations and its involvement in sporadic PD, endophilin A may prove to be another crucial protein involved in PD.

Autophagy in PD; cause and effect, hand in hand

Neurons need to constantly maintain homeostasis by preserving healthy organelles and functional proteins, while selectively capturing and eliminating damaged organelles and misfolded proteins. Autophagy, an integral component of the E-L system, is an intracellular proteostasis pathway that performs this housekeeping function (Mizushima *et al.* 1998;

Nixon 2013; Suresh *et al.* 2018b) (Fig. 1A, 1). It was initially identified in cell lines to function in maintaining energy homeostasis during critical periods of development and in response to nutrient depletion (Mizushima 2007). Intriguingly, nutrient depletion does not induce autophagy in neurons, but a complete lack of autophagy can cause lethal neurodegeneration, emphasizing its important role in survival (Hara *et al.* 2006; Komatsu *et al.* 2006). Since neurons are post-mitotic and long-lived, they are particularly vulnerable to accumulation of toxic proteins (Nixon 2013). In addition, greater dependency of neurons on mitochondria for energy instead of glycolytic pathways demands constant mitochondrial turnover (Kann and Kovacs 2007), leading to many malfunctioning mitochondria. Both these factors play major roles in the pathogenesis of PD.

PINK1 and Parkin: Role in mitophagy and beyond

Under physiological conditions, autophagy plays a crucial role in clearing unwanted proteins, as well as damaged mitochondria through a targeted mechanism called mitophagy (Nguyen *et al.* 2016). Loss-of-function mutations in *PINK1* and *Parkin* (*PARK2* and *PARK6*), result in recessively inherited early onset PD and significantly, both genes were discovered to be essential for mitophagy (Kitada *et al.* 1998; Mori *et al.* 1998; Valente *et al.* 2004). *PINK1* is a serine/threonine protein kinase that acts as a surveillance molecule for mitochondrial health and accumulates on the outer membrane of abnormal mitochondria (Nguyen *et al.* 2016). *PINK1* attracts recruitment of *Parkin* to damaged mitochondria, which through its E3 ubiquitin ligase activity (Narendra *et al.* 2010; Kane *et al.* 2014), coats the mitochondria with ubiquitin triggering autophagosomes to engulf the mitochondria (Narendra *et al.* 2008), culminating in lysosomal degradation (Fig. 1a1). Discoveries of *PINK1* and *Parkin* mutations were the first to shed light on the direct involvement of autophagy and E-L system in PD pathogenesis (Pickrell and Youle 2015). These observations also helped in validating previous neurotoxic studies (Przedborski *et al.* 2004) which identified mitochondrial dysfunction as one of the primary causes of neurodegeneration. Though the symptoms are indistinguishable from sporadic cases, several studies on brains of PD patients with *Parkin* mutations do not show α -synuclein aggregation and Lewy body pathology (Doherty and Hardy 2013). Only one report suggests *PINK1*-linked parkinsonism to be associated with Lewy pathology (Samaranch *et al.* 2010). This prompts us to speculate if there are alternative neuropathological cascades in PD that are α -synuclein independent but centered around the E-L system.

In addition to their well-defined roles in mitophagy, *Parkin* was identified to modulate E-L system through Rab7 ubiquitination (Song *et al.* 2016), whereas *PINK1* phosphorylates some Rab proteins (Lai *et al.* 2015). It is also worth noting that several of these Rab proteins (for example Rab7) regulate mitochondrial dynamics and quality control independent of

Parkin/PINK1 (Wang *et al.*, 2018). *Parkin* mutations are also known to directly affect retromer recruitment (Williams *et al.* 2018) and interact strongly with VPS35 by ubiquitinating it (Malik *et al.* 2015; Martinez *et al.* 2017). *Parkin* binds to endophilin A and functions in its ubiquitination (Cao *et al.* 2014). *Parkin* also ubiquitinates EPS15 to regulate epidermal growth factor receptors and phosphoinositide 3-kinase (PI(3)K)-Akt signaling that plays a role in neuronal survival (Fallon *et al.* 2006). These developments suggest mitophagy-independent routes these proteins may take toward PD. Further investigations of *Parkin* and *PINK1* in this direction might provide crucial insights in understanding neurodegenerative mechanisms that are Lewy-body independent.

