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MINI REVIEW
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Why Malformations of Cortical Development Cause Epilepsy

*Alfonso Represa**

INSERM, Institut de Neurobiologie de la Méditerranée, Aix-Marseille University, Marseille, France

Malformations of cortical development (MCDs), a complex family of rare disorders, result from alterations of one or combined developmental steps, including progenitors proliferation, neuronal migration and differentiation. They are an important cause of childhood epilepsy and frequently associate cognitive deficits and behavioral alterations. Though the physiopathological mechanisms of epilepsy in MCD patients remain poorly elucidated, research during the past decade highlighted the contribution of some factors that will be reviewed in this paper and that include: (i) the genes that caused the malformation, that can be responsible for a significant reduction of inhibitory cells (e.g., ARX gene) or be inducing cell-autonomous epileptogenic changes in affected neurons (e.g., mutations on the mTOR pathway); (ii) the alteration of cortical networks development induced by the malformation that will also involve adjacent or distal cortical areas apparently sane so that the epileptogenic focus might be more extended that the malformation or even localized at distance from it; (iii) the normal developmental processes that would influence and determine the onset of epilepsy in MCD patients, particularly precocious in most of the cases.

Keywords: cortical malformation, epileptogenesis, developmental disorder, ARX, mTOR, focal cortical dysplasia, gray matter heterotopia

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Showa University, Japan
Stéphanie Baulac,
INSERM U1127 Institut du Cerveau et de la Moelle épinière, France

***Correspondence:**
Alfonso Represa
alfonso.represa@inserm.fr

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INTRODUCTION

Malformations of cortical development (MCD) are a complex family of rare disorders that result from alterations of one or combined developmental steps, including proliferation of neural progenitors, migration of neuroblasts to the cortical plate, layer organization and neuronal maturation (Barkovich et al., 2012). Thus, in general, alterations of neuronal and glial proliferation associating neuronal dysgenesis are a cause of focal cortical dysplasia type II (FCD-type II); alterations of neuronal migration leading to ectopic localization of neurons are a cause of periventricular nodular heterotopia (neurons accumulate along the ventricular walls) and subcortical band heterotopia (SBH; neurons accumulate in the white matter between the cortex and the ventricular wall); alterations on processes subsequent to neuronal migration are at the origin of polymicrogyria, characterized by a cortex organized in multiple small gyri (Barkovich et al., 2012).

Malformations of cortical development are an important cause of childhood epilepsy. Though the precise incidence of MCDs is not known, it is estimated that they account for up to 40% of cases of intractable or medication-resistant childhood epilepsies (Barkovich et al., 2012; Guerrini and Dobyns, 2014) and that at least 75% of the patients with MCDs will have epilepsy (Leventer et al., 1999). Associated to seizures, the patients display different comorbidities, particularly cognitive deficits, that are more frequent and severe when epilepsy begins early in life (Berg et al., 2017). MCDs consequently represent a severe burden for patients, families and society.

Though the physiopathological mechanisms of epilepsy in MCD patients remain unclear, clinical and experimental data suggest that epileptogenesis results from diverse developmental processes that can be cell autonomous or not, directly imputable to the genetic cause of the malformation or linked to an abnormal development of neuronal networks. In this review, we will discuss the pathophysiological mechanisms involved in MCD epileptogenesis through the analysis of three distinct matters: the genetic causes of MCDs, the localization of the epileptogenic focus and the age at the epilepsy onset in patients and animal models.

CAUSATIVE GENES AND EPILEPTOGENESIS

The classification of cortical malformations according to the developmental step involved (proliferation, migration or differentiation) or the type of malformation generated (e.g., gray matter heterotopia, lissencephaly, focal cortical dysplasia) does not provide clear indications about the epileptogenic process. However, the analysis of causative genes and their respective cellular roles, provided in some cases interesting data that would help the understanding of the epileptogenic process.

The pathogenesis of MCDs is multifactorial involving different genes and environmental factors. There is a large and increasing number of genes identified during the past decade as causative of MCD (Guerrini and Dobyns, 2014). This is an exciting research field that is also contributing to our knowledge of genetic factors controlling brain development. However, the objective of this report is not to provide a complete overview of the genetic causes of MCDs, but to discuss some examples that help our understanding of epileptogenesis.

One of the more fascinating genes causing brain malformation is ARX (aristaless related homeobox gene). *In vitro* and *in vivo* studies have shown that the Arx gene is both a positive and a negative regulator of gene transcription important for brain development (Collombat et al., 2003; Seufert et al., 2005; McKenzie et al., 2007; Fullenkamp and El-Hodiri, 2008). Among the roles of this gene are the regionalization of the brain, the proliferation of cortical progenitors, the migration of interneurons and early commitment of cholinergic neurons (Colombo et al., 2004; Marsh et al., 2009, 2016; Friocourt and Parnavelas, 2010). Numerous mutations of the ARX gene have been reported in more than a dozen different early neurological disorders, where intellectual disability is associated or not with epileptic seizures (Bienvenu et al., 2002; Kitamura et al., 2002; Stromme et al., 2002). These conditions do or do not associate brain malformations during embryonic development (Shoubridge et al., 2010). Phenotypic heterogeneity may, in part, be explained by the nature and location of ARX mutations (Kato et al., 2004; Olivetti and Noebels, 2012). Indeed, phenotypes without malformation are mainly caused by mutations that are in the polyalanine domains and outside the homeodomain. Conversely, the more severe phenotypes with brain malformation are mostly associated with mutations leading to protein truncation or located in the homeodomain; this is the case for the XLAG syndrome characterized by a severe lissencephaly, agenesis of the corpus callosum and abnormal genitalia. Animal models so far generated have shown that Arx deficient mice or Knockin mice displaying Arx mutations associate a more or less pronounced reduction of cortical GABAergic and cholinergic neurons (reviewed in Olivetti and Noebels, 2012) and the analysis of post-mortem brain tissue reported a three-layered cortex containing exclusively pyramidal neurons in XLAG patients from three different families (Bonneau et al., 2002). In the context of developmental malformations, ARX related syndromes can be thus considered as "interneuronopathies" and the epilepsy and cognitive deficits reported in patients and animal models, would be directly related to the reduction of inhibition. Glutamatergic neurons do not express ARX and are not directly affected by the mutation of ARX but support the consequences and are thus responsible for the expression of epileptic seizures.

Mutations affecting the activation of the mammalian target of rapamycin (mTOR)-signaling pathway have been identified in focal malformations of cortical development associating alterations of progenitor cell proliferation, defective neuronal migration and lamination and the presence of cytomegalic neurons and balloon cells as a result of a defective differentiation program of cortical cells. These malformations include FCD type II and Hemimegalencephaly (Harvey et al., 2008; Blümcke et al., 2011; D'Gama et al., 2017). Tuberous sclerosis, a rare multisystem genetic disease condition that in the brain generates cortical tubers (focal distortions in cellular organization and morphology which extend into the subcortical white matter) is also caused by a hyperactivation of mTORC1, due to mutations in either TSC1 or TSC2 genes (European Chromosome 16 Tuberous Sclerosis Consortium, 1993). This disorder presents intractable epilepsy, cognitive disability, and autism spectrum disorders. Interestingly, tuberal lesion display cellular features similar to FCD type II (i.e., cytomegalic neurons and balloon cells). Therefore, collectively, these disorders might be referred to as "mTORopathies" (Reviewed by Crino, 2015; Marsan and Baulac, 2018). During the past decade somatic activation mutations in mTOR itself have been identified in these syndromes (Lim et al., 2015; Mirzaa et al., 2016; Møller et al., 2016; D'Gama et al., 2017; Ribierre et al., 2018). In addition, positive (e.g., gain of function mutations in Akt1 or AKT3; Lee et al., 2012; Poduri et al., 2012; Jansen et al., 2015) or negative (e.g., TSC2, or DEPDC5; Baulac et al., 2015; D'Gama et al., 2017; Lim et al., 2017) regulators of mTOR have been implicated in FCD type II and Hemimegalencephaly.

Because some patients with FCD are surgically treated, there have been opportunities for investigating on resected tissue neuronal properties (reviewed by Abdijadid et al., 2015). These investigations described for example an abnormal expression of glutamate and GABA receptor in dysplastic and heterotopic neurons (Crino et al., 2001; Lozovaya et al., 2014), a reduction of $GABA_A$ -receptor-mediated inhibition (Calcagnotto et al., 2005), an altered pattern of expression and distribution of synaptic protein SV2 (Toering et al., 2009). Carlos Cepeda and coworkers nicely evaluated the electrophysiological properties of cytomegalic neurons and balloon cells and identified in dysplastic areas the presence of neurons with immature cellular

and synaptic properties (Cepeda et al., 2007); these observations gave origin to the hypothesis that local interactions of dysmature cells, that would be directly affected by the mutations, with normal postnatal neurons produce seizures (Cepeda et al., 2007). Though these investigations did not elucidate the exact mechanisms of epileptogenesis in FCD, they indicate clearly that different cell types, molecular changes and cellular interactions are contributing factors.

Many neuronal changes reported on neurons from FCD type II or tuber resections would be considered as cell autonomous as they indicate that the hyperactivation of the mTOR pathway leads to neuronal and synaptic dysfunctions, that contribute to epilepsy, independently from the cytoarchitectonic alteration. This notion has been confirmed by Hsieh et al. (2016) when investigating a murine model of type II FCDs by increasing mTOR activity in layer 2/3 neurons of the medial prefrontal cortex. When the hyperactivation of mTOR was induced in cortical progenitors by in utero electroporation, animals displayed dyslamination and cytomegalic neurons; associated to these changes, animals developed spontaneous tonic-clonic seizures. However, when mTOR hyperactivation was induced after corticogenesis, thanks to the use of inducible vectors, animals still displayed an epileptic condition without neuronal misplacement and dysmorphogenesis. In agreement with this, my lab has shown that heterozygous mice from a Tsc1-KO line, develop spontaneous seizures during the first month of life in the absence of any apparent cortical dysplasia (Lozovaya et al., 2014). In this study we demonstrated that Tsc1+/−neurons, particularly L4 spiny stellate cells, display an anomalous expression of NR2C receptors resulting in a change on the kinetics of NMDA currents and that this change was sufficient to induce the epileptic condition. Indeed, the treatment of pups with antagonists specific for NR2C transiently abolished seizures (Lozovaya et al., 2014). We also demonstrated in this report that treatment of newborn mice with rapamycin was sufficient to reverse the phenotype confirming the link between mTORC1 hyperactivation and NMDA receptor changes. Interestingly, cortical resections from patients with tuberous sclerosis and FCD type II also demonstrated higher expression levels of NR2C as compared with control fetal or adult samples (Lozovaya et al., 2014); in addition to this, patch-clamp recordings on these cortical resections demonstrated the contribution of NR2C to NMDA currents confirming the potential role of NR2C to epileptogenesis in mTORpathies.

It is also important to remind, however, that human samples investigated were obtained from severely affected, pharmaco-resistant patients with resection of the epileptogenic zone being the only therapeutic option. The epileptic process by itself can be cause of many subsequent alterations, including an excitatory/inhibitory imbalance. Thus, based on investigations of a Tsc1-KO mice line, Bateup et al. (2013) reported that many biochemical, transcriptional and functional changes in Tsc1 neurons arise secondarily, due to increased network activity. To study the epileptogenic process itself, we must develop appropriate experimental paradigms in order to evaluate the changes that increase the excitability of neurons and networks and that take place before epilepsy onset.

In conclusion, the two paradigmatic examples discussed before, suggest that the initial genetic alteration that yields cytoarchitectonic disruptions of cortical development, might also be responsible for the clinical manifestations. However, these investigations also highlighted to a certain extent the importance of cellular interactions and the possibility that developmental changes, that might involve non-mutated neurons during a particularly vulnerable developmental period, could also contribute to the emergence of epilepsy and/or cognitive deficits.

THE EPILEPTOGENIC NETWORK

Investigations combining EEG and functional imaging have demonstrated that patients with focal cortical malformations display interictal or ictal events not only in the affected area (the malformation itself) but also in more or less distal cortical areas. Thus, reports of patients with SBH or periventricular nodular heterotopia indicate that the epileptogenic network is restricted to the heterotopia, or involves both the heterotopia and the surrounding cortex or localizes out of the heterotopia (Mai et al., 2003; Tassi et al., 2005; Kobayashi et al., 2006; Tyvaert et al., 2008; Valton et al., 2008; Christodoulou et al., 2012; Shafi et al., 2015; Pizzo et al., 2017). In patients with FCD, it has also been reported that epileptogenicity extends beyond the limit of the malformation in many patients (Aubert et al., 2009). Another pathological conditions in which the adjacent or even more distal cortex can be the primary origin of epileptiform activity is the tuberous sclerosis. Although tubers are thought to be the initial heart of the epileptogenic zone, electrocorticographic recordings of some patients revealed epileptiform activities and ictal onsets in the perituberal cortex (Madhavan et al., 2007; Major et al., 2009; Ma et al., 2012).

Though conclusions are hampered by the diversities of clinical courses, that includes variabilities on epilepsy onset, type of seizures, efficiency of AEDs, cortical area affected, etc., these data tend to support the notion that epileptic network frequently involves supposedly "healthy" cortical areas that are affected by the presence of a cortical malformation. We investigated this notion in a rat model of SBH induced by in utero (by embryonic day 15) knockdown (KD) of Dcx, the main causative gene of this condition (des Portes et al., 1998; Gleeson et al., 1998; Pilz et al., 1998). In Dcx-KD rats, affected neurons fail to migrate to the cortical plate and form a band of ectopic neurons in the white matter of the electroporated hemisphere (Bai et al., 2003). Dcx-KD rats display altered neocortical excitability already present at the second postnatal week, resulting in an increased propensity for convulsant-induced seizures and spontaneous absence-like seizures in adulthood (Ackman et al., 2009; Manent et al., 2009; Lapray et al., 2010).

We first investigated in Dcx-KD juvenile rats the phenotype of ectopic neurons and found that they displayed a reduced dendritic tree as compared with control (mismatch) neurons and a reduced density of dendritic spines (Ackman et al., 2009; Martineau et al., 2018). We also performed genetic labeling of scaffolding proteins PSD-95 and gephyrin for quantifying, respectively, glutamatergic and GABAergic synapses

in ectopic neurons and observed that they were severely reduced (Martineau et al., 2018). These structural changes were associated with a decrease of the frequency of glutamatergic and GABAergic synaptic currents in patch-clamp recordings (Ackman et al., 2009; Martineau et al., 2018). Though DCX also plays a role on neuron maturation (Martineau et al., 2018), the ectopic position of neurons is mainly responsible for the impaired development and synaptogenesis.

Reports on other animal models of cortical migration defects revealed similar features: neurons displayed a simplification of their dendritic arbors in ectopic gray masses induced by either fetal irradiation (Ferrer et al., 1984) or treatment of pregnant rats with methylazoxymethanol (MAM) (Singh, 1980; Chevassus au Louis et al., 1998; Rafiki et al., 1998; Sancini et al., 1998); a significant diminution in spine numbers was also reported in MAM treated offspring (Colciaghi et al., 2014). All together these observations confirm that the positioning of neocortical neurons significantly impacts the subsequent development of dendrites and synapses impairing their integration in functional networks.

In contrast to these dendritic and synaptic defects, ectopic neurons develop axonal projections toward targets that are considered to be "normal" for cortical neurons. In DCX-KD rats, ectopic neurons form cortico-cortical projections like Layer II/III neurons: they reach layers V-VI in the ipsilateral cortex and send axonal projections to the contralateral cortex (Ackman et al., 2009); they also form cortico-striatal connections like Layer V neurons. Similarly, ectopic neurons in irradiated rats and in Tish rats, respectively, lesional and genetic models of SBH, project as normal Layer V neurons to the spinal cord and thalamus (D'Amato and Hicks, 1980; Jensen and Killackey, 1984; Lee et al., 1997). Therefore, ectopic neurons, though poorly innervated as compared with their normotopic counterparts, are in the position of spreading signals to typical cortical targets and participating in the propagation of epileptic activities.

We then analyzed the synaptic properties of normotopic neurons from DCX-KD rats to evaluate if the SBH altered the development of normal positioned neurons. Our studies revealed that normotopic neurons displayed an increased frequency of spontaneous glutamatergic post-synaptic currents while GABAergic currents were unaffected (Ackman et al., 2009). As a consequence, the excitatory/inhibitory ratio of synaptic inputs was increased in normotopic neurons (Ackman et al., 2009). Calcium imaging analysis also revealed that more neurons were active in the cortex overlaying SBH than in the cortex of control rats and that they displayed higher frequency of spontaneous events. In addition, more neurons exhibited synchronized events (Ackman et al., 2009). More recently, in a rat model of bilateral double cortex we also observed that the strength of excitatory L4 to L2/3 synapses (**Figure 1**), the intrinsic properties of L4 glutamatergic cells and the excitation/inhibition ratio in L2/3 converge into making the early stage of cortical sensory integration abnormally strong in somatosensory cortex adjacent to heterotopia, thus demonstrating developmental alterations of cortical functional circuits that likely play a major role in the cortical dysfunction of the malformed brain (Plantier et al., 2018). Investigations of animal models of periventricular heterotopia induced by irradiation (Zhu and Roper, 2000) or treatment with carmustine (Benardete and Kriegstein, 2002) also found an excitatory/inhibitory misbalance in normotopic cortex.