Synaptic autophagy and axonal transport: a road less travelled in PD research

α -Synuclein aggregates initially in pre-synaptic termini, where the protein is enriched (Kramer and Schulz-Schaeffer 2007). α -Synuclein aggregates are a major target for autophagy-mediated degradation (Xilouri *et al.* 2008). In both genetic and sporadic PD, aggregated α -synuclein is likely to enhance autophagosome formation to meet the higher demand for its degradation (Wong and Holzbaur 2015). Once threshold reaches beyond compensatory limits, mutant/over-expressed α -synuclein starts to accumulate, turning detrimental to cellular homeostasis. In fact, A53T and A30P α -synuclein mutants blocked their own uptake and other substrates by lysosomes for degradation through the chaperone-mediated autophagy pathway *in vitro* (Cuervo *et al.* 2004). Dopamine-modified α -synuclein is identified to further exacerbate this process, which may explain the selective vulnerability of dopaminergic neurons in PD (Martinez-Vicente *et al.* 2008). A recent study on mammalian cells and transgenic mice shows that α -synuclein over-expression mediated autophagy defects are exerted through Rab1a inhibition (Winslow *et al.* 2010). Further understanding of the mechanisms for α -synuclein-autophagy crosstalk in PD might have crucial therapeutic applications. Backing this notion, both *in vitro* and *in vivo* studies show that enhancing autophagy and autophagosome-lysosome fusion could ameliorate PD-like features by clearing aggregated α -synuclein (Suresh *et al.* 2017; Suresh *et al.* 2018a).

Several other PD causing genes associated with the E-L system exert their pathological effects through impairing synaptic autophagy. The R258Q SynJ1 mutation directly affects autophagosome biogenesis and maturation at synaptic terminals of flies and neurites of human iPSC-derived neurons. The observation of dopaminergic neuronal loss in these flies suggests autophagosome generation defects as one of the possible mechanisms through which SynJ1 mutations lead to dopaminergic neurodegeneration and PD (Vanhouwaert *et al.* 2017). Pre-synaptic endocytic membrane bending protein endophilin A also has functions in providing docking sites for autophagy proteins and facilitating autophagosome expansion.

This is achieved by phosphorylation of the endophilin A-BAR domain by LRRK2, as noted in a fly model (Soukup and Verstreken 2017). Congruent to these observations, mice lacking endophilin A revealed deficiency in autophagosome formation and developed a parkinsonian phenotype (Murdoch *et al.* 2016). These studies, along with recent finding of clathrin involvement in autophagy regulation (Rong *et al.* 2012), suggest a cooperative cross-talk between SV endocytosis and autophagy.

Macromolecules and organelles in the pre-synaptic terminal travel a long distance from the soma, making them old and vulnerable. Rapid turnover of proteins, membranes, receptors, and vesicles in the pre-synapse assigns autophagy a major role in maintaining synaptic homeostasis. This tasks synaptic autophagy with quality control responsibilities. Though there is compelling evidence that compromised autophagy contributes to PD, these studies primarily looked at the soma from a proteinopathy or mitophagy perspective (Lynch-Day *et al.* 2012). Selective inactivation of autophagy by conditional knockout of autophagy protein atg7 in dopaminergic neurons leads to enhanced dopamine release and rapid pre-synaptic recovery with abnormal axonal profiles (Hernandez *et al.* 2012). Conversely, autophagy enhancement through mTOR inhibition slows dopamine release, reduces the number of synaptic vesicles, and increases autophagosome density in the axons (Hernandez *et al.* 2012). These results emphasize the importance of synaptic autophagy in normal dopamine transmission and further evaluation is required to understand if additional synaptic autophagy defects exist, which may lead to PD pathogenesis.

Another unique and stressful situation for neurons is that autophagosomes loaded with unwanted materials from an active pre-synaptic terminal, need to be transported a long distance to the soma for lysosomal degradation (Fig. 1b). Microtubule and scaffolding proteins aid in this retrograde axonal transport of autophagosomes and are crucial for the normal functioning of E-L system (Birdsall and Waites 2019). During their transport, autophagosomes mature into autolysosomes by fusing with late-endosomes/lysosomes towards the proximal end of axon (Nikolietopoulou and Tavernarakis 2018). There are recent studies suggesting lysosomal degradation, especially of mitophagosome mediated by Parkin-PINK1, occurs at the distal end. This is predicted to provide rapid neuroprotection against oxidative stress by damaged mitochondria, without requirement for retrograde transport (Ashrafi *et al.* 2014; Ashrafi and Schwarz 2015). Axonal transport aids this function through anterograde transport of E-L system components required for degradation from soma towards the distal end. Any defect in this to-and-fro transport machinery can completely clog the E-L system and other processes. Considering its functional significance in neuronal survival, axonal transport might hold clues for understanding pathological mechanisms. Abnormal

axonal swelling with lysosomal accumulation has been noted in Alzheimer's disease, blocking the retrograde transport of autophagosomes (Nixon *et al.* 2005; Gowrishankar *et al.* 2015). Similar understanding on the health of axonal transport machinery and its dynamics in PD is crucial toward finding commonalities amongst neurodegenerative diseases.

Sometimes, there are traffic issues: E-L system trafficking in PD

The multi-compartmentalized nature of neurons puts tremendous pressure on the endosomal sorting and trafficking machinery, which is also true in case of nigrostriatal neurons. The vacuolar protein sorting (VPS) group of proteins that form retromer complex comprising of VPS35, VPS26, and VPS29 are crucial in retrograde trafficking and sorting of protein cargos from endosomes to the TGN, facilitating their recycle and reuse (Fig. 1a) (Seaman *et al.* 1998; Haft *et al.* 2000). The retromer complex also functions in sorting proteins/cargo to regulate endosomal and lysosomal maturation (Vagnozzi and Pratico 2018). Pedigrees with single allelic mutation in *VPS35* revealed autosomal dominant, late-onset, L-dopa-responsive PD, with striking similarities to idiopathic PD (Wider *et al.* 2008; Vilarino-Guell *et al.* 2011). Broadly, it is proposed that the mutant *VPS35* fails to associate with other components of the retromer complex, resulting in impaired retrograde trafficking (Williams *et al.* 2017). Induced loss-of-function mutations of *VPS35* *in vitro* resulted in enlargement of late-endosomes and aggregation of α -synuclein oligomers (Follett *et al.* 2016). Another study on flies revealed ceramide accumulation because of *VPS35* deficiency, suggesting lysosomal dysfunction (Lin *et al.* 2018). α -Synuclein accumulation in nigral dopaminergic neurons, loss of dopaminergic neurons, reduced dopamine transporter levels in the striatum, and locomotory deficits were noted in *VPS35*-deficient mice (Tang *et al.* 2015a; Ishizu *et al.* 2016). Further *in vitro* studies of *VPS35* (D620N) mutant cells revealed dysfunction in endosome-to-Golgi retrieval of lysosomal membrane proteins (LAMP2a) and accelerated lysosomal degradation of LAMP2a (Tang *et al.* 2015a). LAMP2a is considered important in directing misfolded α -synuclein to lysosomes, suggesting a mechanism for α -synuclein accumulation in *VPS35* deficiency (Tang *et al.* 2015a). *VPS35* mutations also affect autophagy and dendritic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking (Zavodszky *et al.* 2014; Munsie *et al.* 2015). *VPS35* mutations bring severe abnormality in mitochondrial dynamics, as seen in mouse dopaminergic neuronal cultures and in PD patient derived fibroblasts (Tang *et al.* 2015b). Oxidative stress implicated in sporadic PD might mediate pathogenesis through *VPS35*-driven mitochondrial defects (Wang *et al.* 2016). *VPS35* also has a role in biogenesis of mitochondria-derived vesicles,

further suggesting that its dysfunction causes mitochondrial pathology and neurodegeneration (Sugiura *et al.* 2014). VPS35 is functionally linked to LRRK2, boosting its kinase activity (Mir *et al.* 2018). In fact, decreased VPS35 levels were noted in the SNpc of PD patients with *LRRK2* mutations (Zhao *et al.* 2018). Interestingly, over-expression of VPS35 ameliorated locomotor deficits and enhanced lifespan in *LRRK2* mutant models (MacLeod *et al.* 2013; Linhart *et al.* 2014), which was also seen in *Parkin* mutant models (Malik *et al.* 2015). Neuroprotection was also noted against MPP⁺ induced toxicity in dopaminergic neurons (Bi *et al.* 2013). Along with its E-L sorting functions, these links between VPS35 and α -synuclein, LRRK2, Parkin, lysosomal dysfunction, autophagy, mitochondrial perturbations, and oxidative stress strongly suggest VPS35 as a good therapeutic target.