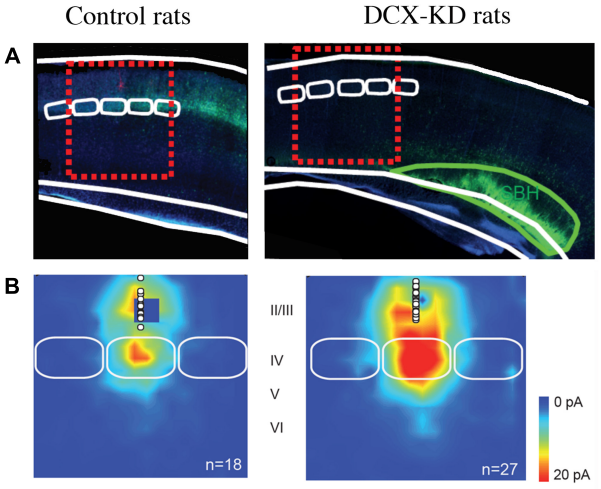

FIGURE 1 | Collateral Alterations of Functional Cortical Circuits in a Rat Model of Subcortical Band Heterotopia. **(A)** Representative examples of across-barrel brain slices from control (Mismatch) and Dcx-KD rats. Fluorescence microphotographs of electroporated cells are superimposed on schematized views of barrels (in white) and SBH (in green). Red dashed line rectangles illustrate the cortical columns evaluated in the study. **(B)** Excitatory synaptic input maps for L2/3 neurons in mismatch and Dcx-KD rats. Colors indicate the mean amplitude of excitatory synaptic responses in a 100-ms time window. White circles show soma positions of recorded L2/3 neurons. Solid white lines delineate barrels. Note that in DCX-KD rats the strength of excitatory inputs to L2/3 neurons are dramatically increased (for more details see Plantier et al., 2018).

Taken together these data support the notion that normotopic cortex becomes hyper excitable during postnatal development and that it would be responsible for epileptogenesis in DCX-KD rats. Interestingly, the suppression of excitability of ectopic neurons by their transfection with potassium channels Kir2.1, did not alter the high propensity of DCX-KD rats to experience seizures, while the transfection of ectopic and normotopic neurons significantly reduces it (Petit et al., 2014). These observations prompted us to evaluate the origin of interictal events on acute slices from DCX-KD rats using 60-channels microelectrode arrays (Petit et al., 2014). Our data demonstrated that most of interictal discharges initiated in normotopic cortex and propagated secondarily to the SBH. *In vivo* recording with deep electrodes in Tish rats (Chen et al., 2000) also indicated that normotopic neurons were more prone to exhibit epileptiform activities than heterotopic neurons and that blocking the connectivity between the two fields by a local TTX injection inhibited the firing of ectopic but not that of normotopic neurons.

Another pathological condition in which the dysplastic or malformed brain area is not the primary origin of epileptiform activities is provided by the rat model of microgyria induced in rats by freeze-lesioning of deep layer neurons at neonatal stage. Though injured animals did not display spontaneous

epileptic events, it has been proven that there is a focal region of hyperexcitability around the lesion (Jacobs et al., 1996); In the cortex lateral to the microgyria, an increased excitation of L5 neurons and an increased inhibition of L5 and L2/3 neurons were observed (Jacobs and Prince, 2005; Brill and Huguenard, 2010; Jin et al., 2014). In addition to this, a rather widespread cortical reduction of $GABA_A$ receptor subunits α1, α2, α3, α5, and γ2 expression has been reported (Redecker et al., 2000). These alterations together would account for the increased excitability of an apparently normal cortex lateral to the microgyria.

Collectively, these clinical and experimental observations support the notion that anatomically unaltered cortical regions surrounding the malformation are included in a large epileptogenic network. We propose that developmental changes in these areas play a major role in the generation of more or less large epileptogenic networks.

THE TIME OF ONSET OF EPILEPSY

The age at epilepsy onset in patients with MCD is largely variable ranging from newborn to adulthood. This is observed even within the same type of cortical malformations. For example, in a series of 132 patients with polymicrogyria (Leventer et al., 2010), the mean age of epilepsy onset was 4.9 ± 6.7 years but ranging from 1 day to 34 years of age. However, 43% of the patients had the first seizures during the first year of life, coinciding with an important period of synaptogenesis. In a similar way, the analysis of 86 female patients with mutations of DCX (Bahi-Buisson et al., 2013) indicate an early onset, during the first year of life, in 55% of cases, but the age at onset varied between the first month of life to 17 years.

These data indicate that the majority of patients with MCD develop the first clinical manifestations of epilepsy during the first year of life, a period of brain development characterized by an intense neuronal maturation and synaptogenesis. It is thus plausible that epilepsy onset is facilitated in these patients by the increasing weigh of maturing synaptic inputs and/or the maturation of the axonal initial segment responsible for the genesis of action potentials and/or other molecular and synaptic changes linked to neuronal maturation. Interestingly, in Dravet syndrome (Dravet, 1978), a severe encephalopathy due to *de novo* loss-of-function mutations in the SCN1A gene, leading to haploinsufficiency of NaV1.1 channel (reviewed by Brunklaus and Zuberi, 2014), epilepsy typically presents around 6 months of age. Expression analysis on human temporal cortex and hippocampus demonstrated that Na(v)1.1 immunoreactivity increased significantly during the late fetal and postnatal periods, reaching peaks 7–9 months after birth (Wang et al., 2011). Hence, epilepsy onset in Dravet patients compares with the developmental course of the affected channel. It is not unlikely that such developmental pattern plays a role on the onset of epileptic manifestations in Dravet and would be also participating in other epileptic syndromes.

It is well established that GABAergic synapses play a major pathophysiological role in epilepsy and thus GABAergic transmission is targeted by many antiepileptic drugs. Synaptic currents induced by the activation of $GABA_A$ receptors are carried by chloride and consequently the intracellular concentrations of this anion determine the type of response evoked by the transmitter. In adult neurons, the potassium-chloride cotransporter KCC2 usually extrudes chloride promoting hyperpolarizing, inhibitory, responses. In immature neurons the Na^+-K^+-$2Cl^-$ cotransporter NKCC1 loads them with chloride and favors depolarizing responses to GABA so

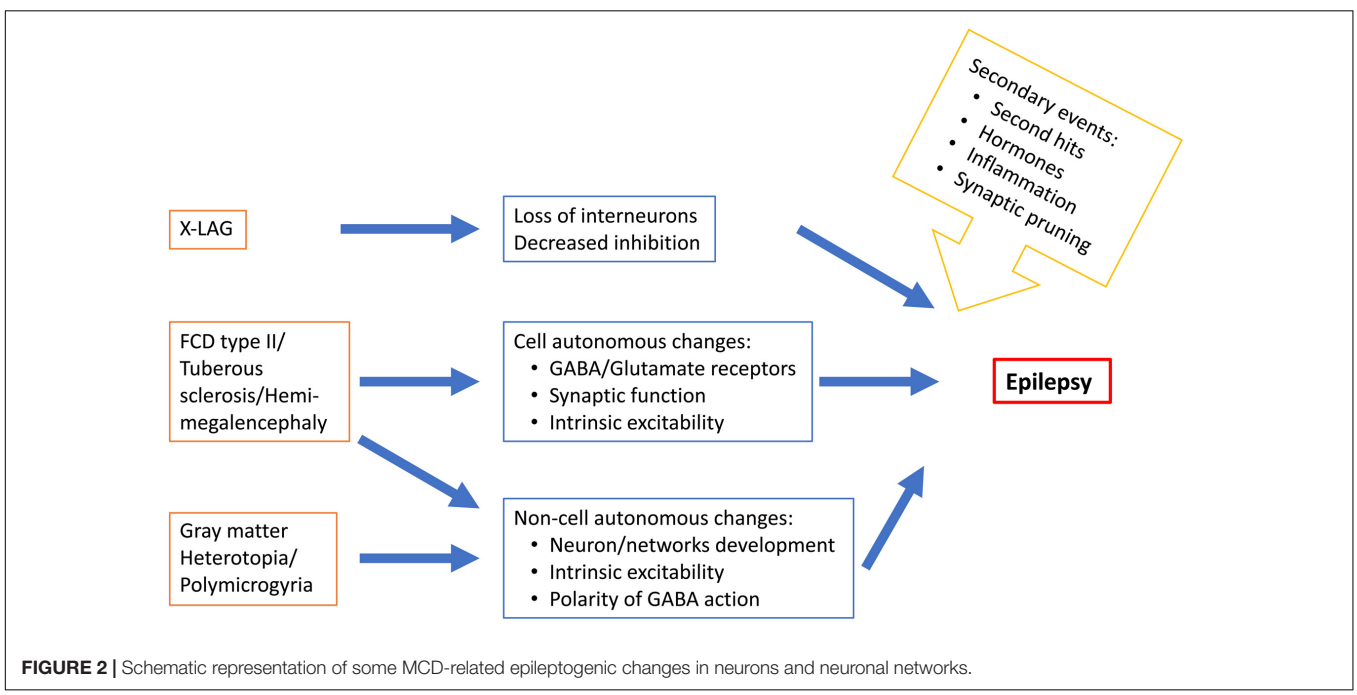

FIGURE 2 | Schematic representation of some MCD-related epileptogenic changes in neurons and neuronal networks.

that the transmitter might have in developmental brain an excitatory effect (Ben-Ari et al., 1989). However, though the topic remains controversial, the action of GABA on developing cortical neurons *in vivo* seems to be inhibitory (Tyzio et al., 2008; Kirmse et al., 2015; Valeeva et al., 2016), maybe because of its shunting effect (Staley and Mody, 1992) that is independent from the polarity of GABAergic signals. Interestingly, in epileptic tissue from patients with temporal lobe epilepsy it has been shown that changes in chloride homeostasis switch GABAergic signaling from hyperpolarizing to depolarizing (Cohen et al., 2002; Khalilov et al., 2003; Huberfeld et al., 2007). It is therefore likely that GABA may play an important role in childhood epilepsy and that its depolarizing actions in immature neurons would contribute to epilepsy onset. However, in cortical resections from pediatric (6 to 14 months old) Sturge-Weber patients, a severe epileptogenic neurocutaneous syndrome, we found that GABA played mainly an inhibitory and anticonvulsive role (Tyzio et al., 2009). On the other hand, investigations on resections from pediatric patients with FCD, Hemimegalencephaly and tuberous sclerosis (Aronica et al., 2007; Talos et al., 2012) suggest a possible dysregulation on the expression of cation-chloride co-transporters. These changes may be activity-dependent and secondary to the epileptic process itself (Puskarjov et al., 2012); they would thus participate more in the expression and evolution of the epileptic disease than at its onset.

The occurrence of a large time window between the initial insult and the onset of clinical manifestations is a common feature for many neurological disorders, including Parkinson and Alzheimer diseases. In these neurodegenerative diseases the latency window can be related to the evolution of the disease, for example the progressive degeneration of dopaminergic neurons in Parkinson disease. Following this reasoning, the late onset of epilepsy reported in some patients might be due to developmental apoptotic processes and/or synaptic (Bourgeois and Rakic, 1993) and dendritic (Zehr et al., 2006) pruning. It is interesting to note that synaptic pruning is particularly important around puberty. For example, electron microscopy studies in primates (Bourgeois and Rakic, 1993) showed a significant reduction in the density of synapses in cortex and hippocampus, during this period of time and imaging analysis of human cortex depicted a reduction in cortical thickness during equivalent periods (Giedd et al., 2006; Raznahan et al., 2011). Would these changes contribute to the peak of incidence of epilepsy that was observed at this period of life (Doherty et al., 2003)? this including juvenile myoclonic epilepsy, emerging mainly in mid-to-late childhood (Martínez-Juárez et al., 2006; Kasteleijn-Nolst Trenité et al., 2013; Ochoa-Gómez et al., 2017)? We do not have an obvious answer on this, but it is clear that we are facing a particular vulnerable period of brain life and that a deregulation of axonal pruning processes, for example affecting more inhibitory synapses or stabilizing recurrent axonal loops, could reveal at last a neuronal excitability status concealed until then.

Alternatively, the intervention of additional precipitating factors during specific time windows, can be at play. Among the factors that would increase neuronal excitability one can mention hormones like estrogens (Zehr et al., 2006) or progesterone (Smith et al., 2002) and inflammation (Bartolini et al., 2018). There is an increasing interest for the role of inflammation and glia in neurodevelopmental disorders like autism spectrum disorders (ASD), schizophrenia, cerebral palsy, cognitive impairment, epilepsy and depression (Galic et al., 2012; Devinsky et al., 2013; Vezzani, 2013; Knuesel et al., 2014; Marchi et al., 2014; Rosenblat et al., 2014; Jiang et al., 2018). Indeed, microglia could have a significant physiological role, contributing to the regulation of cell death/survival, synapse pruning and neurogenesis (Stolp et al., 2011; Vukovic et al., 2012; Kettenmann et al., 2013; Wake et al., 2013). Microglia thus contribute to the regulation of maturation and plasticity of developing neuronal circuits. Furthermore, it has been suggested that microglia would act as a versatile modulator of neurogenesis depending on its activation state: pro-inflammatory microglia would reduce neurogenesis while anti-inflammatory microglia could increase neurogenesis through release of trophic factors (e.g., Kyritsis et al., 2012) and it is suspected that similar dual action of microglia applies to synaptic functioning, plasticity and stability (Marin and Kipnis, 2013; Nisticò et al., 2017).

The contribution of inflammation and glial cells in epilepsy has been particularly investigated (reviewed by Devinsky et al., 2013; Vezzani, 2013) and it has been proposed that inflammation plays an important role in the onset of pediatric seizures (Bartolini et al., 2018). While some infections (e.g., bacterial meningitis, herpes virus, toxoplasmosis) are known to cause acute seizures (Lowenstein et al., 2014; Vezzani et al., 2016) an actual epileptogenic process would take place in some patients after an initial infection. The precise mechanisms remain to be elucidated but they seem to depend on "*the pathogen itself, the developmental stage, the degree of cytokine-mediated inflammatory response and the genotype-phenotype of the person concerned*" (Vezzani, 2013). It can be thus proposed that in a patient with a susceptible brain condition like MCDs, inflammation-induced responses would act as a second-hit, a trigger or an aggravating factor. Interestingly, signs of activation of both innate and adaptative immunities have been found in dysplastic tissue from FCD type II patients (Iyer et al., 2010), suggesting that at least in this type of malformation an inflammatory process is engaged. To note, however, that some of these changes can be related to the action of mTOR on glial cells or be a consequence of the epileptic activity per se. Future research is required for better understanding this important question.

CONCLUSION

In conclusion, epileptogenesis in MCDs occur during a period of brain development characterized by many molecular, cellular and structural changes (**Figure 2**) that determine the features of brain operation and functioning and impact epileptogenic processes and epilepsy expression. Epileptogenesis in MCDs involves complex multifactorial causes that would relate to

the type of gene or insult involved in the malformation, normal developmental processes and developmental adaptative or reactive changes in cortical circuitry. The emergence of new animal models reproducing focal mosaic lesions associating manifestation reminiscent of human clinical symptoms, open promising vistas for better understanding the physiopathology of these disorders and testing new therapeutic options.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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New Neurons in the Post-ischemic and Injured Brain: Migrating or Resident?

*Nikolai M. Nemirovich-Danchenko and Marina Yu. Khodanovich**

Laboratory of Neurobiology, Research Institute of Biology and Biophysics, Tomsk State University, Tomsk, Russia

The endogenous potential of adult neurogenesis is of particular interest for the development of new strategies for recovery after stroke and traumatic brain injury. These pathological conditions affect endogenous neurogenesis in two aspects. On the one hand, injury usually initiates the migration of neuronal precursors (NPCs) to the lesion area from the already existing, in physiological conditions, neurogenic niche – the ventricular-subventricular zone (V-SVZ) near the lateral ventricles. On the other hand, recent studies have convincingly demonstrated the local generation of new neurons near lesion areas in different brain locations. The striatum, cortex, and hippocampal CA1 region are considered to be locations of such new neurogenic zones in the damaged brain. This review focuses on the relative contribution of two types of NPCs of different origin, resident population in new neurogenic zones and cells migrating from the lateral ventricles, to post-stroke or post-traumatic enhancement of neurogenesis. The migratory pathways of NPCs have also been considered. In addition, the review highlights the advantages and limitations of different methodological approaches to the definition of NPC location and tracking of new neurons. In general, we suggest that despite the considerable number of studies, we still lack a comprehensive understanding of neurogenesis in the damaged brain. We believe that the advancement of methods for *in vivo* visualization and longitudinal observation of neurogenesis in the brain could fundamentally change the current situation in this field.

Keywords: neurogenesis, neural stem cells, migration, stroke, traumatic brain injury, subventricular zone, striatum, cortex

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Benedikt Berninger,
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Federico Luzzati,
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Sophie Peron,
Johannes Gutenberg University
Mainz, Germany
Maryam Faiz,
University of Toronto, Canada

***Correspondence:**
Marina Yu. Khodanovich
khodanovich@mail.tsu.ru

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INTRODUCTION

At present, it is well known that the production of new neurons in the mammalian brain is not restricted to the embryonic and early postnatal development stages, but occurs throughout the lifespan of animals. However, not all brain regions are equally capable of generating new neurons. There are two main neurogenic niches where neurogenesis persists: the ventricular-subventricular zone (V-SVZ) and the subgranular zone of the hippocampus (SGZ). By the term V-SVZ we mean a complex of brain regions that includes: the subventricular zone (SVZ) – the area stretching along the lateral wall of the lateral ventricles, the ventral extension of the lateral ventricles, and the posterior periventricular region (PPV). Several researchers have also considered these areas as

Abbreviations: V-SVZ, ventricular-subventricular zone.

a single neurogenic zone (Lim and Alvarez-Buylla, 2016; Mizrak et al., 2019). During adulthood, neurogenesis also occurs in several other brain regions, including the striatum (Luzzati et al., 2006, 2014) and cortex (Gould et al., 2002; Dayer et al., 2005). At the same time, in mice, the most well-studied mammalian species, under physiological conditions no neurogenesis occurs in the cortex (Ehninger and Kempermann, 2003) and striatum (Teramoto et al., 2003; Luzzati et al., 2011; Nato et al., 2015) or, probably, it occurs but only at a very low rate.

Some types of brain damage, such as ischemia or traumatic brain injury (TBI), stimulate neurogenesis in the brain (Sun, 2016; Marques et al., 2019). On the one hand, we know that the proliferation of neuronal precursors (NPCs) in the V-SVZ (the most active proliferative region of the brain) increases, and that these precursors migrate from their place of origin to the injury site (Nakatomi et al., 2002; Jin et al., 2003; Yamashita et al., 2006). On the other hand, many studies have shown that when the brain is damaged, new neurons are generated from local neural stem cells (NSCs) at the injury site (Ohira et al., 2010; Magnusson et al., 2014; Nato et al., 2015). This raises the question: do the NPCs that migrate from the V-SVZ play a major role in the injury-related neurogenesis, or is the role of resident NPCs more important? The answer to this question is of huge clinical significance, because it will determine treatment methods targeted either at the increase in localized neurogenesis near the injury site or at the increase in neurogenesis in – and migration from – the V-SVZ. Consequently, numerous studies have been carried out to establish the dynamics of proliferation and migration of NPCs in the brain in various conditions. In the present review we consider the studies with a focus on the features of neurogenesis in conditions of ischemia and TBI.

METHODS FOR STUDYING THE PRODUCTION AND MIGRATION OF NPCs

Here we consider approaches to studying neurogenesis, paying particular attention to how the origin of new neurons can be established. In other words, whether new neurons have originated in the same area where they localize, or if they/their precursors have migrated there from another area. Methods for defining migration pathways of NPCs are also reviewed here.

Research Into Spatial Distribution of Neuronal Precursors in Fixed Brain Tissues

Historically, the first studies that established the production of new neurons in the mammalian brain were performed using autoradiographic investigation of 3H-thymidine-labeled dividing cells, in particular, newborn neurons (Altman, 1962; Kaplan and Hinds, 1977; Goldman and Nottebohm, 2006). However, neurogenesis in the adult mammalian brain has not been acknowledged by the scientific community for a long time. The existence of neurogenesis was recognized only after methodological improvement, which promoted further studies. This improvement was replacement of radioactive analog of thymidine with bromodeoxiuridine (BrdU), which can be detected by immunohistochemistry (Abrous et al., 2005).

This approach, based on the administration of such proliferation markers as BrdU and other analogs of thymidine, still remains among the most widely used methods. BrdU can be administered intraperitoneally or with drinking water. It incorporates into the cells that are in S-phase at the time of administration. The timing of BrdU administration and subsequent killing of animals affect the interpretation of results. When BrdU is given systematically for several days or weeks, labeled cells born at different time points cannot be distinguished. Alternatively, a single dose (pulse) of BrdU labels the cells that are born at the time of the pulse. Then animals can be sacrificed either shortly after administration, or at a delayed time point (pulsed and pulse-chase paradigm, respectively). This approach allows for the tracing of a short-term or a long-term fate of a particular group of cells that are born at a certain time point (Dayer et al., 2005; Sundholm-Peters et al., 2005; Shapiro et al., 2009; Lugert et al., 2012).

In addition to BrdU, other nucleotide analogs can be used: -5-chloro-20-deoxyuridine (CldU), 5-iodo-20-deoxyuridine (IdU), and 5-ethynyl-20-deoxyuridine (EdU). The use of combination of several thymidine analogs within the same experiment gives additional possibilities. Labeling of dividing cells with two (Encinas et al., 2011) or even three (Podgorny et al., 2018) nucleotide analogs allows for measuring cell-division kinetics, and identifying and tracing subclasses of NSCs or NPCs. Assessment of the fraction of CldU/IdU double-labeled NPCs enabled Encinas et al. (2011) to determine the number of divisions, the length of S-phase, and the length of the division cycle of amplifying and quiescent neural progenitors in the dentate gyrus (DG).

The immunodetection of BrdU or other thymidine analogs in fixed brain slices, in combination with labeling the specific markers of immature (doublecortin or DCX) and mature (NeuN, MAP2, NSE) neurons, as well as NSCs (Nestin, SOX2), allows for indirect tracking of NPCs. Additionally, Ki67, a marker of cell divisions, is used to confirm the proliferation status and to identify the location of non-migrating progenitors (Abrous et al., 2005; Kuhn et al., 2016).

Although this approach facilitated the estimation of the proliferation and differentiation of NPCs, it provided only indirect evidence of neuroblast migration. The signs of migration can be: a distribution pattern of migrating neuroblasts (markers – DCX and sometimes PSA-NCAM) from the place of origin toward the place of destination (Palma-Tortosa et al., 2017), a density gradient of neuroblasts with high density at the presumed place of origin (Thored et al., 2006; Fukuzaki et al., 2015), as well as the change in this gradient at subsequent time points (Yan et al., 2007; Oya et al., 2009; Li et al., 2011). Also, in their works researchers pay due attention to the orientation of migrating cells. Neuroblasts stretch out their leading processes in the direction of the zone to which they are moving (Parent et al., 2002; Zhang et al., 2004; Dayer et al., 2005; Osman et al., 2011; Nakaguchi et al., 2012; Moraga et al., 2014). Moreover, migrating

neuroblasts often integrate into chains, and the orientation of the chain also corresponds to the direction of migration (Parent et al., 2002; Zhang et al., 2004; Wang et al., 2007; Kuge et al., 2009; Yan et al., 2009b; Kazanis et al., 2013; Wan et al., 2016; Song et al., 2017). Alternatively, clustering cells with round cell bodies, lacking long leading processes and co-expressing DCX with proliferation marker Ki67 are considered as non-migrating local NPCs (Magnusson et al., 2014; Nato et al., 2015).

Studies of postmortem tissues are of great importance in research on human neurogenesis because this is the only way it can be investigated. BrdU labeling in combination with other neuronal markers enabled scientists to prove for the first time the existence of neurogenesis in humans (Eriksson et al., 1998). A more sophisticated technique of ^{14}C-dating was proposed by Jonas Frisen's group (Bhardwaj et al., 2006; Spalding et al., 2013). The technique is based on the fact that the ^{14}C levels in the atmosphere have been decreasing at a known rate since a sharp rise in the ^{14}C content caused by the extensive testing of nuclear weapons in the middle of the 20th centuary. Thus, comparison of the ^{14}C-content in DNA with that in the atmosphere enabled the assessment of the age of cells. Isolation of cell nuclei from brain tissue with subsequent labeling with NeuN and the ^{14}C content measuring by accelerator mass spectrometry allowed for evaluation of the turnover rate of neurons in the human hippocampus, cortex, olfactory bulb (OB), and striatum (Bhardwaj et al., 2006; Spalding et al., 2013; Ernst et al., 2014).

The above-mentioned studies have investigated neuroblasts distribution in brain slices. The limitation of this approach is a lack of information about whole brain tissues. In recent years, completely new techniques for *ex vivo* studies which potentially expand possibilities for the investigation of new neuron generation have been devised.

A promising technique called CLARITY (Chung and Deisseroth, 2013) was developed by the Deisseroth lab. This technique makes the entire brain transparent for any optical imaging via special chemical transformations of intact brain tissue. These transformations aim to remove the lipid component of the brain while retaining the protein and nucleic components in their native state. After transformation, the preserved components can either be immunohistochemically stained or initially fluorescent-labeled by genetic modification, thereby facilitating the performance of precise whole-brain imaging without dividing into slices. Imaging of the whole brain rather than of just separate brain slices could provide an entire picture of the spatial distribution and orientation of migrating NPCs.

Despite the unique possibility to obtain 3D images of brain structure at the molecular level, we did not find any research works that have used this method since it was published. The major limitations of this method are the complexity and timescales involved in the brain processing and immunostaining, as well as the toxicity of the reagents used (Jensen and Berg, 2017). Significant efforts have been made to improve this technique (Jensen and Berg, 2017) in order that it might be more extensively used in studies – including NPC tracking.

Another powerful technique, single-cell transcriptomics, is based on measuring gene expression at the level of individual cells in certain cell populations. It helps clarify the mechanisms of cell reprogramming into NSCs in non-neurogenic zones after injury and promotes a better understanding of the balance of NSC activation and quiescent state in the neurogenic niches (Llorens-Bobadilla et al., 2015). Cell subgroups identified by this technique can be compared to the known cell types using previously established marker genes; however, novel cell subtypes can also be discovered using single-cell data (Liu and Trapnell, 2016). Unlike immunofluorescent detection with antibodies, which is usually limited in the number of markers, single-cell transcriptomics allows for the simultaneous investigation of hundreds, or even thousands, of genes. The main limitation of this technique in studies on cell migration is the loss of the original spatial context. Currently, efforts are being made to overcome this limitation by using computational methods of 3D reconstruction (Satija et al., 2015). Combining single-cell transcriptomics with other single-cell techniques, such as fluorescent RNA FISH, provides an orthogonal method of quantifying transcript levels, and is often used to independently validate results from scRNA-seq data (Liu and Trapnell, 2016).

In general, neuroblast distribution, orientation, morphology and clustering are only indirect signs of migration. Additionally, this approach is limited only to estimation of the origin and migration direction of cells, which at a certain time point are migrating neuroblasts. However, we lack information on the origin of new cells that have already become mature neurons. In the next section, we will discuss methods that allow us to specifically trace the fate of cells of a particular origin, i.e., the cells that originated from the SVZ or cortex.

Determining the Place/Time of NPCs Production by Chemical and Genetic Labeling

Local labeling of the cells within a particular region of the brain is a powerful method for tracing the migration of NPCs that are produced there. After labeling, the fate of the NPCs and their offspring can be examined at different time points. For example, these labeled cells can be found in a different brain region some time later, or they can stay at their place of origin. To determine a phenotype of stained cells, immunohistochemical detection of such cell type-specific proteins as DCX, calretinin, NeuN, MAP2, NSE, and Nestin is carried out (Nakatomi et al., 2002; Jin et al., 2003; Zhao et al., 2003; Goings et al., 2004; Ramaswamy et al., 2005; Zhang et al., 2005, 2011; Ohab et al., 2006; Yamashita et al., 2006; Kolb et al., 2007; Yang et al., 2007, 2008; Faiz et al., 2008, 2015; Hou et al., 2008; Lai et al., 2008; Liu et al., 2009; Kreuzberg et al., 2010; Li B. et al., 2010; Li L. et al., 2010; Ohira et al., 2010; Shimada et al., 2010; Yoshikawa et al., 2010; Bi et al., 2011; Grade et al., 2013; Saha et al., 2013; Magnusson et al., 2014; Duan et al., 2015).

Local injection of various tracers into the brain is widely used. In this method, the cells residing in the zone of injection, absorb the tracer; this then allows for tracking the fate of these cells, or their offspring at different post-injection time points. Tracers can be either a stain (Nakatomi et al., 2002; Jin et al., 2003; Zhao et al., 2003; Ramaswamy et al., 2005; Zhang et al., 2005; Faiz et al., 2008; Hou et al., 2008; Shimada et al., 2010), or a genetic vector,

which after entering the cell causes expression of the marker in it (Nakatomi et al., 2002; Hou et al., 2008; Lai et al., 2008; Magnusson et al., 2014; Duan et al., 2015).

For labeling brain cells, such stains as 1,1′-dioctadecyl-6,6′-di- (4-sulfophenyl)-3,3,3′,3′ tetramethylindocarbocyanine (DiI), and fluorescent latex microspheres are used. For detection of the labeled cells, fluorescent microscopy is used (Nakatomi et al., 2002; Jin et al., 2003; Zhao et al., 2003; Ramaswamy et al., 2005; Zhang et al., 2005; Faiz et al., 2008; Hou et al., 2008; Shimada et al., 2010). However, the major drawback of this approach is that a cell gets a very limited amount of the label, which can then be lost either due to cell metabolism, or via cell divisions, if labeled cells are proliferating as in the case of NPCs. In this regard, a genetic vector injection is a more appropriate method because even one copy of a reporter gene entering the cell may be enough to cause a stable synthesis of labeling substance in it. Moreover, the application of such genetic vectors as retrovirus or lentivirus, which integrate into the host cell genome, ensures the transmission of the reporter gene to all offspring of the infected cell: this is an advantage for the staining of proliferating cells, for example, NPCs.

Genetic vectors for *in vivo* cells tracing encode many different reporter genes that allow for the identification of targeted cells. These include the green fluorescent protein (GFP) gene, the yellow fluorescent protein (YFP) gene, the DSRed, mCherry, and mKate for detection using fluorescence microscopy; the alkaline phosphatase (AP) gene for immunodetection; the galactosidase (GLA) gene for histochemical detection using transmitted light microscopy (Nakatomi et al., 2002; Goings et al., 2004; Ohab et al., 2006; Kolb et al., 2007; Yang et al., 2007, 2008; Hou et al., 2008; Lai et al., 2008; Liu et al., 2009; Li B. et al., 2010; Ohira et al., 2010; Yoshikawa et al., 2010; Grade et al., 2013; Saha et al., 2013; Magnusson et al., 2014; Müller-Taubenberger and Ishikawa-Ankerhold, 2014; Vandeputte et al., 2014; Duan et al., 2015; de Jong et al., 2017).

Another important question concerns promoters that drive reporter gene expression in vectors for NPCs labeling. The use of general promoters (CMV-promoter, CAG-promoter) in the construct results in non-specific expression of the reporter gene in the cells that have been successfully infected with a genetic vector (Grade et al., 2013; Saha et al., 2013; Magnusson et al., 2014; Duan et al., 2015). Nevertheless, this promoter-related non-specificity may at least in part be compensated by the use of viral serotypes that preferentially target specific cell types (Aschauer et al., 2013), as well as by the use of retroviruses that transduce specifically mitotic cells (Roe et al., 1993). Non-specific expression is in fact an advantage, if the aim of investigation is to trace the cells' fate throughout their differentiation – when changes in expression of many cell type-specific genes occur. The use of cell type-specific promoter drives expression of the reporter gene in a particular group of cells. It can be a promoter of the gene, the expression of which is a reliable indicator of a certain cell group; for example, DCX – a protein of young neurons, Nestin – of NSCs, GFAP – of NSCs and astroglia. This approach has only a limited potential for studying neurogenesis as it does not allow for the detection of neural cells at different stages of their development.

Interestingly, there is still at least one work in which this approach was applied to investigate the origin of new neurons in the brain. Duan et al. (2015) injected a plasmid carrying the GFP gene under the control of the GFAP gene promoter into the striatum of mice and showed that after ischemia new striatal neurons express the GFP-reporter, despite the lack of GFAP gene expression. The authors concluded that these new neurons in the striatum stem from local GFAP-expressing astrocytes. However, since it remains unclear what could be the cause of GFP expression in these new neurons, the results of the research cannot be unambiguously interpreted.