Another VPS family member associated with PD is VPS13C. Five allelic variations in the *VPS13C* gene can lead to autosomal recessive, early-onset PD, primarily because of premature termination of VPS13C (Lesage *et al.* 2016; Schormair *et al.* 2018). Patients were noted with cardinal PD symptoms and extrapyramidal signs. Post-mortem studies revealed α -synuclein/ubiquitin positive-Lewy bodies in the brainstem, limbic system, hippocampus, and cortical associative areas (Lesage *et al.* 2016). A few studies reveal that VPS13 functions in the TGN-endosomal cycle (De *et al.* 2017), whereas recent discoveries have also located it on the outer mitochondrial membrane (Lesage *et al.* 2016). In fact, siRNA-mediated silencing of VPS13C resulted in lower mitochondrial membrane potential, mitochondrial fragmentation, increased respiratory rates, exacerbated Parkin-dependent mitophagy, and up-regulation of Parkin in response to mitochondrial damage (Lesage *et al.* 2016). A new study has revealed VPS13C's role in forming membrane contact sites and facilitating lipid transfer between the ER and mitochondria (Kumar *et al.* 2018). This is noteworthy, considering lipid transfer's increasing recognition in disease pathogenesis.

Lysosomes: PD may also begin at the end of the tunnel

Since lysosomes are the destination for most E-L system vesicles carrying a variety of cargos for degradation and recycling (Fig. 1a), dysfunction in any of its components results in lysosomal storage disorders, severely perturbing cellular homeostasis. A growing body of evidence suggests lysosomal storage disorders increase risk for PD pathogenesis (Robak *et al.* 2017). Among them, Gaucher disease (GD) confers the greatest risk, where 5–15% of GD patients or carriers develop PD later in life, to an extent where causal *GBA* mutations are the most common genetic risk factor for PD (Table 1) (Aharon-Peretz *et al.* 2004; Simon-Sanchez *et al.* 2009; Bultron *et al.* 2010). *GBA* encodes glucocerebrosidase 1 (Gcase1), which facilitates breakdown of glucosylceramide (GlcCer) into

glucose and ceramide. In its absence, GlcCer accumulates and undergoes partial enzymatic deacylation to glucosylsphingosine and other more complex glycosphingolipids, which mediate disease pathogenesis. *GBA* is a relatively well-studied E-L system gene with regards to PD, in terms of post-mortem brains, clinical investigations, and basic research. Reduced Gcase1 activity in substantia nigra, putamen, and other brain areas of PD patients with *GBA* mutations have been noted along with GlcCer and glycosphingolipids accumulation (Gegg *et al.* 2012; Alcalay *et al.* 2015). Interestingly, similar observations were made even in sporadic PD patients (Alcalay *et al.* 2015; Rocha *et al.* 2015), suggesting an even broader role for *GBA* than just mutations and potentially involve epigenetic and environmental modifiers of *GBA*. Gcase1 levels decrease with aging, the major risk factor for PD (Rocha *et al.* 2015; Hallett *et al.* 2018), indicating *GBA*'s possible role in lowering the nigral neurons' threshold to develop PD. Cognitive areas such as hippocampus, frontal cortex, and amygdala also showed relative decrease in Gcase1 activity, along with a significant elevation of hippocampal glucosylsphingosine in sporadic cases (Gegg *et al.* 2012). This correlates with a higher Lewy body burden identified in the hippocampus and medial temporal regions, as well as in a diffused cortical Lewy body pathology in *GBA*-linked PD (Watson and Leverenz 2010; Hallett *et al.* 2018). These pathological observations strengthen clinical findings that cognitive deterioration is severe in PD patients with *GBA* mutations compared to that of sporadic cases (Liu *et al.* 2016; Mata *et al.* 2016). These features indicate that mouse models of PD with *GBA* mutations are important in studying lipid dyshomeostasis and mechanisms of cognitive dysfunction in PD.

A recent study by Taguchi *et al.*, provides evidence for an association between loss of Gcase1 activity, glycosphingolipids accumulation, and α -synuclein aggregation, both *in vitro* and *in vivo* models of *Gba* mutation (Taguchi *et al.* 2017). Glucosylsphingosine which promoted pathological α -synuclein aggregates, capable for further self-templating, was shown to accumulate in the brains of young homozygous *Gba* mutant and KO mice. This strongly implicates glucosylsphingosine in PD pathogenesis. Even in heterozygous *Gba* mutations, the Gcase1 activity decreased with age to an extent where mice reached threshold for haploinsufficiency, mimicking the human condition. Furthermore, the *Gba* mutant mice, when crossed with well-characterized α -synuclein transgenic PD mice, revealed accelerated PD progression including α -synuclein aggregation and motor deficits (Taguchi *et al.* 2017). α -Synuclein aggregation has also been seen in human iPSCs-derived neurons from GD and PD patients that are exposed to Gcase1 inhibitors, and in those carrying *GBA* mutations (Zunke *et al.* 2018). Heterozygous mutations in *GBA* also cause misprocessing of Gcase1 in the ER, leading to ER retention of Gcase1. This produces ER stress and auto-lysosomal perturbations causing an abnormal lipid profile and increased α -synuclein