A completely different approach involves the use of transgenic animals already carrying a reporter gene in their cells. As a rule, the reporter gene is initially inactive because of an insertion containing a stop signal. It can then be restored (the restoration mechanisms are described below), thus facilitating the reconstruction of the reporter gene in the cells residing in a specific brain region at a specific time point and of a specific phenotype. Later, the cells where the reporter gene was restored and their offspring can be detected (Li L. et al., 2010; Bi et al., 2011; Zhang et al., 2011; Magnusson et al., 2014; Faiz et al., 2015).

The reporter gene can be restored by the use of site-specific Cre-recombinase that removes an insertion from the reporter gene (Magnusson et al., 2014; Nato et al., 2015). A specific variant of this recombinase is a CreERT2 recombinase that requires the presence of tamoxifen for activity (Li L. et al., 2010; Zhang et al., 2011; Faiz et al., 2015). Tamoxifen can be either administered intraperitoneally (Li L. et al., 2010; Zhang et al., 2011) or added to daily ration (Faiz et al., 2015). The gene of Cre-recombinase recombinase can be initially present in the animal genome, together with the reporter gene (Li L. et al., 2010; Zhang et al., 2011; Faiz et al., 2015). Alternatively, it can be introduced into the animal brain via vector injection (Magnusson et al., 2014). Additionally, to achieve cell-type specificity of the reporter gene restoration, the recombinase gene can be placed under the control of a cell-specific promoter, for example, the promoter of the GFAP gene (Magnusson et al., 2014; Nato et al., 2015). As a result of these manipulations, reporter gene restoration occurs in a particular brain region, or at least at a specific time point, and in the cells having a particular phenotype.

Lastly, Kreuzberg et al. (2010) used animals carrying the reporter gene under the control of a promoter of the 5HT3A gene, which is active in all, or the majority, of neuroblasts originating from the SVZ, as well as in some mature interneurons. In combination with BrdU administration, it allows the distinguishing of SVZ-derived newborn cells.

Another principally new technique to have been recently developed is the Brainbow strategy (Livet et al., 2007) which aims to identify single cells of one type and trace their connections and lineages. The strategy is based on Cre-Lox recombination that allows the Brainbow transgene to cause expression of three different fluorescent proteins in random combinations and obtain multicolor micrographs of brain sections. Recently, this approach has been successfully applied to trace lineages of NSCs and NPCs in the DG and SVZ for 6 months (Gomez-Nicola et al., 2014). Obviously,

this technique has huge potential since it helps distinguish ramified processes of neighboring cells and thereby trace their lineage. However, we believe that the advantages of the Brainbow technique are also its limitations because multicolor single-cell labeling cannot be combined with another type of fluorescent labeling.

Thus, local *in vivo* cell labeling is the most reliable method for establishing the origin of new neurons in brain regions. Rapid development in genetic engineering led to widespread using of plasmid libraries designed for new viral vector production. To increase efficiency of the use of libraries, a novel tool of viral barcoding was suggested (Davidsson et al., 2016). The method of *in vivo* cell labeling is often used together with detection of labeled cells in fixed brain sections. Additionally, this method facilitates observation of the migration of labeled neuroblasts in real time, as described in the section "Real-Time Observation of NPCs Migration."

Suppression of NPC Production and Migration

In this section we describe approaches that diminish or exclude any participation of one brain region in the replenishment of neurons in another region. Two methods are applicable here: either locally suppressing the production of new NPCs in one region or preventing the migration of NPCs from one region to another.

Inhibition of neurogenesis in a particular brain region can be achieved by local injection of the antimitotic agent Ara-C, which kills cycling cells. In a number of studies, Ara-C was administered into the lateral ventricle. Thus the role of SVZ neurogenesis in the addition of new neurons in the damaged cortex was examined (Leker et al., 2007; Li B. et al., 2010; Yoshikawa et al., 2010; Faiz et al., 2015). In some works, migration from the SVZ toward the cortex was prevented by surgical separation (Shapiro et al., 2009; Ahmed et al., 2012).

However, it should be mentioned that some issues have complicated the interpretation of the results obtained with the use of these approaches. In particular, antimitotic drugs can diffuse in the brain tissue from the region of injection to remote brain areas. Thus, spatial specificity of antimitotic effect should be carefully verified. Additionally, both local antimitotic drug administration and surgical isolation of brain areas are highly invasive approaches, potentially causing unwanted side effects.

Almost all the above-mentioned studies have demonstrated that the suppression of proliferation in, or migration from, one region affects the number of new neurons in the other; therefore, the results of these experiments cannot be unequivocally interpreted (Leker et al., 2007; Shapiro et al., 2009; Li B. et al., 2010; Yoshikawa et al., 2010; Faiz et al., 2015).

Real-Time Observation of NPCs Migration

In the last few decades, new methods for longitudinal *in vivo* tracking of NPCs have been devised, while some existing ones have been considerably improved. One of those more recently developed approaches involves MRI-based labeling of migrating NPCs, while others are based on fluorescence or bioluminescence detection.

The most common MRI-based labeling technique uses iron oxide particles as MRI-negative contrast agent visible in T2-weighted images (Norman et al., 1992; Bulte et al., 1999). Several efforts have been made to visualize NPCs by injecting iron oxide nanoparticles directly into the lateral ventricles or the SVZ (Shapiro et al., 2006; Panizzo et al., 2009; Nieman et al., 2010; Sumner et al., 2010; Vreys et al., 2010). However, this method has several significant limitations: cells can release iron oxide particles that can then be absorbed by non-proliferating cells, including macrophages, which are present at the site of damage, thus indicating low specificity of the method; furthermore, neuroblasts can die before reaching the target regions (Winner et al., 2002). Consequently, MRI detection by iron-based nanoparticles does not provide precise information about the state, location and survival of new neurons.

Attempts to overcome low specificity of paramagnetic nanoparticles have been made in a number of works (Elvira et al., 2012; Zhong et al., 2015; Zhang et al., 2016), in which the animal brain was injected with paramagnetic particles conjugated with antibodies to specific cell-surface antigens of NPCs. Another type of cell labeling for MRI is based on reporter genes coding paramagnetic proteins, mainly ferritin (Iordanova and Ahrens, 2012; Vande Velde et al., 2012). This method, though less liable to imaging artifacts compared to iron oxide labeling, shows relatively low MR signal intensity from ferritin overexpression (Naumova et al., 2014). Thus, the reporter gene design and MRI protocols need improving. A similar methodological approach utilizing specific reporter genes was applied for NPC visualization in other modalities. Introduction of the luciferase gene allowed the tracing of NPCs using bioluminescence detection (Reumers et al., 2008; Vandeputte et al., 2014).

Multiple works at different levels of observation were performed with the use of fluorescence. Neuroblast migration dynamics was directly observed on acute brain slices using time-lapse microscopy (Landecker, 2009). In these works, migrating cells are also labeled either by prior administration of fluorescent particles (Zhang et al., 2007) or a vector with a reporter gene (Inta et al., 2008; Kojima et al., 2010; Le Magueresse et al., 2012; Grade et al., 2013) into the animal brain, or by using transgenic animals carrying the reporter gene under the control of a neuron-specific promoter (Dayer et al., 2008; Zhang et al., 2009). The advantage of studies on brain sections, when compared to research into the whole brain, is greater resolution and the possibility of a detailed estimation of particular cells' behavior. The limitation of this method is the short lifespan of a brain slice – usually studies last up to one day. However, the living whole brain is preferred to long-living brain slices for observations at the molecular level.

The most promising strategy is a recently developed technique of *in vivo* cell tracking with intravital microscopy through a cranial window. Two-photon microscopy is a further improvement of confocal laser scanning microscopy due to its

deeper tissue penetration, efficient light detection, and reduced photobleaching (Denk et al., 1990). These benefits allowed for the combination of this technique with intravital observation of living neural cells that had been previously labeled using reporter genes of fluorescent proteins. Recently this technique has been used for the investigation of hippocampal neurogenesis in the DG (Pilz et al., 2016).

Table 1 summarizes the advantages and limitations of the methods described in this section. From our point of view, the methods for local cell labeling and the methods for direct observation of cells, described in the sections "Determining the Place/Time of NPCs Production by Chemical and Genetic Labeling" and "Real-Time Observation of NPCs Migration," are the most promising, informative, and precise.

EARLY POSTNATAL AND ADULT NEUROGENESIS IN THE NORMAL BRAIN

In this section we summarize the data accumulated to date about the origins of NPCs and their migration pathways in the normal mammalian brain during the early postnatal and adult periods.

The Ventricular-Subventricular Zone

The V-SVZ is the main neurogenic niche and the major source of new neurons in the postnatal brain, both in the early postnatal period and throughout adulthood. This is a place where NSCs reside and NPCs originate. Many authors have recently used the general term, V-SVZ, to describe several regions located in the walls of the lateral ventricles (Lim and Alvarez-Buylla, 2016). The SVZ, which stretches along the lateral wall of the lateral ventricles, is a major focus of studies (Doetsch et al., 1997, 1999; Bernier et al., 2000; Gage, 2000; Kornack and Rakic, 2001; Pencea et al., 2001a; Temple, 2001; Alvarez-Buylla et al., 2002; Doetsch, 2003; Ming and Song, 2005; Duan et al., 2008). Additionally, there are data on NSCs in a ventral extension of the lateral ventricle (Zhao et al., 2003) and in the PPV region (Nakatomi et al., 2002; Bull and Bartlett, 2005; Abdipranoto-Cowley et al., 2009; Saha et al., 2013).

Neural stem cells in the V-SVZ, the so-called type B1 cells, have many characteristics of parenchymal astrocytes because they express GFAP, glutamate aspartate transporter (GLAST), and brain lipid-binding protein (BLBP) (Lim and Alvarez-Buylla, 2016). However, unlike parenchymal astrocytes, type B1 cells have a direct contact with the ventricle. A subset of these cells is quiescent NSCs, whereas others are mitotically active (Llorens-Bobadilla et al., 2015). In the activated state, type B1 cells also express Nestin and produce transit-amplifying precursors (type C cells) that produce NPCs (type A cells) expressing the markers of immature neurons (Lim and Alvarez-Buylla, 2016). The NPCs that originate in the V-SVZ migrate to other brain regions where they finally differentiate into neurons and may integrate into local neural networks (Lledo and Saghatelyan, 2005; Sakamoto et al., 2014).

In the early postnatal brain, several migratory pathways stretching from the V-SVZ to other brain regions have been described. Inta et al. (2008) showed in fixed and cultivated mouse brain slices that in the first weeks of life, neuroblasts travel along four different pathways – rostral, ventral, external, and dorsal – from the lateral ventricles. The rostral migratory stream (RMS) flows from the SVZ to the OB; the ventral stream flows from the SVZ to the striatum and the nucleus accumbens; the external migratory pathway emerges from the anterior parts of the SVZ and extends along the external capsule toward the latero-dorsal regions; the dorsal stream flows from the PPV along the superior border of the hippocampus toward the occipital cortex. Other authors also report on the massive migration of neuroblasts from the SVZ as well as from the RMS via the corpus callosum to the cortex (Dayer et al., 2008; Le Magueresse et al., 2012). In the cortex, migrating neuroblasts mainly reach the lower layers (Dayer et al., 2008; Inta et al., 2008; Le Magueresse et al., 2012), but can be also found in the upper layers (Dayer et al., 2008; Inta et al., 2008).

De Marchis et al. (2004) injected CellTracker Green into the SVZ of newborn mice and showed three migratory pathways for neuroblasts originating in the SVZ: primarily, the RMS and two additional pathways – ventral migratory mass (VMM) and ventrocaudal migratory stream (VMS). Within the VMM, neuroblasts migrate from the SVZ to the basal forebrain and populate the islands of Calleja, while the VMS deviates from the anterior part of the RMS to flow in the ventrocaudal direction, when neuroblasts reach the pyramidal layer of the olfactory tubercle.

In the adult brain, the V-SVZ remains the main zone of neurogenesis. However, its intensity decreases soon after birth and the majority of pathways become largely quiescent (Fuentealba et al., 2015). The exception is NPC migration from the SVZ to the OB, which remains active in adulthood, at least in rodents (Lim and Alvarez-Buylla, 2016). Cells migrate in chains and form an RMS stretching from the SVZ to the OB. In the OB, neuroblasts then migrate radially and turn into mature neurons in the granular and periglomerular layers (Sun et al., 2011).

The migration from the V-SVZ to other brain regions substantially decreases compared with the early postnatal period, and a range of works have confirmed this. De Marchis et al. (2004) found that migration via the VMS to the olfactory tubercle, which was observed in neonatal mice, is less intense during adulthood, whilst migration within the VMM stops completely. Several other studies report on the occurrence of neuroblasts and new mature neurons in different areas of the adult mouse and rat brains, including the striatum, nucleus accumbens, ventral septum, corpus callosum, olfactory tubercle, anterior olfactory nuclei, tenia tecta, islands of Calleja, amygdala and lateral entorhinal cortex (Dayer et al., 2005; Sundholm-Peters et al., 2005; Shapiro et al., 2009). The origin of these neurons has not been established. However, the authors posit that they are produced in the SVZ (Dayer et al., 2005; Sundholm-Peters et al., 2005; Shapiro et al., 2009). Zhao et al. (2003) labeled NPCs localizing near the lateral ventricles with DiI injection into the lateral ventricle, and demonstrated that neuroblasts migrate from a ventral extension of the lateral ventricle to the substantia nigra, where they differentiate into dopaminergic

TABLE 1 | Methods for estimation of NPCs migration in the brain.

Method	Description of the method	Advantages	Limitations
Estimation of neuroblasts migration patterns in fixed brain slices.	In fixed brain slices signs of neuroblasts migration are observed: distribution of cells from the supposed place of origin to the supposed destination; orientation of chains of cells and cell processes in the direction toward the destination.	It is a non-invasive and simple research method that facilitates the estimation of cell migration in the brain at the time of animal euthanasia.	This approach facilitates the estimation of the direction of migration only of the cells migrating at the time of animal euthanasia. Furthermore, although the distribution of neuroblasts in the fixed brain slice describes the general direction of migration, it fails to prove the origins and direction of migration of each cell.
Local administration of antimitotic agents.	The Ara-C antimitotic agent is administered into a particular brain region to suppress neurogenesis in it.	The method facilitates the reduction or exclusion of any participation of a brain region in replenishment with neurons in another region.	The method is invasive and potentially causes adverse effects. The results of such experiments can be unequivocally interpreted only in the absence of any effects, i.e., when killing proliferating cells in one region doesn't affect the number of new neurons in the other. Moreover, antimitotic drugs can diffuse in brain tissue from the region of injection to the remote brain areas. Thus, spatial specificity of antimitotic effect should be carefully verified.
Surgical separation of brain regions.	Surgical separation prevents migration from one brain region into another.	This method facilitates the exclusion of any participation of a brain region in replenishment with neurons in another region.	The method is invasive, and potentially causes adverse effects.
Local labeling of cells in the brain.	Local administration of stains or genetic vectors carrying the reporter gene. Alternatively, transgenic animals can be administered with special agents that induce expression of a reporter gene in them (tamoxifen or a genetic vector with the recombinase gene can serve as an inductor).	This method helps prove the origin of a particular neuron from a particular brain region. Moreover, the method is suitable for direct observation of cell migration.	The method is invasive, and potentially causes adverse effects.
Direct observation of cell migration in live brain slices.	Preliminary *in vivo* staining of neuroblasts, obtaining of live brain slices and detection.	The method facilitates a detailed observation of individual cell behavior.	The conditions in the live brain slice cannot be equal to the conditions in the live animal brain: cutting the brain into slices results in damage to many cells, and isolating a slice from the whole brain removes potential influences from other cells in the whole brain. Moreover, brain slices remain viable for quite a short time (usually studies last up to one day), which is not enough to observe the long-term fate of migrated cells.
Direct observation of *in vivo* cell migration.	Preliminary *in vivo* staining of neuroblasts and subsequent bioluminescent or MRI detection. Alternatively, two-photon microscopy through a cranial window.	The method facilitates the tracing of long-term cell migration in the live brain.	In general, the method has a low-resolution capacity, as it facilitates the observation of the migration of only large cell groups. However, a recently developed technique of intravital microscopy through a cranial window, in combination with two-photon microscopy, facilitates the tracing of individual cells deep within the brain tissue. Still, cranial window is a highly invasive approach.

neurons. Nakatomi et al. (2002) administered DiI or a genetic vector with a GFP gene into the lateral ventricle of a normal animal, and showed negligible migration of stained neuroblasts from the PPV toward CA1 of the hippocampus.

Despite the fact that these pathways remain largely quiescent (Fuentealba et al., 2015), they might be important for response to injury. Multiple studies on the injured brain showed significant intensification of NPC migration in these conditions (Arvidsson et al., 2002; Nakatomi et al., 2002; Thored et al., 2006).

In humans, the amount of neuroblasts migrating to the OB drops rapidly in the early postnatal period. Spalding et al. (2013) using the ^{14}C technique showed that in the OB,

adult neurogenesis can be negligible. As in animal models, neurogenesis in the SVZ in humans declines over a lifetime (Dennis et al., 2016).

The Hippocampus

The DG of the hippocampus ranks second after the V-SVZ where proliferation of NPCs continues throughout the whole life of animals. According to the classical point of view, the dentate neuroepithelium gives rise to both granular neurons and NSCs in the prenatal period (Gonçalves et al., 2016). However, recent studies of Li et al. (2013) showed that the NSCs in the DG may originate from the population in the most ventral part of the hippocampus, close to the lateral ventricle. These cells migrate to the DG at the late stage of gestation and become the source of NSCs of the SGZ in adulthood. Proliferation in the DG is limited to the SGZ from the second postnatal week (Urbán and Guillemot, 2014). Radial glia-like cells (RGL), also called type 1 cells that express GFAP, Nestin, and SOX2, are considered as genuine NSCs in the SGZ (Gonçalves et al., 2016). Most of these cells remain quiescent and eventually divide (Encinas et al., 2011).

Unlike the SVZ (Obernier et al., 2018), neurogenic potential of NSCs in the SGZ is highly debated. Bonaguidi et al. (2011) showed self-renewal properties and multipotent capacity of RGL cells, using cloning analysis. At the same time, the studies of Encinas and Enikolopov, based on the Nestin-GFP transgenic mice (Mignone et al., 2016), showed restriction of the NSC pool and its inability to self-renew. Moreover, the authors, using multiple S-phase labeling, observed that RGL cells in the SGZ make 2-3 asymmetric divisions and then lose their RGL morphology. Further they move to the hilus where they start expressing S100ß (a marker of mature astrocytes) and continue to express GFAP but not Nestin (Encinas et al., 2011). The number of RGL cells dramatically drops with age (Gage, 2000; Encinas et al., 2011; Ming and Song, 2011; Bergami et al., 2015).

Asymmetric division gives rise to bipolar neural progenitors that differentiate into neuroblasts after several divisions. During their maturation these NPCs, unlike NPCs from the V-SVZ, migrate short distances – from the subgranular to the granular layer of the DG – and become mature glutamatergic granular neurons (Gage, 2000; Alvarez-Buylla et al., 2001; Temple, 2001; Doetsch, 2003; Ming and Song, 2005; Duan et al., 2008; Imayoshi et al., 2008).

In adult humans, hippocampal neurogenesis in the DG is more prominent than in the OB (Gage, 2000; Spalding et al., 2013; Jessberger and Gage, 2014). Spalding et al. (2013) showed two different types of neuronal populations in the human hippocampus, one of which is renewed continuously, unlike the other. The renewing cell population accounts for approximately one third of the hippocampal neurons and exceeds similar cell population in rodents (Snyder and Cameron, 2012). In humans, as in rodents, hippocampal neurogenesis declines over the years (Ihunwo et al., 2016). It is worth noting that despite the large number of research works, inconsistency still exists concerning the abundance of adult human hippocampal neurogenesis. For example, two recent studies have provided quite contradictory results. Moreno-Jiménez et al. (2019) found thousands of cells of immature neuronal phenotype within the DG of healthy adult subjects, aged between 43 and 87. In sharp contrast, Sorrells et al. (2018) report that hippocampal neurogenesis declines in children and no immature neurons are found within the DG in adult subjects. Moreno-Jiménez et al. (2019) along with Kempermann et al. (2018) consider that such discrepancies may stem from the differences in the details of post-mortem tissue processing, and conclude that the level of human hippocampal neurogenesis may in fact be underestimated in some studies due to the loss of the detectable immature neuronal markers in post-mortem brain tissues. Thus, a consensus about the level of adult human hippocampal neurogenesis is yet to be achieved.

The Cortex

Neurogenic potential of the cerebral cortex in physiological conditions is considerably smaller in comparison to the V-SVZ and to the SGZ of the DG.

The majority of cortical neurons and glial cells originate from the radial glia stem cells that located along lateral ventricles. These cells generate cortical neurons and direct their migration to the cortical layers at the embryonic stage (Rakic et al., 2009). This process is almost complete prior birth. However, the mouse cortex retains its neurogenic potential for a short period after birth. For almost 10 days after birth, there exist in the mouse cortex multipotent astrocytic stem cells, which, having been translated into cell culture, form neurospheres and differentiate into neurons, astrocytes and oligodendrocytes (Laywell et al., 2000; Ahmed et al., 2012). From day 10 neurogenesis in the cortex stops; however, traumatic injury of the cortex at day 15 causes its restoration (Ahmed et al., 2012).

Almost no neurogenesis occurs in the cortex of the normal adult brain in rodents, but systematic analysis shows that small numbers of NeuN/BrdU double positive new neurons still continue to be generated there (Dayer et al., 2005). Their origin has yet to be established, but the authors posit that these neurons might be produced from local precursors. Interestingly, these new neurons seem to be generated not from DCX-expressing cells but, rather, from those that express NG2, a protein that is usually associated with oligodendrogenesis. There were no DCX-expressing cells in the cortex, but instead there were proliferating (BrdU-labeled) NG2-positive cells. Moreover, some NeuN/BrdU positive cells also co-expressed NG2, suggesting that NG2-precursors are the source of new neurons in the cortex. Given that DCX is a marker of migrating neuroblasts, the authors concluded that the observed cortical DCX-negative progenitors are of local origin (Dayer et al., 2005). Gould et al. (2002) similarly showed rare cells incorporating BrdU and co-expressing the neuronal markers NeuN and TuJ1 in the anterior rat neocortex. It remains unclear whether these cells are of local origin or they arise from the SVZ. At the same time, some other studies failed to discover any newly generated neurons in the adult rodent cortex (Ehninger and Kempermann, 2003; Madsen et al., 2005). However, analysis performed in the review by Cameron and Dayer (2008) suggests that the causes for such negative results may stem from an extremely low level of cortical neurogenesis and the small diameter of their nuclei.

A number of studies in primates and humans mainly report on the absence or a low level of adult neurogenesis in the cerebral cortex. Longitudinal observations by Gould et al. (2002) revealed a small number of cells that co-expressed the neuronal markers NeuN and TuJ1 with BrdU. The authors concluded that these *de novo* generated neurons have transient existence because their number declines after 9 weeks of observation. The origin of these cells has not been studied in this work. In humans, Eriksson's studies (Eriksson et al., 1998) showed a non-neuronal phenotype of BrdU-labeled cells in the cortex. An extremely sensitive analysis, which included both BrdU labeling and ^{14}C incorporation, estimated the exchange rate of neurons in the adult human neocortex at 1/1,000 neurons every fifth year (Bhardwaj et al., 2006).

For several decades, the piriform cortex has attracted significant attention due to its ability to express the markers of immature neurons DCX and PSA-NCAM, which was revealed in most mammalian species in adulthood. Nevertheless, numerous attempts to achieve evidence of neurogenesis in this area using BrdU labeling failed. It was concluded that the expression of DCX and PSA-NCAM could be explained by this particular population of neurons retaining its structural plasticity (Nacher and Bonfanti, 2015).

The Striatum

The adult striatum, at least in some mammalian species, also retains the potential to generate new neurons. Ontogenically, the striatum originates from the ganglionic eminence located in the ventral part of the developing telencephalon. The most active striatal neurogenesis, which proceeds in two phases that result in a different functionality of striatal neurons, almost ends with birth (Pauly et al., 2012). In adults the striatum consists of about 95% GABAergic medium spiny neurons and less than 5% aspiny interneurons (Tepper and Bolam, 2004; Pauly et al., 2012).

Unlike the cerebral cortex, there is significantly more evidence of the generation of new neurons in the adult striatum under physiological conditions. Several works did not reveal new neurons in the unlesioned striatum (Pencea et al., 2001b; Teramoto et al., 2003; Luzzati et al., 2011). Nevertheless, some studies clearly demonstrated that neuroblasts and new neurons continued to arise in the striatum of adult rats (Dayer et al., 2005), rabbits (Luzzati et al., 2006), guinea pigs (Luzzati et al., 2014) and humans (Ernst et al., 2014). However, adult-born new neurons were not found in mice in physiological conditions (Nato et al., 2015), suggesting a potentially important difference between the species. In humans, unlike in other species, Ernst et al. (2014) showed substantial neurogenesis in the striatum: a subpopulation of interneurons, mainly expressing the marker calretinin, is exchanged at a rate of 2.7% per year in adulthood.

The origin of NPCs in the adult striatum has been intensively studied. Some works have largely clarified the question concerning "local vs. SVZ" source of these neurons. Luzzati et al. (2006), analyzing spatial distribution of proliferating NPC clusters in rabbits, have demonstrated that new neurons in the striatum, at least partially, are of local origin. The authors also showed that NPCs differentiate into mature neurons; however, only a small amount of them survive during maturation. Similarly, NSCs of astroglial nature, like NSCs in the SVZ and DG, were found in the external capsule and lateral striatum of juvenile guinea pigs (Luzzati et al., 2014). These cells proliferate and give rise to new neurons that have existed transiently and have not contributed to the population of mature functional neurons. At the same time, they expressed Sp8, a transcription factor associated with neuroblasts migrating to the OB, and showed tropism for white matter tracts. Collectively, these studies suggest that the striatum, at least in rabbits and guinea pigs, is able to generate new neurons but that their survival is negligible.

Various pathologic changes and injuries strongly affect neurogenesis and migration of NPCs in the adult brain. Though the V-SVZ is still considered the main source of new neurons, migration pathways and destinations of NPCs can change depending on lesion area. At the same time, there is much evidence that new neurons can originate from different sources outside the known and proven neurogenic zones. In the next section we consider several works investigating these changes.

NEUROGENESIS IN THE INJURED BRAIN

In this section we describe changes in proliferation and migration of NPCs in the injured brain. In general, injury can stimulate V-SVZ neurogenesis accompanied by the migration of V-SVZ-derived neuroblasts to the site of injury, as well as local generation of new neuroblasts near the damage area. In the latter case, new neuronal progenitors are found to arise from local astrocytes, which gain NSCs properties. The significance of SVZ-derived versus local progenitors is a highly debated question. **Table 2** summarizes the research described in the following sections.

New Neurons in the Damaged Hippocampus – Resident and Migrating From the PPV Region

As has already been mentioned, under physiological conditions significant neurogenesis occurs in the DG of the hippocampus. Under ischemic conditions, mainly in the model of global ischemia, the number of neuroblasts in the DG can grow (Schmidt and Reymann, 2002; Bendel et al., 2005; Wojcik et al., 2009; Khodanovich et al., 2016, 2018a).

Additionally, after global ischemia new neurons are detected in the CA1 region of the hippocampus, where in these conditions a massive death of pyramidal neurons occurs (Nakatomi et al., 2002; Schmidt and Reymann, 2002; Daval et al., 2004; Bendel et al., 2005; Oya et al., 2009; Wojcik et al., 2009). The PPV is probably the major source of these new neurons in CA1. This supposition is lent credence by the work of Nakatomi et al. (2002) who, having labeled the cells from the PPV by intraventricular administration of DiI or of a vector with a GFP gene, proved that under total ischemic conditions neuroblasts migrate from the PPV. Moreover, the authors showed that a small number of neuroblasts from the PPV also migrate to the DG. Some authors revealed post-ischemic distribution of

TABLE 2 | Data on the origins of new neurons in the damaged brain.

Model of brain injury	Brain region						References
	CA1 region of the hippocampus		Striatum		Cortex		
	Resident	Migrating from the V-SVZ	Resident	Migrating from the V-SVZ	Resident	Migrating from the V-SVZ	
Global ischemia		Yes					Nakatomi et al., 2002
		Yes					Oya et al., 2009
MCAO			No	Yes			Yamashita et al., 2006
			Yes				Magnusson et al., 2014; Duan et al., 2015
				Yes			Arvidsson et al., 2002; Zhang et al., 2004, 2007, 2009, 2011; Thored et al., 2006; Hou et al., 2008; Liu et al., 2009; Kojima et al., 2010; Li L. et al., 2010; Nakaguchi et al., 2012; Grade et al., 2013; Kazanis et al., 2013
Neonatal ischemia/hypoxia				Yes			Yang and Levison, 2006; Yang et al., 2008
Global ischemia				Yes			Yoshikawa et al., 2010
Hemorrhagic stroke				Yes			Yan et al., 2009a
MCAO				Yes		Yes	Parent et al., 2002; Jin et al., 2003; Zhang et al., 2005; Yan et al., 2007
Global ischemia					Yes	Yes	Ohira et al., 2010
					Yes		Fukuzaki et al., 2015
Neonatal hypoxia					Yes		Bi et al., 2011
MCAO					Yes	No	Kuge et al., 2009
Chemical injury of the cortex					No	Yes	Faiz et al., 2015
						Yes	Magavi et al., 2000; Chen et al., 2004; Faiz et al., 2008
Global ischemia						Yes	Li et al., 2011
MCAO						Yes	Leker et al., 2007; Wang et al., 2007; Lai et al., 2008; Li B. et al., 2010; Kreuzberg et al., 2010; Moraga et al., 2014; Palma-Tortosa et al., 2017; Pradillo et al., 2017
MCAO+global ischemia						Yes	Ohab et al., 2006; Song et al., 2017; Tseng et al., 2018
Photothrombotic stroke (cortex)						Yes	Osman et al., 2011; Vandeputte et al., 2014
Thermocoagulation of pial vessels (cortex)						Yes	Gotts and Chesselet, 2005
Cortical devascularization						Yes	Kolb et al., 2007; Wan et al., 2016
Neonatal ischemia/hypoxia						Yes	Yang et al., 2007
Mechanical injury of the cortex						Yes	Goings et al., 2004; Ramaswamy et al., 2005; Saha et al., 2013; Yi et al., 2013

neuroblasts from the PPV to CA1 in fixed slices (Oya et al., 2009; Khodanovich et al., 2018b). Furthermore, it is likely that the same migration was being reported in the work by Daval et al. (2004), wherein the authors discovered an increase, after neonatal hypoxia, in the number of neuroblasts in the SVZ and the PPV, where labeled cells formed a migrating chain toward the periventricular region located above the hippocampus. Many works that have demonstrated the appearance of young neurons in CA1 after ischemia still provide no information about their origins (Schmidt and Reymann, 2002; Bendel et al., 2005; Wojcik et al., 2009; Niv et al., 2012; Ortega et al., 2013). Bendel et al. (2005) emphasized that after ischemia NPCs proliferation increased both in the PPV and the SGZ of the DG, and both these regions could be the source of new neurons in CA1.

Thus, these data testify that new neurons in the damaged CA1 region originate from the PPV. However, there is no proof that resident NPCs cannot occur in this region after an injury, because this possibility has not been specifically examined.

New Neurons in the Damaged Striatum – Resident and Migrating From the SVZ

In the damaged striatum, many young neurons can be detected, as has been shown on models of ischemia (Jin et al., 2003; Yamashita et al., 2006; Hou et al., 2008; Kojima et al., 2010; Grade et al., 2013; Magnusson et al., 2014) and neonatal ischemia-hypoxia (Yang and Levison, 2006; Yang et al., 2008).

Some works do not specify the origins of these cells (Kobayashi et al., 2006; Lee et al., 2006; Zhang et al., 2006, 2010; Masuda et al., 2007; Yoo et al., 2008; Cui et al., 2009; Yan et al., 2009a; Shimada et al., 2010; Li et al., 2011; Ortega et al., 2013; Cheyuo et al., 2015; Fujioka et al., 2017; Wang et al., 2017). However, some proved their origins from the SVZ using different methods by:

- labeling cells in the SVZ (Jin et al., 2003; Zhang et al., 2005, 2007, 2011; Yamashita et al., 2006; Hou et al., 2008; Yang et al., 2008; Liu et al., 2009; Kojima et al., 2010; Li L. et al., 2010; Yoshikawa et al., 2010; Grade et al., 2013);
- direct observation of migration in living brain slices (Zhang et al., 2007, 2009; Kojima et al., 2010; Grade et al., 2013);
- intraventricular administration of the antimitotic agent Ara-C (Yoshikawa et al., 2010);
- or estimation of neuroblasts distribution in fixed brain slices (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003; Zhang et al., 2004, 2005, 2007, 2009, 2011; Thored et al., 2006; Yang and Levison, 2006; Yan et al., 2007, 2009b; Yang et al., 2008; Liu et al., 2009; Li L. et al., 2010; Yoshikawa et al., 2010; Nakaguchi et al., 2012; Kazanis et al., 2013).