accumulation and secretion (Ron and Horowitz 2005; Fernandes *et al.* 2016). This study along with a few other observations suggest dopaminergic dysfunction because of gain-of-function mutations in *Gba* (Cullen *et al.* 2011; Sidransky and Lopez 2012; Fernandes *et al.* 2016). However, therapeutic strategies based on loss-of-function observations have been successful, arguing against a gain-of-function model. Activation of residual Gcase1 by small molecules and chaperones and ameliorating glycosphingolipid accumulation by inhibiting GlcCer synthase lowered α -synuclein pathology and reinstated lysosomal health (Richter *et al.* 2014; Sardi *et al.* 2017; Charvin *et al.* 2018). These strategies are already being tested in clinics (Richter *et al.* 2014).

ATPase cation transporting 13A2 (ATP13A2), another lysosomal protein which localizes on its membrane (Fig. 1a) with unknown function, has been implicated in PD (*PARK9*, Table 1). Autosomal recessive, early-onset parkinsonism because of *ATP13A2* mutations first noted in Kufor-Rakeb Syndrome, followed by recent studies in other forms of parkinsonism (Ramirez *et al.* 2006; Di Fonzo *et al.* 2007; Park *et al.* 2011; Eiberg *et al.* 2012). Fibroblasts from these patients showed impaired lysosomal acidification and proteostasis, leading to inefficiency in substrate degradation and autophagosome clearance. These observations were replicated in *ATP13A2* knockdown dopaminergic cell lines, where its restoration re-established lysosomal homeostasis and ameliorated cell death (Dehay *et al.* 2012). Down-regulation of *ATP13A2* was also seen in nigral neurons of sporadic PD patients, along with *ATP13A2* traces in Lewy bodies (Dehay *et al.* 2012). A loss-of-function *ATP13A2* mutant mouse model revealed age-related lysosomal dysfunction, protein aggregation, and gliosis leading to motor abnormalities. Interestingly, the levels of total and oligomeric α -synuclein was normal, despite ubiquitinated protein aggregation, cathepsin-D dysfunction, and autophagy defects (Kett *et al.* 2015). These observations suggest that *ATP13A2* mutations may lead to PD through an α -synuclein independent mechanism.

E-L system; a key target of key PD-associated proteins?

α -Synuclein

Misfolded α -synuclein can trigger PD pathogenesis in the pre-synaptic terminals of nigrostriatal neurons (Kramer and Schulz-Schaeffer 2007; Spinelli *et al.* 2014; Imbriani *et al.* 2018). This may well be one of the earliest pathogenic events in PD, where α -synuclein aggregates target SV endocytosis, SV trafficking, and autophagy components of E-L system to initiate or exacerbate dopaminergic neuronal dysfunction.

α -Synuclein and other family members function in pre-synaptic terminals, where they modulate SV distribution and neurotransmission (Gretchen-Harrison *et al.* 2010; Burre 2015;

Vargas *et al.* 2017) (Fig. 1c). The biophysical properties of synucleins reveal their ability to sense and modify membrane curvature (Middleton and Rhoades 2010; Shen *et al.*, 2012; Westphal and Chandra 2013). These functions suggest that synucleins regulate SV exocytosis and endocytosis (Gretchen-Harrison *et al.* 2010; Vargas *et al.* 2014). Studies in KO mice in fact show that synucleins normally function post SV exocytosis, in fusion pore expansion and/or control the kinetics of SV endocytosis by facilitating membrane curvature (Vargas *et al.* 2014; Lautenschlager *et al.* 2017; Logan *et al.* 2017). α -Synuclein also appears to control SV availability for release by regulating SV clustering (Diao *et al.* 2013; Vargas *et al.* 2017).

On a pathological level, several studies show that over-expression of α -synuclein inhibits neurotransmission. The locus of this consistent effect is not clear. Studies have shown that elevated α -synuclein levels and oligomeric α -synuclein inhibit SV exocytosis in a SNARE-dependent manner or by affecting SV clustering (Nemani *et al.* 2010; Huang *et al.* 2019). On the other hand, considerable evidence suggests that aggregated α -synuclein primarily impacts SV endocytosis, which is of relevance to E-L system genes linked to PD. In lamprey giant synapses, injection of aggregated α -synuclein inhibit clathrin uncoating as evidenced by the accumulation of CCVs when SV recycling was evoked by high-frequency stimulation (Busch *et al.* 2014). A recent study also suggests that extra synuclein might sequester Hsc70, thus affecting clathrin uncoating and subsequently causing SV recycling defects (Banks *et al.*, 2019). A similar observation was made in mammalian Calyx of Held synapse, where acute elevation of monomeric and mutant α -synuclein levels decreased SV endocytosis with a frequency-dependent effect on SV exocytosis. This was also seen upon transgenic expression of α -synuclein (Xu *et al.* 2016; Eguchi *et al.* 2017). Over-expression of mutant α -synuclein also affects dopamine transporter trafficking on the pre-synaptic membrane, leading to dopamine dyshomeostasis (Kisos *et al.* 2014). The observations that over-expression and deletion of synucleins have similar effects on SV exo- and endocytosis (Lautenschlager *et al.* 2017) suggest that SV cycling is exquisitely sensitive to α -synuclein levels. Further understanding of the precise mechanisms for interactions of α -synuclein and synaptic E-L system proteins involved in PD will provide crucial insights on early causal pathogenic events in PD.

At the soma, aggregated α -synuclein mainly targets E-L system trafficking, especially the retromer pathway. Recent studies propose that aggregated α -synuclein disrupts the retromer complex by interacting through VPS26 or VPS29, and thus preventing the recruitment of adaptors like Snx3 and SNX-BAR dimers (Chung *et al.* 2017; Patel and Witt 2018). Deficiency of VPS35 in dopaminergic neurons impairs endosome-to-Golgi retrieval of receptors like LAMP2a,

enhancing its lysosomal degradation. LAMP2a is a critical receptor for chaperone-mediated autophagy of misfolded α -synuclein, so its deficiency leads to a vicious cycle of α -synuclein accumulation (Tang *et al.* 2015a), which might be the case in patients with VPS35 mutations. Over-expression of α -synuclein also directly compromises macroautophagy through inhibition of Rab1a (Winslow *et al.* 2010). It is also pertinent to note that aggregated α -synuclein in the soma is a key target of treatment strategies in development through induction of macroautophagy (Suresh *et al.* 2017; Suresh *et al.* 2018a).

Recent developments suggest that α -synuclein pathology spreads in a prion-like manner through synaptically connected regions in a manner consistent with Braak staging (Braak *et al.* 2003; Luk *et al.* 2012). The E-L system plays a prominent role in cell-to-cell transmission of preformed α -synuclein filaments, leading to the spread of phospho- α -synuclein pathology. Internalization of fibrillar α -synuclein occurs via endocytosis (as well as tunnelling nanotubules) and follows the neuronal endosomal pathway to lysosomal degradation (Rodriguez *et al.* 2018). Heparan sulfate proteoglycans, lymphocyte-activation gene 3, $\alpha 3$ isoform of Na^+/K^+ ATPase, and a few endocytic vesicle receptors are predicted to act as receptors for α -synuclein preformed fibrils (Holmes *et al.* 2013; Shrivastava *et al.* 2015; Mao *et al.* 2016; Ihse *et al.* 2017). *In vitro* studies suggest that internalized α -synuclein fibrils are localized in both early endosomes and late endolysosomes (Lee *et al.* 2008b; Karpowicz *et al.* 2017). The lipid bilayer of endosomes restricts cytoplasmic spillage, so most endocytosed α -synuclein is degraded by lysosomes. How a fraction of α -synuclein preformed fibrils escapes this barrier and goes on to initiate seeding still remains unclear (Bieri *et al.* 2018). Studies of α -synuclein axonal transport predict that retrograde transport of α -synuclein preformed fibrils is mediated by endocytic vesicles and is twice as fast and more efficient than anterograde transport (Brahic *et al.* 2016). This strengthens the notion that PD pathology spreads retrogradely from the synaptic terminal to soma. The E-L system is also involved in exocytosis of fibrillized α -synuclein, facilitating its prion-like spread. An ER-bound enzyme with chaperone activity USP19 (ubiquitin-specific protease 19) has been shown to deliver misfolded α -synuclein to late endosomes, which then fuses with the plasma membrane through a unique process called misfolding-associated protein secretion (Lee *et al.* 2016). Another recent study suggests that the DNAJ/Hsc70 complex could facilitate α -synuclein exocytosis (Fontaine *et al.* 2016). Over-expression of *ATP13A2* and *GBA* have been shown to increase exocytosis of α -synuclein (Tsunemi *et al.* 2014; Fernandes *et al.* 2016). Together, these observations identify the E-L system as the primary conduit for the spread of α -synuclein-mediated pathology in PD.