Additionally, data provided by Yamashita et al. (2006) suggest that SVZ-derived NPCs can be the main source of new neurons in the damaged striatum after transient MCAO, with no, or minor, participation of local striatal progenitors. For labeling cells that originate from the striatum, a genetic vector carrying the GFP gene was injected into the striatum. As a result, GFP expression was induced in some striatal cells (in the majority of cells near the injection site), but not in the SVZ. Then, ischemia was induced, and none of the detected DCX-positive neuroblasts in the damaged striatum expressed GFP. However, given that only a subset of striatal cells was infected with a GFP-expressing vector, the generation of new neurons from some unlabeled striatal progenitors cannot be entirely excluded. At the same time, DCX-positive neuroblasts in the damaged striatum expressed GFP, if the vector was injected into the SVZ. This points to a significant role for SVZ-derived progenitors.

At the same time, three works proved that after lesion, a number of new neurons in the striatum are generated from local precursors (Magnusson et al., 2014; Duan et al., 2015; Nato et al., 2015). Nato et al. (2015) found local neurogenesis in the damaged striatum after excitotoxic lesion. By injecting a vector carrying Cre-recombinase under the control of a GFAP promoter into the striatum of transgenic R26R reporter mice, the authors induced YFP expression in striatal – but not subventricular – astrocytes. After subsequent lesion, about 85% of clustering Ki67 and DCX-positive cells in the damaged striatum co-expressed a YFP reporter, indicating that the majority of lesion-induced neuroblasts are of local origin. Additionally, the induction of a YFP reporter in the SVZ revealed that only a small number of these striatal neuroblasts derived from the SVZ. Similarly, Magnusson et al. (2014) induced the expression of the YFP gene in the striatal astrocytes and their offspring (but without induction of YFP expression in the SVZ or the RMS). After ischemia, which was later induced, many cells that had expressed YFP were also colocalized with DCX and with NeuN. Duan et al. (2015) induced GFP expression in astrocytes of the striatum by injecting a vector carrying a GFP gene under the control of a GFAP promoter. After ischemia they discovered NeuN- and Nestin-positive cells among those that had expressed GFP. The authors concluded that these cells are GFP-positive because they had generated from striatal GFAP-expressing astrocytes. However, it is unclear whether cells are able to retain a detectable amount of GFP in the absence of GFP-gene expression for an any significant length of time. Thus, the exact mechanism of GFP appearance in GFAP-negative neurons and NPCs remains an open question and the results of Duan et al. (2015) are difficult to interpret.

It should be noted here that the majority of the above-mentioned works estimated the role of either the SVZ or only of the striatum in the production of new neurons in the damaged striatum. Only the two works investigated both and obtained contradictory results (Yamashita et al., 2006; Nato et al., 2015). In particular, Yamashita et al. (2006) failed to discover new striatal neurons of local origin at all, whereas Nato et al. (2015) found that the majority of lesion-induced striatal neuroblasts are of local origin. It is unclear whether these contrasting results stem from the difference in the nature of the lesion (transient local ischemia versus chemical lesion), or from some other methodological aspects of the studies. At the same time, the work by Li L. et al. (2010), who labeled the cells originating from the SVZ, showed that labeled cells from the SVZ account for only one third of all neuroblasts in the damaged striatum. However, it remains unclear whether this value is indicative

of the actual proportion of subventricular/striatal precursors or of insufficient labeling of precursors in the SVZ. Thus, it remains to be elucidated which plays a more important role in the restoration of the damaged striatum – the SVZ or the striatum.

New Neurons in the Damaged Cortex – Resident and Migrating From the SVZ/PPV Region

Damage to the cortex of the adult animal brain enhances neurogenesis in it, and this has been shown in different models of brain damage, such as ischemia (Jin et al., 2003; Ohab et al., 2006; Kolb et al., 2007; Kreuzberg et al., 2010; Ohira et al., 2010), neonatal ischemia-hypoxia (Yang et al., 2007), neonatal hypoxia (Bi et al., 2011), and mechanical (Saha et al., 2013), chemical (Faiz et al., 2008, 2015) and thermal (Covey et al., 2010; Ajioka et al., 2015) damage. Some of the above works do not include an investigation into the origins of new neurons in the damaged cortex (Zhang et al., 2006; Yoo et al., 2008; Covey et al., 2010; Ortega et al., 2013; Ajioka et al., 2015).

The majority of works establish their origin from the SVZ or from the PPV. These findings were obtained with the use of different approaches based on:

- distribution pattern of neuroblasts migrating from the SVZ/PPV to the damaged area in fixed brain slices (Magavi et al., 2000; Parent et al., 2002; Jin et al., 2003; Chen et al., 2004; Goings et al., 2004; Gotts and Chesselet, 2005; Ramaswamy et al., 2005; Zhang et al., 2005; Ohab et al., 2006; Kolb et al., 2007; Leker et al., 2007; Wang et al., 2007; Yan et al., 2007; Faiz et al., 2008, 2015; Li et al., 2008, Li B. et al., 2010; Li et al., 2011; Kreuzberg et al., 2010; Osman et al., 2011; Saha et al., 2013; Yi et al., 2013; Moraga et al., 2014; Vandeputte et al., 2014; Wan et al., 2016; Palma-Tortosa et al., 2017; Pradillo et al., 2017; Song et al., 2017; Tseng et al., 2018);
- suppression of cell proliferation in the SVZ by intraventricular administration of Ara-C (Leker et al., 2007; Li B. et al., 2010; Faiz et al., 2015);
- labeling cells from the lateral ventricles (Jin et al., 2003; Goings et al., 2004; Ramaswamy et al., 2005; Zhang et al., 2005; Ohab et al., 2006; Kolb et al., 2007; Yang et al., 2007; Faiz et al., 2008, 2015; Lai et al., 2008; Kreuzberg et al., 2010; Li B. et al., 2010; Saha et al., 2013; Vandeputte et al., 2014);
- and direct observation of migration in the live brain (Vandeputte et al., 2014).

At the same time, some studies do in fact suggest that the production of new neurons can occur in the injured cortex (Gu et al., 2000; Kuge et al., 2009; Ohira et al., 2010; Shimada et al., 2010, 2012; Bi et al., 2011; Nakagomi et al., 2011, 2015; Fukuzaki et al., 2015). Bi et al. (2011) showed by special labeling that, after neonatal hypoxia, cortex astrocytes in neonates gain the properties of NSCs and differentiate into new neurons both in the damaged cortex and after having been translated into cell culture.

Ohira et al. (2010) discovered, in the first layer of the normal animal cortex, proliferating cells that express the GAD67 neuronal marker. Mild ischemia, which was achieved by 10-min cross-clamping of the two major carotid arteries, caused an increase in the number of these cells, their migration into the lower layers of the cortex and differentiation into functionally mature neurons (revealed by expression of the TuJ1, HuC/D, MAP2, c-Fos markers, and electrophysiological activity). A vector with the GFP reporter gene was administered before ischemia either into the cortex or into the SVZ to determine the source of new neurons in the cortex. The cortex appeared to be the main source of new neurons itself, with only a few having migrated to the cortex from the SVZ.

Kuge et al. (2009) report in their work that after transient MCAO new neurons were generated in the cortex. The authors emphasize that they have not detected any patterns of neuroblasts migration from the SVZ via the corpus callosum to the cortex. Therefore, they assume that these new neurons in the cortex are of local origin.

Fukuzaki et al. (2015) described in their work that after 10-min cross-clamping of the two carotid arteries they observed proliferation of GAD67-positive NPCs in the cortex. The fact that these cells localized mainly in the first layer showed that they were local precursors.

Gu et al. (2000) reported in their work that after phototrombotic stroke new neurons (colocalization of bromodeoxyuridine with MAP2 and NeuN) started generating in the damaged cortex 72 h after surgery. The authors concluded that this early appearance of new neurons in the cortex proves that it is very unlikely that they could have originated in the SVZ. At the same time, it is questionable that even local progenitors in the cortex can generate mature NeuN-expressing cortical neurons within 3 days. Thus, the mechanism of new neuron generation in this study remains unclear.

Some works described that after permanent MCAO in adult animals, cortical astrocytes (Buffo et al., 2008; Shimada et al., 2010, 2012), as well as pericytes of leptomeningeal and cortical vessels (Nakagomi et al., 2011, 2015), gained NSC properties: in cell culture they differentiate into neurons, astrocytes and oligodendrocytes. At the same time, in the damaged cortex these precursors exhibited a more limited capacity to differentiate – differentiating only into astrocytes and oligodendrocytes and never into neurons (Buffo et al., 2008; Shimada et al., 2010).

Despite a large number of studies, it is still difficult to get a full picture of neurogenesis in the damaged cortex since the majority of researchers estimated the role of either only the cortex or of the SVZ in the production of new cortical neurons. The work by Ohira et al. (2010) is a rare exception. The authors labeled both cells of the cortex and cells of the SVZ, and demonstrated that after a mild cortical ischemia a larger part of new cortical neurons originated in the cortex, not in the SVZ. At the same time, Faiz et al. (2015) showed that after chemical cortical damage, all of the NPCs that were generated in the cortex had originated from the SVZ. The different results allow us to assume that the role of local and SVZ precursors in the

restoration of the cortex can substantially vary depending on the conditions of the experiment, particularly on the type and degree of damage.

Interhemispheric Migration

In this section we consider several works that describe interhemispheric migration of neuroblasts after cortical injury (Ramaswamy et al., 2005; Wan et al., 2016). The authors discovered that damage to the cortex may result in migration of neuroblasts from one hemisphere to the other. While Wan et al. (2016) reported on migration from the healthy hemisphere to the damaged one, Ramaswamy et al. (2005) discussed migration from damaged to healthy.

Wan et al. (2016) observed that after cortical devascularization, migration occurs not only from the SVZ of the same hemisphere, but also from the contralateral SVZ, to the injured cortex. In fixed brain slices, the authors observed distribution of cells migrating from the contralateral SVZ via the midline, further to the ipsilateral corpus callosum, and finally to the lesion area in the cortex. Some authors report on the enhancement of proliferation in the contralateral SVZ after ischemic or mechanical brain damage but they have not found any signs of migration from the contralateral SVZ to ipsilateral (Leker et al., 2007; Kreuzberg et al., 2010; Li B. et al., 2010; Saha et al., 2013).

Ramaswamy et al. (2005) obtained more unusual results. They discovered that after mechanical damage to the cortex, neuroblasts migrate from the ipsilateral SVZ not only to the lesion area in the cortex, but also to the contralateral (non-damaged) hemisphere. The origin of neuroblasts migrating from the SVZ has been proved by preliminary administration of fluorescent microspheres into the lateral ventricle. The authors hypothesized that migration of neuroblasts to the contralateral hemisphere may functionally compensate for the damage to the ipsilateral cortex, hence the healthy cortex in the contralateral hemisphere may take over the functions of the damaged cortex. The results obtained by the two mentioned groups (Ramaswamy et al., 2005; Wan et al., 2016) are extraordinary, and have not been reported elsewhere. Still, it remains unclear whether migration from the damaged hemisphere to the health one or vice versa occurs only under some specific conditions set by researchers, or is an ordinary process in the injured brain that has just been overlooked by other investigators.

Functional Relevance of Injury-Induced Neurogenesis

Although this review focuses mainly on the anatomical origin of new neurons appearing in the damaged brain areas, the question about their functional relevance cannot be ignored due to its high importance when seeking out new directions for future therapies. Here we discuss some related issues. For more information about the survival, differentiation and functional integration of new neurons within the injured brain tissue, we direct the reader to the reviews by Lindvall and Kokaia (2015) and Marlier et al. (2015).

Relatively few works have addressed the study of structural and functional integration of new neurons into the existing neural networks after brain injury. Mainly, such integration has been shown in the damaged striatum and cortex.

At least two studies provided evidence that both SVZ-derived and locally generated neurons may successfully integrate into the pre-existing circuit within the damaged cortex. Lai et al. (2008) used lentiviral labeling of SVZ-derived progenitors in combination with patch-clamp recordings on the labeled neurons within the cortex. They showed that after MCAO, new SVZ-derived neurons in the damaged cortex fired the induced and spontaneous action potentials, which suggests that these neurons are synaptically integrated. However, the results of Lai et al. (2008) should be interpreted with consideration for the fact that a lentiviral vector could have infected non-dividing cells in the cortex. Ohira et al. (2010) retrovirally labeled the local progenitors within the cortex, and after global ischemia indirectly examined the functional integration of new neurons by evaluation of the immediate early gene c-Fos expression. They found that c-Fos expression by the new neurons was much higher in the enriched environment when compared with the control conditions, which suggests that the new neurons in the cortex are synaptically active. It was also concluded that the new neurons are GABAergic interneurons.

Several studies showed structural and functional integration of new neurons into the existing striatal networks. Yamashita et al. (2006), using electron microscopy, showed that newly generated neurons form synapses with neighboring cells in the post-ischemic striatum. Additionally, a retrograde tracing study by Sun et al. (2012) showed that a subpopulation of new neurons in the damaged striatum re-establishes long connections with the substantia nigra. Moreover, Hou et al. (2008) provided both structural and electrophysiological evidence of synaptic integration of new striatal neurons after MCAO. Strong evidence in support of the regenerative role of lesion-induced neurogenesis comes from the work by Jin et al. (2010). The authors used transgenic animals expressing herpes simplex virus thymidine kinase under the control of the promoter for DCX to selectively ablate new neurons in the brain through a ganciclovir injection. They showed that neurogenesis ablation worsens the outcome after MCAO at both histological and behavioral levels.

Despite these optimistic results, the majority of these studies showed two important limitations in the restoration of neuronal circuits by adding newly generated neurons: the low survival rate of these neurons and the poor scope of their differentiation potential. Most new neurons within the ischemic striatum die, possible due to unfavorable environment in the damaged tissue (Arvidsson et al., 2002; Parent et al., 2002; Thored et al., 2006). Since generation and migration of new neurons can last for months (Thored et al., 2006; Leker et al., 2007) and even a year (Kokaia et al., 2006; Osman et al., 2011) after injury, we can assume that mature neurons may, at least partially, compensate for their high mortality within the ischemic tissue.

Another important limitation is a relevance of functional features of integrated new neurons to the pre-existing functionality of the network. Studies on a phenotype of

newly generated neurons within the ischemic striatum provided contradictory results regarding a percentage of newly generated neurons expressing DARPP-32, a marker of medium-sized spiny neurons – the major class of striatal neurons. Arvidsson et al. (2002) and Parent et al. (2002) showed that after MCAO a substantial number of new striatal neurons are DARPP-32-positive. In contrast, on the same model of ischemia, Teramoto et al. (2003) and Liu et al. (2009) failed to find any newly generated DARPP-32-positive neurons at all. Teramoto et al. (2003) found that about 65% of newly generated striatal neurons are parvalbumin-positive interneurons, which normally comprise a minor subpopulation of striatal neurons. Liu et al. (2009) reported that nearly all new neurons in the damaged striatum are calretinin-positive interneurons, which are extremely rare in the striatum under physiological conditions. In addition, these new striatal neurons express the transcription factor Sp8, which is expressed in most newly generated neurons in the OB under physiological conditions. The results of Liu et al. (2009) suggested that SVZ-derived progenitors had strictly limited differentiation potency, and that after MCAO they cannot generate the majority of neuronal classes, which are lost within the damaged striatum. This point of view may be further strengthened by the work of Merkle et al. (2007). The authors found that, despite the diversity of the newly generated OB interneurons, their stem progenitors within the SVZ have a strictly restricted potency of differentiation. In fact, the SVZ stem cell niche has a mosaic organization, where NSCs from SVZ subregions vary in their differentiation potential. NSCs from a particular SVZ-subregion may give rise only to a particular type of interneurons, even when cultivated *in vitro*. It seems unlikely that these strictly predetermined cells can produce other types of neurons necessary for brain recovery.

Identification of the causes of the above-mentioned discrepancies is one of the directions for future studies. Thus, more research needs to be done to comprehensively evaluate the regenerative potential of adult neurogenesis and to develop optimal strategies for its enhancement.

CONCLUSION

Up to the present time, a substantial amount of research material concerning the origins and migratory pathways of NPCs in the brain under physiological and pathological conditions has been accumulated. In the healthy brain, the majority of NPCs originate in the V-SVZ located in the walls of the lateral ventricles (Lim and Alvarez-Buylla, 2016). In the embryonic and early postnatal brain, NPCs migrate from the V-SVZ to the OB, striatum, and cortex. In adulthood, these pathways – except for migration to the OB in rodents (Lim and Alvarez-Buylla, 2016) and, probably, to the striatum in humans (Ernst et al., 2014) – remain substantially quiescent but can intensify in conditions of brain injury (Nakatomi et al., 2002; Yamashita et al., 2006; Faiz et al., 2015). Among other regions, at the very least, the adult striatum maintains the capacity to generate new neurons in both physiological (Dayer et al., 2005; Luzzati et al., 2006, 2011) and pathological (Magnusson et al., 2014; Nato et al., 2015) conditions. The fate and functionality of local newborn striatal neurons have been intensively studied (Lindvall and Kokaia, 2015; Marlier et al., 2015). Adult neurogenesis in the cortex and migration of NPCs from the V-SVZ to this area in physiological conditions are less evident (Gould et al., 2002; Dayer et al., 2005; Bhardwaj et al., 2006; Cameron and Dayer, 2008). However, whilst brain injury provokes the generation of new cortical neurons, the survival and functionality of these neurons are commonly questioned (Ohira et al., 2010; Faiz et al., 2015).

Human neurogenesis, which has been also shown in the studies with the comprehensive ^{14}C-content assessment (Spalding et al., 2013), substantially differs from neurogenesis in rodents. Using this technique in studies on rodents, which are more extensively used in research, could help clarify these differences.

Despite the impressive array of research works, at present there is no full picture of neurogenesis and NPC migration in the brain. Most research works address either a single neurogenic region or a single migratory pathway of neuroblasts. Future studies on the injured brain could examine the following aspects: firstly, the local or migratory nature of NPCs after brain injury could be confirmed by novel techniques of real-time observations; secondly, since the presence of newly generated neurons does not ensure their functionality, the question about the integration of new neurons into the existing networks and their phenotypes could be further investigated; thirdly, comparative studies are needed to understand the difference between human and rodent neurogenesis for future translation of new therapies to clinic.

It is likely that in the future, methods for longitudinal observation capable of capturing new neuron production, migration, and functional integration in the same animal brain, will be improved. We believe that the advancement of methods for *in vivo* visualization of neurogenesis in the brain could fundamentally change the current situation in this field.

AUTHOR CONTRIBUTIONS

NN-D wrote the manuscript. MK conceived the presented idea and critically revised the manuscript. Both authors contributed to the literature search and analyses.

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REVIEW
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Role of the Immune System in the Development of the Central Nervous System

*Keiko Morimoto and Kazunori Nakajima**

Department of Anatomy, Keio University School of Medicine, Tokyo, Japan

The central nervous system (CNS) and the immune system are both intricate and highly organized systems that regulate the entire body, with both sharing certain common features in developmental mechanisms and operational modes. It is known that innate immunity-related molecules, such as cytokines, toll-like receptors, the complement family, and acquired immunity-related molecules, such as the major histocompatibility complex and antibody receptors, are also expressed in the brain and play important roles in brain development. Moreover, although the brain has previously been regarded as an immune-privileged site, it is known to contain lymphatic vessels. Not only microglia but also lymphocytes regulate cognition and play a vital role in the formation of neuronal circuits. This review provides an overview of the function of immune cells and immune molecules in the CNS, with particular emphasis on their effect on neural developmental processes.

Keywords: MHC, complement, T cell, central nervous system, immune system

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Ola Hermanson,
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Mladen-Roko Rasin,
Rutgers University, The State University of New Jersey, United States

***Correspondence:**
Kazunori Nakajima
kazunori@keio.jp

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INTRODUCTION

The central nervous system (CNS) and the immune system have much in common. The most prominent characteristic of either system is their ability to transmit information to distant parts of the body with extraordinary specificity and diversity. In the immune system, the diversity of acquired immune cells–T cells and B cells–is generated by the stochastic VDJ recombination of T cell receptors (TCRs) and immunoglobulin (Ig) genes, and somatic hypermutation of TCRs (at least in the shark) (Ott et al., 2018) and immunoglobulins. For example, the human heavy chain region contains 38–46 variable (V) gene segments, 23 diversity (D) gene segments, and 6 joining (J) gene segments, and one segment of each type is selected in each lymphocyte by a mechanism called VDJ recombination. Moreover, the many different combinations of heavy- and light-chain variable regions that pair to form the antigen-binding site result in at least 10^{11} different receptors. The diversity of immunoglobulins is magnified by somatic hypermutation that occurs after the initiation of immune response and introduces point mutations into the rearranged variable region to enhance the reactivity to antigen. As for T cells, theoretically, 10^{15} TCRs can be produced by using almost the same mechanism as for immunoglobulins. This mechanism is critical for the evolution of the vertebrate adaptive immune system, because the genome with its limited size (approximately 3 billion nucleotides) could not directly encode all the possible antigen receptors. On the other hand, the human brain contains approximately 10^{11} neural cells that are classified into hundreds of different neuronal subtypes based on cell morphology, gene expression profile, and axon/dendrite projection patterns. For example, 21 neuronal subtypes are identified in the human frontal cortex by single-cell methylomes (Luo et al., 2017). Each neuron collects inputs from and sends outputs to many other specific neurons–on average, 10^3 for both inputs and

outputs for a mammalian neuron. In addition, glial cells, which outnumber neurons approximately 10 times, cover synapses and control the neural network. It is known that each human astrocyte can contact and encompass nearly 2×10^6 synapses (Oberheim et al., 2006). In this way, specific and diverse neural networks are established, although the precise underlying molecular mechanisms have not been completely illustrated. During the generation of diversity, some non-functional or autoreactive TCR-expressing T cells and undesirable neurons could emerge. These T cells undergo apoptosis in the thymus by a mechanism known as positive and negative selection, and some neurons are removed by apoptosis or lose their synaptic connections through synaptic pruning. Moreover, both systems possess memory mechanisms. In the immune system, after invasion of bacteria, viruses, and other disease-causing organisms, the appropriate acquired immune cells that can respond to specific antigens are expanded and stored as memory T and B cells, so they can immediately generate an accelerated and more robust antibody-mediated immune response when the pathogen is encountered again. On the other hand, synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD), underlie memory in the nervous system. In addition, both systems use the mechanisms of accelerators and brakes. In the immune system, there are immunosuppressive T/B cells (Treg, Breg) (Sakaguchi et al., 2008; Rosser and Mauri, 2015) and their imbalance results in allergy and autoimmunity. On the other hand, CNS neurons are classified into excitatory and inhibitory neurons, and the appropriate balance between these two populations is critical for neuronal networks to function normally (Yizhar et al., 2011). During the formation of neural circuits, both excitatory neurons and inhibitory interneurons undergo extensive cell death in the critical window of postnatal development (Southwell et al., 2012) and the survival of interneurons depends on the activity of excitatory pyramidal neurons (Wong et al., 2018). Of note, acquired immune system cells and highly developed myelination in the nervous system appeared at nearly the same time during evolution, around the evolution from jawless to cartilaginous fish (Zalc et al., 2008). It would be interesting if this was not mere coincidence but the two phenomena were causally linked. In addition, our understanding of the CNS has recently dramatically changed from an "immune privileged site" to a "special immune-controlled site." In 2015, it was discovered that functional lymphatic vessels line the dural sinuses, and are able to carry both fluid and immune cells from the cerebrospinal fluid to the deep cervical lymph nodes (Louveau et al., 2015). The importance of meningeal lymphatic vessels for waste clearance was confirmed because impairment of meningeal lymphatic function caused cognitive impairment in mice (Da Mesquita et al., 2018). These discoveries shed more light on the interaction between the CNS and the immune system. Moreover, emerging evidence suggests that an increasing number of molecules that are typically associated with the immune system are also expressed in various CNS regions and play crucial roles in brain development. This review summarizes the reports on the function of immune cells (**Figure 1**) and immune molecules (**Table 1**) mainly in CNS development during the embryonic and early postnatal periods, with some attention paid to their function in more mature brains.

CONTRIBUTION OF IMMUNE CELLS TO CNS FUNCTION

In the steady state, many lymphocytes reside mostly in the meninges and choroid plexus; however, a few lymphocytes are also found in the brain parenchyma, such as in the fimbria of the dorsal hippocampus and anterior olfactory nucleus, as clearly illustrated by reconstitution of green fluorescent protein-expressing lymphoid cells in *Rag2*$^{-/-}$ mice (Song et al., 2016). The most dominant immune cells in the brain are microglia, which comprise 80% of brain immune cells. Other immune cells identified in the brain include myeloid cells, monocytes/macrophages, dendritic cells, T cells, B cells and natural killer (NK) cells (Korin et al., 2017). Lymphocytes (including T cells, B cells, and NK cells), which are identified as a CD45^{hi} population, are scarce in the CNS, with approximately 10,000 per hemisphere in adult naïve mice (Pösel et al., 2016). However, it is now clear that these limited numbers of immune cells have a significant impact on brain function. In particular, T cells have been implicated in complex brain processes including spatial learning, memory, emotional behavior, and stress responsiveness. For example, in mice undergoing the Morris-water-maze test (MWM), CD4$^+$ T cells (helper T cells), but not CD8$^+$ T cells (cytotoxic T cells), are recruited to the meninges, and secrete interleukin (IL)-4. IL-4 skews macrophages and microglia to an M2 (anti-inflammatory) phenotype, and induces the production of brain-derived neurotrophic factor by astrocytes, leading to the improvement of spatial learning and memory (Kipnis et al., 2004; Ziv et al., 2006; Wolf et al., 2009; Derecki et al., 2010; Radjavi et al., 2014). Previous studies have also demonstrated that B cells are not required for these processes because B cell-deficient μMT mice do not exhibit learning disabilities (Wolf et al., 2009; Radjavi et al., 2014).

In contrast to the adult brain, data regarding the interaction of immune cells and neural cells— except for microglia—during developmental stages are quite limited. However, epidemiological studies have demonstrated a link between maternal infection and the onset of neurodevelopmental disorders, such as autism spectrum disorder (ASD), schizophrenia, epilepsy, cerebral palsy, anxiety, and major depressive disorder, pointing to the association between the immune system and neural development (reviewed in Knuesel et al., 2014; Estes and McAllister, 2016). Animal models using rodent and non-human primates have also clearly demonstrated a causal relationship between maternal infection and ASD- and schizophrenia-related behavioral abnormalities. It is widely accepted that a major consequence of maternal immune activation (MIA) are changes in microglial morphology, distribution, and the expression level of several marker proteins. Moreover, it is known that microglia have multifaceted functions during normal brain development. It

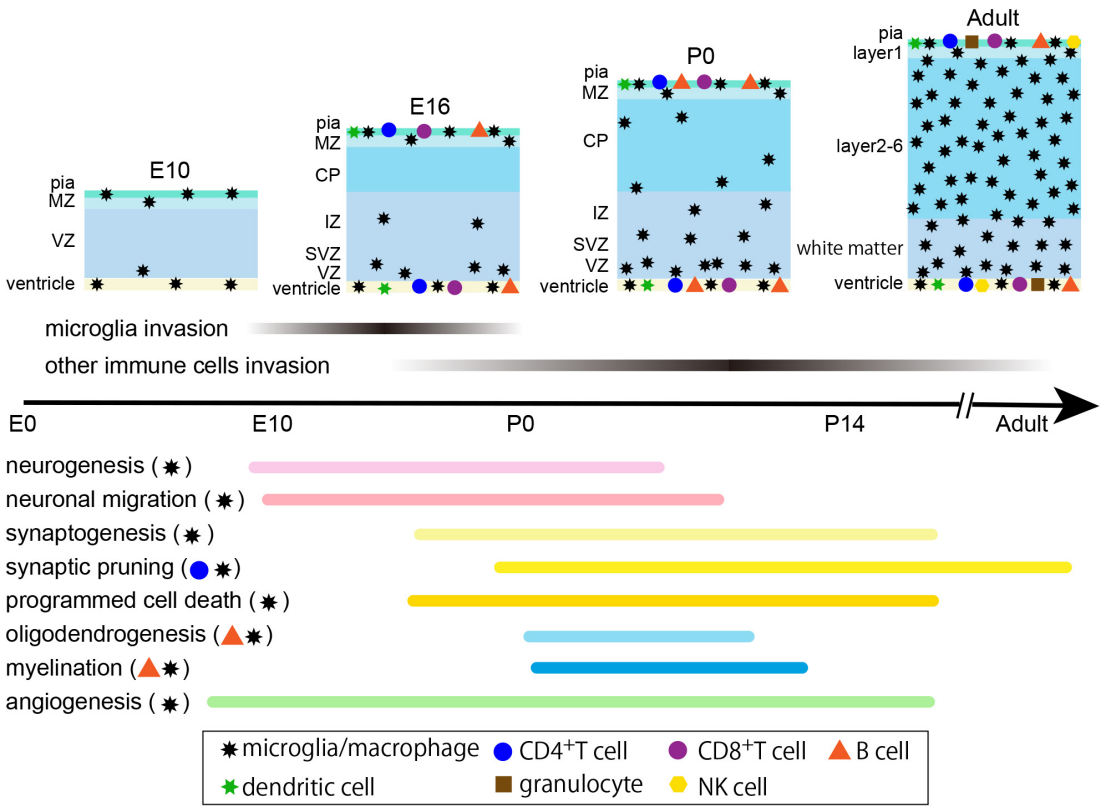

FIGURE 1 | Timeline of cerebral cortex development and distribution of immune cells in mice. Microglia begin to enter the brain at E9.5, and other immune cells, such as T cells, B cells and dendritic cells, infiltrate the brain at least by E16. No data regarding the distribution of granulocytes and NK cells at developmental stages are available; however, they exist in the adult brain. Immune cells, except microglia, are mostly located at the pial surface, ventricle and choroid plexus, and a few cells enter the brain parenchyma. The lower part indicates the time course of major developmental events and the marks on the right illustrate the related immune cells for each process. E, embryonic; P, postnatal; MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

has recently been reported that acquired immune cells are also engaged in the developmental processes of the CNS.

Neonatal Immune Cell Population

Analyses of embryonic and neonatal immune populations in the CNS remain limited; however, one report illustrated that a small number of lymphocytes infiltrated the developing mouse brain even at embryonic day 16 (E16) and, among the investigated cell types, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells, B cells are the most abundant in the CNS, peaking at approximately 5% of total lymphocytes (Tanabe and Yamashita, 2018b). Another study, using *CD11c/EYFP* transgenic mice, clearly illustrated that $CD11c^+$ (also known as integrin αX and complement component 3 receptor 4 subunit) cells—which include monocytes, macrophages, dendritic cells, granulocytes, and NK cells—were present along the ventricles and within the adjacent parenchyma at E16 and postnatal day 2 (Bulloch et al., 2008). However, there have been no detailed reports describing the subpopulation of immune cells in the developing brain.

Microglial Function in CNS Development

Microglia are tissue-resident macrophages that play essential roles in innate immunity and have an origin that is different from other immune cells. Hoxb8-negative microglia arise from erythromyeloid precursors in the yolk sack during primitive hematopoiesis and infiltrate the brain at E9.5 in mice (Ginhoux et al., 2010), immediately after the onset of angiogenesis and neurogenesis. In contrast, Hoxb8-positive microglia infiltrate the brain at E12.5 (De et al., 2018). Although microglia are initially located along the meninges and ventricles, after E14 they distribute broadly throughout the cortex and then change their distribution to avoid the cortical plate. After E18, they again enter the cortical plate and begin to distribute to the entire cortex and increase their numbers dramatically (Reemst et al., 2016). During these dynamic changes in microglial distribution, the neural system undertakes highly orchestrated processes, including angiogenesis/vascularization, proliferation and migration of neurons and glia, programed cell death of neural stem cells and neurons, formation of synapses, myelination and establishment of neuronal circuits. Microglia contribute virtually to all of these events (reviewed in Kettenmann et al., 2013; Wu et al., 2015). For example, microglia regulate angiogenesis/vascularization by clearing excess vessels and participating in vessel anastomosis (Fantin et al., 2010), control the number of neural stem cells by phagocytosis (Cunningham et al., 2013), and regulate the survival of neurons in layer 5 via insulin-like growth factor 1

TABLE 1 | Molecules that are expressed both in the nervous system and immune system, and their reported/potential functions.