LRRK2

Leucine-Rich Repeat Kinase 2 (LRRK2) is a multidomain large protein with both GTPase and kinase activity along with other domains for protein-protein interactions (Kett and Dauer 2012). Epidemiological, histopathological, and clinical investigations have all established *LRRK2* gene mutations as the most common genetic cause of PD (*PARK8*, Table 1) (Paisan-Ruiz *et al.* 2004; Zimprich *et al.* 2004), with a considerable contribution to sporadic cases (Kett and Dauer 2012). Over eight identified *LRRK2* mutations cause PD. The mutation at G2019S is the most common and is known to increase LRRK2 kinase activity (West *et al.* 2005; Smith *et al.* 2006; Kett and Dauer 2012). LRRK2 functions have been explored through PD-related studies, which have revealed its close association with the E-L system. LRRK2's role in vesicular trafficking, lysosomal homeostasis, and autophagy are well-accepted. Phosphorylation by LRRK2 is essential for a subset of RAB GTPases' function of E-L vesicular sorting and trafficking (Steger *et al.*, 2016; Steger *et al.*, 2017). In an over-expression system, RAB7L1 was noted to interact strongly with LRRK2 at the switch-II site (Steger *et al.*, 2017), unlike as seen in *in vitro* kinase assays (Steger *et al.*, 2016). Several *LRRK2* mutations significantly affect RAB pathways, where variations in Rab7L1's interactions with LRRK2 have been identified to increase the risk of sporadic PD. In fact, recent GWAS identify *RAB29* encoding RAB7L1 (*PARK16*, Table 1) as an independent risk gene for PD (Satake *et al.* 2009). Rab7L1 up-regulation could attenuate LRRK2-induced toxicity in dopaminergic neurons (MacLeod *et al.* 2013). This is predicted to be functioning through VPS35 and VPS26 (Linhart *et al.* 2014). VPS35 also cooperates with LRRK2 to regulate SV recycling and dopaminergic synaptic release (Inoshita *et al.* 2017), thus suggesting a prominent role for the retromer complex in LRRK2-mediated PD pathogenesis.

LRRK2 regulates lysosomal protein trafficking and morphology (Kuwahara *et al.* 2016; Eguchi *et al.* 2018). In *LRRK2* mutants, perinuclear lysosomal clustering was noted, mediated by increased phosphorylation of RAB7 (Hockey *et al.* 2015). Two-pore channels are the calcium channels present on acidic vesicles of the E-L system and help in regulating their trafficking and fusion of lysosomes with autophagosomes/endosomes (Calcraft *et al.* 2009). Abnormal lysosomal morphology and density seen in fibroblasts from patients with LRRK2 mutations could be reversed by inhibiting these two-pore channels (Hockey *et al.* 2015). Thus, these channels might be one potential mediator of E-L system dysfunction in LRRK2-associated PD. Lysosome-related proteins involved in PD such as Gcase1, LAMP2A, and ATP13A2 are also down-regulated in PD patients with *LRRK2* mutations (Zhao *et al.* 2018), suggesting that lysosomes are one of LRRK2's prime targets.

Mechanisms for LRRK2-mediated autophagy defects are still elusive. The ability of LRRK2 kinase inhibitors to reverse autophagy dysfunction suggest that kinase activity may be significant (Manzoni *et al.* 2013; Saez-Atienzar *et al.* 2014). Mutant *LRRK2* is also a substrate for chaperone-mediated autophagy, impairment of which could exacerbate pathological implications (Orenstein *et al.* 2013). Accumulation of MVBs and malformed autophagosomes were noted in *LRRK2* R1441C mutations (Alegre-Abarrategui *et al.* 2009). Intra-axonal autophagic vacuole defects in nigral dopaminergic projections were seen in mice expressing human *LRRK2* (Li *et al.* 2009; Tagliaferro *et al.* 2015). *LRRK2* also regulates synaptic autophagy through phosphorylation of endophilin A (Soukup *et al.* 2016). G2019S mutations are known to induce autophagy through MAPK/ERK kinases (Bravo-San Pedro *et al.* 2012; Bravo-San Pedro *et al.* 2013), and some studies suggest that *LRRK2*-mediated autophagy regulation is largely independent of mTOR (Manzoni *et al.* 2013). Since RAB-mediated trafficking is crucial for autophagy, which is also affected in *LRRK2* mutations, it is important to evaluate if autophagy defects are the consequences of RAB dysregulation or a direct effect of *LRRK2* mutations.

LRRK2 regulates the SV cycle by interacting with E-L system proteins that are also implicated in PD: auxilin, SYNJ1, and endophilin A. *LRRK2* overactivity phosphorylates auxilin at its clathrin binding domain, making their interaction weaker. This leads to disruption of SV endocytosis (Nguyen and Krainc 2018). This was seen in patient iPSC-derived dopaminergic neurons, along with accumulation of oxidized dopamine, increased α -synuclein levels, and decreased Gcase1 activity (Nguyen and Krainc 2018). Hyperphosphorylation of endophilin A and synJ1 by *LRRK2* significantly altered synaptic endocytosis (Matta *et al.* 2012; Islam *et al.* 2016; Pan *et al.* 2017). A recent study showed that *LRRK2* can directly bind to clathrin-light chains through its GTPase domain, affecting Rac1-mediated endosomal dynamics (Schreij *et al.* 2015). *LRRK2* also perturbs synaptic exocytosis through phosphorylation of N-ethylmaleimide-Sensitive Factor which up-regulates SNARE disassembly (Belluzzi *et al.* 2016). Furthermore, *LRRK2* is highly expressed in dorsal striatal medium spiny neurons (MSNs), the post-synaptic compartment of nigrostriatal pathway. G2019S mutation of *LRRK2* leads to abnormally high activity of MSNs and morphological perturbations during development (Matikainen-Ankney *et al.* 2016). Abnormal synaptogenesis and dopamine receptor activation in the developing MSNs mediated by dysfunctional protein kinase A was seen in *LRRK2* R1441C mutation (Parisiadou *et al.* 2014). These defects can permanently modify the circuit structure and function, predisposing the individuals to both motor and non-motor dysfunctions of PD. *LRRK2* mutation-mediated endocytosis defects were noted predominantly in these MSNs, along with nigral dopaminergic

neurons and not in cortical or hippocampal neurons (Pan *et al.* 2017; Maas *et al.* 2017). This suggests a heightened vulnerability in the E-L system of nigrostriatal circuit for *LRRK2* mutations.

Increased *LRRK2* kinase activity mediated elevation of exocytosis may also be relevant for cell-to-cell transfer of misfolded α -synuclein (Lin *et al.* 2009; Bae *et al.* 2018; Schapansky *et al.* 2018). *LRRK2* can be selectively recruited to overloaded lysosomes and divert undegraded materials out of the kidney cells, along with secretion of α -synuclein from endosomes (Eguchi *et al.* 2018). Studies also demonstrate heightened uptake of α -synuclein fibrils through endocytosis in the presence of pathogenic *LRRK2* (Bae *et al.* 2018). However, *LRRK2*'s role in prion-like spreading of α -synuclein remains speculative, until confirmation that similar mechanisms exist in neurons.

Future directions

Clinical description and diagnosis of PD has changed little since James Parkinson's characterization of the disease 200 years ago (Parkinson 2002). In the 1960s, identification of dopamine deficiency as the cause of motor abnormalities led to symptomatic relief for patients (Carlsson *et al.* 1957). Although some success has been noted with deep brain stimulation (Limousin *et al.* 1998), we are still relying on symptomatic relief. The last three decades of basic research have enriched our understanding of PD's cellular and molecular mechanisms, bringing tangible hope for disease modifying treatment strategies. Identification of risk genes and conceptual shifts in our understanding through genetic models point to E-L system dysfunction as the key mediator of PD pathogenesis. Further strengthening this concept, PD pathogenesis appears to begin at synaptic terminals where endo- and exocytotic machinery is most active and where α -synuclein and *LRRK2* exert their effects through the E-L system and vice-versa. Studies so far suggest that altered flux through the E-L system in nigral dopaminergic neurons that have lesser threshold for degeneration is the key trigger for neurodegeneration. Moving forward, critical elucidation of the mechanisms behind sorting, trafficking, and recycling defects demands detailed studies of auxilin and other DNAJ family proteins, along with SYNJ1, endophilin A, and the retromer complex. Studying these proteins/genes will also provide crucial insights into less explored roles of synaptic autophagy, axonal transport, and clathrin-independent endocytosis in PD. Recent surveys of PD patients note preservation of cognitive functioning as a major unmet need. Further investigation of *GBA* mutations implicated in PD is a promising avenue considering *GBA*'s known involvement in cognitive disorders like Dementia with Lewy Bodies. Along with *GBA*, studying *ATP13A2* and other lysosomal genes will enrich our understanding of lysosomal dysfunction, potentially bringing us closer to repurposing drugs used

in lysosomal storage disorders for PD. Affirming ongoing clinical trials targeting autophagy, α -synuclein, and LRRK2, an enhanced understanding of E-L system-mediated pathogenesis will prove crucial in developing novel treatment strategies and diagnostic tools for PD.

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