Neuro-immune common molecule		Nervous system			Immune system		
		Expression	**Function**	**References (bold: review)**	**Expression**	**Function**	**References (bold: review)**
Major histocompatibility complex (MHC) class I	H2-Kb, H2-Db	Neuron, glial cells	Regulate axonal and dendritic growth, synaptic density, synaptic transmission, activity-dependent refinement and plasticity	**Elmer and McAllister, 2012; McAllister, 2014**	All nucleated cells, platelet	Present antigen to T cells, activate NK cells if missing or changed	**Blum et al., 2013; Vivier et al., 2011**
Complement family	C1q, C2-9	Neuron, glial cells	Regulate activity-dependent synaptic pruning, related to neurogenesis, migration, and neuronal survival	**Veerhuis et al., 2011; Stephan et al., 2012**	Epithelial cell, monocyte/macrophage, fibroblast, hepatocyte	Eliminate cellular debris and infectious microbes, orchestrate immune responses	**Ricklin et al., 2010**
	CR3	Microglia			neutrophil, macrophage, NK cell		
Fc receptor	FcγR II B	Purkinje cell	Regulate the development of Purkinje cell	Nakamura et al., 2007	B cell, monocyte/macrophage, neutrophil, dendritic cell, basophil	Low affinity receptor for the Fc region of IgG and negatively regulate receptor-induced signaling	**Bruhns, 2012**
	Fcα/μR	Oligodendrocyte precursor cell (OPC)	Regulate proliferation and maturation of OPC	**Tanabe and Yamashita, 2018a**	B cell, macrophage	Work as a receptor for the Fc region of IgA and IgM	Shibuya et al., 2000
CD3 family	CD3ϵ	Purkinje cell	Regulate the development of Purkinje cell	Nakamura et al., 2007	T cell	Work as a co-receptor for TCR	**Kuhns et al., 2006**
	CD3ζ	dLGN, hippocampal neuron	Regulate activity-dependent synapse formation of RGCs in retina, LTP and LTD, and promote axon pruning	Huh et al., 2000; Baudouin et al., 2008; Xu et al., 2010, **Elmer and McAllister, 2012**			
Cytokine	IL-1β, IL-6, TNF-α, TGF-β	Neuron, microglia, astrocyte	Regulate cell survival, proliferation and differentiation, axonal growth and synaptogenesis	**Bauer et al., 2007; Knuesel et al., 2014**	Several immune cells, fibroblast, endotherial cell	Play key roles in mediating inflammatory and anti-inflammatory reactions	**Arango Duque and Descoteaux, 2014**
Chemokine	CXCL1 (fractalkine)	Neuron	Regulate microglial recruitment, neuronal survival, synaptic maturation, activity and plasticity, synaptic pruning	**Paolicelli et al., 2014**	Monocyte/macrophage, fibroblast, epithelial cell, endothelial cell	Survival, migration and adhesion of monocyte	Imai et al., 1997

(Continued)

TABLE 1 | Continued

Neuro-immune common molecule		Nervous system			Immune system		
		Expression	**Function**	**References (bold: review)**	**Expression**	**Function**	**References (bold: review)**
	CX3CR1	Microglia			Monocyte/macrophage, T cell subset, platelet, NK cell		
	CXCL12 (SDF-1)	Cerebral cortex (subplate,ventricular surface)	Enhance migration of microglia, NPC, cortical interneuron and Cajal Retzius cell, related to axon guidance, neurite outgrowth	**Li and Ransohoff, 2008; Zhu and Murakami, 2012; Guyon, 2014**	Bone marrow	Essential for development of B cell and homing of hematopoietic stem cell to the bone marrow	**Nagasawa, 2015**
	CXCR4	Neuron			Several immune cells including hematopoietic stem cell		
TLR	TLR2, 3, 4, 8, 9	Neuron, neuronal progenitor cell (NPC), microglia, astrocyte, oligodendrocyte	Related to axon outgrowth, NPC proliferation, cognition, sensory and motor behaviors	**Kioussis and Pachnis, 2009; Okun et al., 2011; Khariv et al., 2013**	Monocyte/macrophage, dendritic cell, B cell, NK cell, regulatory T cell, neutrophil, basophil, fibroblast, epithelial cell, endothelial cell	Key molecules for innate immune system, work as a receptor for peptidoglycan (TLR2), dsRNA (TLR3), LPS (TLR4), ssRNA (TLR8), CpG DNA (TLR9)	**Kawai and Akira, 2007**
Pentraxin	PTX3	Astrocyte	Modulate phagocytic functions of microglia, induce functional synapse formation	Jeon et al., 2010; Fossati et al., 2019	Dendritic cell, macrophage, neutrophil	Activate complement, facilitate pathogen recognition by phagocytes	**Garlanda et al., 2005**
Pcdh	Pcdh18	Ventricular zone in the forbrain and midbrain	Involved in neural circuit formation	**Kim et al., 2011**	Activated CD8+ memory T cell	Function as an inhibitory signaling receptor and restrict the effector phase	Vazquez-Cintron et al., 2012
Dscam	Dscam	Neuron	Specify neuronal wiring, regulate axon guidance and retinal lamination	**Boulanger, 2009; Schmucker and Chen, 2009**	Hemolymph (in flies)	Bind directly to bacteria	Watson et al., 2005
Eph/Ephrin	Ephrin-B1	Neuron	Axon guidance during development, synaptic plasticity	**Klein, 2009**	Germinal center B cell, memory precursor B cell	Inhibit GC recruitment and retention of Tfh cells, promote IL-21 production	Laidlaw et al., 2017; Lu et al., 2017
Semaphorin	Sema3A	Olfactory neuron, cerebral cortex, corpus callosum	Inhibit axon branching in the cortical neurons, regulate pre-target axon sorting of olfactory system	**Tran et al., 2007**; Imai et al., 2009	Dendritic cell, T cell	Inhibit monocyte migration, inihibit T cell activation	**Kumanogoh and Kikutani, 2013; Nishide and Kumanogoh, 2018**

secretion (Ueno et al., 2013). They also modulate major events in forebrain wiring. These include the regulation of invasion of tyrosine hydroxylase-positive dopaminergic interneurons into the subpallium, the laminar positioning of parvalbumin-positive cortical interneurons (Squarzoni et al., 2014), and the control of axon projection through the corpus callosum (Pont-Lezica et al., 2014). Moreover, microglia regulate synaptic formation and synaptic pruning (through activation of the classical complement cascade) (Stevens et al., 2007; Miyamoto et al., 2016); they also regulate myelination by promoting the survival and differentiation of oligodendrocyte precursor cells (OPCs) and the maturation of oligodendrocytes (Pang et al., 2013; Shigemoto-Mogami et al., 2014).

Role of T Cells During CNS Development

In contrast to the contribution of T cells in adult brain function, very little is known about their function during embryonic and neonatal stages. One of the few explored contributions is their involvement in the pathophysiology of neonatal brain injury. Using postmortem brains from human preterm infants with periventricular leukomalacia, and animal models of preterm brain injury and sepsis-induced white matter brain injury, it was shown that γδ T cells—which have a distinctive TCR and have features of non-MHC-restricted antigen recognition and abundant cytokine secretion capacity—were responsible for injury in the developing brain, and that depletion of γδ T cells resulted in protection from injury (Zhang et al., 2017; Albertsson et al., 2018; Nazmi et al., 2018). Moreover, other groups have also demonstrated that T-helper 17 (Th17) lymphocytes coordinate neuroinflammatory responses in lipopolysaccharide (LPS)-sensitized hypoxic-ischemic injury in neonates (Yang et al., 2014). It has been also reported that *TCR*β $^{-/-}$γ$^{-/-}$ mice, which lack both αβ T cells and γδ T cells, exhibit altered size of several areas of the brain and lose sex differences (Rilett et al., 2015). Our knowledge of T cell involvement in CNS development is still fragmented and more work on T cell function in normal CNS development is needed.

B Cells in Oligodendrogenesis

As mentioned above, B cells accumulate in the neonatal brain and decline in number with age. Most of these B cells are IgM^{hi} B-1a cells (Tanabe and Yamashita, 2018b), which have innate-like characteristics and participate in maintaining tissue homeostasis (Baumgarth, 2011). These B-1a cells are suggested to be mature and are recruited to the meningeal space and lateral ventricle depending on the chemokine receptor CXCR5 and in response to CXCL13 secreted from the choroid plexus. B-1a cells secrete natural IgM antibody and promote the proliferation of OPCs through the Fc receptor for IgM (Fcα/μR). Antibody depletion of B-1a cells diminishes the number of oligodendrocytes and results in reduction of myelinated axons in neonatal mouse brains (Tanabe and Yamashita, 2018b). However, depletion of B-1a cells by antibody treatment also resulted in a decrease in the number of microglia in the subventricular zone. Therefore, it has not yet been completely ruled out that B-1a cells may also affect oligodendrogenesis indirectly through microglia. More detail is provided in Tanabe and Yamashita (2018a,b).

MOLECULES THAT PLAY IMPORTANT ROLES IN BOTH THE NERVOUS SYSTEM AND THE IMMUNE SYSTEM

It is known that these two systems share molecular mediators of communication in establishing the ability to monitor and respond to changes in the internal milieu and outside environment. Some of these are discussed in this section.

MHC Class I

MHC class I (MHCI) genes, known to be important for antigen presentation, are polygenic and polymorphic genes, comprising three classes (H2-K, -D, and -L) and multiple variants in mice. They were shown to be expressed in neurons at axons, dendrites and synapses, and in glial cells, especially highly during early postnatal stages. The function of MHCI is well reviewed in Elmer and McAllister (2012) and McAllister (2014). In brief, they are engaged in activity-dependent refinement and plasticity in the visual system, and regulate synaptic plasticity and motor learning in the cerebellum. An important question in this field is whether the diversity of MHCI is necessary for these functions. Synapse elimination and eye-specific axonal segregation in the lateral geniculate nucleus (LGN) were impaired in mice deficient in *H2-K*^{b} and *H2-D*^{b} ($K^{b}D^{b-/-}$), and were rescued by expressing a single MHCI molecule H2-D^{b} in neurons (Lee et al., 2014). However, whether each MHCI has specific functions and whether its polymorphism is related to CNS development and cognitive function related to diseases such as autism and schizophrenia remain to be elucidated.

The other major question concerns the MHCI signaling pathway. The TCR is the most widely known receptor for MHCI; however, no TCR protein has been detected in the CNS (Syken and Shatz, 2003). In contrast, CD3ζ, a component of the TCR, is expressed in the LGN during development (described below). Moreover, messenger RNA for PirB (paired Ig-like receptor B), an innate Ig-like transmembrane receptor for MHCI that antagonizes the integrin and MAP kinase signaling cascades (Takai, 2005), is highly expressed in neurons of the cerebral cortex, olfactory bulb, and granule cells of the cerebellum (Syken et al., 2006). Of note, mice deficient for CD3ζ (Huh et al., 2000) or PirB (Syken et al., 2006) also exhibit defects in the activity-dependent refinement of connections, similar to $\beta_2 m^{-/-} TAP1^{-/-}$ and $K^{b}D^{b-/-}$ mice. Other immune receptors, such as KIR (killer cell immunoglobulin-like receptor) (Bryceson et al., 2005) and Ly49 (a member of the NK family of innate immune receptors) (Zohar et al., 2008), are also believed to be potential neuronal receptors for MHCI. Moreover, whether MHCI molecules in the CNS really present antigens, and if so, what kinds of antigens are presented and whether they are essential for establishing specific neural networks remains to be answered.

The Complement Family

The classical complement family, an immune pathway that functions to eliminate pathogens and apoptotic cells, also plays an important role in synaptic remodeling. Its function has been reviewed several times, such as in Veerhuis et al. (2011) and Stephan et al. (2012). In brief, complement is produced by neurons and glial cells, especially by microglia and astrocytes, from early developmental periods to adulthood. Complement receptors CR3 (also known as CD11b/CD18, Mac-1, and integrin αMβ2) and CR5 are expressed in resident microglia. These complement proteins are engaged in synaptic refinement of retinal ganglion cell (RGC) projections to the dorsal LGN of the visual thalamus and they are also implicated in neurogenesis, migration, and neuronal survival during development and adulthood. The complement family play these important functions by cooperating with other proteins, because C1q co-localizes with H2-D and H2-K at synapses (Datwani et al., 2009), and the antibody and pentraxin families are involved in the complement cascade, as discussed below. C1q homologous proteins, including C1ql2 and Cbln1, are also engaged in synaptic formation, as reviewed in Südhof (2017) and Yuzaki (2017). However, much remains to be resolved: What signals control the activation of the complement cascade? Are they used similarly during development and adulthood? What are the critical molecules for synaptic pruning that might be the potential pharmacological targets of developmental and neurodegenerative diseases and injuries?

Antibody Receptors

The blood-brain barrier prevents large molecules, such as antibodies, from entering the brain parenchyma. However, FcγRIIB, a low-affinity membrane receptor for IgG, which negatively regulates B cell receptor-induced signaling, is expressed in Purkinje cells and Bergmann glia in the developing cerebellum. In addition to FcγR, Fcα/μR, a receptor for the Fc region of IgA and IgM, is expressed in OPCs (Nakahara et al., 2003a,b; Tanabe and Yamashita, 2018b). Although the function of these Fc receptors is not yet fully understood, IgG was reportedly detected in the developing rat cortex, and immunohistochemical signals were observed in subplate and other early-generated cortical neurons as well as in retinal and cerebellar neurons during early developmental stages (Upender et al., 1997). The origin of these IgG proteins remains unclear. Because IgG is actively transferred from the mother to the fetus across the placenta using neonatal Fc receptors, and the barrier function of the blood-brain barrier is not complete until E15, maternal antibodies may leak into the fetal brain through blood vessels.

CD3

CD3 is the most commonly used T cell marker and is composed of four subunits: CD3δ, CD3ε, CD3γ, and CD3ζ. They assemble to form three types of dimers (δε, γε, ζζ), and serve as a co-receptor for MHC-TCR signaling (Call and Wucherpfennig, 2007). CD3ζ is expressed by retinal neurons located in the RGC layer in the developing retina and is localized at synapses in the inner plexiform layer during the period of synapse formation. CD3ζ participates in the eye-specific segregation of RGC axon projections to the dorsal LGN (Huh et al., 2000) and glutamate receptor (GluR)-mediated synaptic activity-dependent synapse formation of RGCs in the retina and dorsal LGN (Xu et al., 2010). CD3ζ is also expressed by hippocampal neurons and the deficiency of CD3ζ results in enhanced LTP and lack of LTD. CD3ζ activation on hippocampal neurons affects cell morphology by promoting dendritic pruning (Huh et al., 2000; Barco et al., 2005; Baudouin et al., 2008). CD3ε is expressed with FcγR II B on Purkinje cells and Bergmann glia in the cerebellar cortex during development, and both CD3ε-deficient mice and FcγR II B-deficient mice exhibit impaired development of Purkinje cells, enhanced paired-pulse facilitation of parallel fiber-Purkinje cell synapses, and poor rotarod performance at high speed (Nakamura et al., 2007). The precise function of these molecules is summarized in a table in Elmer and McAllister (2012). However, the neuron-specific signaling cascade through CD3 and FcγR II B is yet to be uncovered.

Cytokines

Cytokines are small signaling proteins secreted mostly by immune cells that regulate diverse immunological responses. However, many, such as IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-11, IL-13, IL-18, IL-33, TNF-α, TGF-β and IFN-γ are also expressed in the CNS and are involved in cell survival, proliferation and differentiation, axonal growth and synaptogenesis, as reviewed in Bauer et al. (2007). For example, maternal IL-6 is the central molecule that alters social and cognitive behaviors of the offspring of immune-activated mothers (Smith et al., 2007; Wu et al., 2017), and working memory performance of 2-year-old children can be predicted by measuring maternal IL-6 (Rudolph et al., 2018). It is important to take into consideration that both mother and fetus can produce cytokines, and maternal peripheral and placental cytokines can also reach the fetal brain to directly affect CNS development. If pregnant mice colonized with commensal segmented filamentous bacteria undergo immune activation by infection, high amounts of IL-17a is produced by the mother's intestinal Th17 cells, is transferred to the fetal brain and binds to IL-17R expressed on neurons, resulting in behavioral and cortical abnormalities that resemble those observed in autism (Choi et al., 2016; Kim et al., 2017; Shin Yim et al., 2017). Moreover, astrocyte-derived IL-33, which is one of the alarmins released by tissue damage, is used for promoting synapse refinement during development (Vainchtein et al., 2018).

Chemokines

Chemokines are chemotactic cytokines, which direct cell migration, and were originally identified as potent attractants for leukocytes to mediate acute and chronic inflammation. However, accumulating evidence suggests that they also play an essential role in mediating neuron-microglia crosstalk in the developing and mature brains, as illustrated in Ransohoff (2009) and Williams et al. (2014).

One of the most recognized examples is the CX3CL1 (also known as fractalkine) signaling pathway (Paolicelli et al., 2014). Briefly, neuron-derived CX3CL1 and its receptor CX3CR1, which is expressed mostly on microglia, promote microglial recruitment to neuronal circuits by increasing their process movement and cellular migration. This signaling also influences the survival of developing neurons, pruning of synapses, synaptic transmission, synaptic plasticity and connectivity, affecting learning, memory, and behaviors. CXCL12 (also known as stromal cell-derived factor-1, SDF-1)-CXCR4 signaling is also required for the appropriate migration of microglia, neural progenitor cells (NPCs), cortical interneurons, and Cajal Retzius cells. It also controls neurogenesis, axon guidance/pathfinding, neurite outgrowth and maintenance of NPCs, as reviewed in Li and Ransohoff (2008), Zhu and Murakami (2012), and Guyon (2014).

Toll-Like Receptors

Toll-like receptors (TLRs) are pattern recognition receptors involved in the induction of the innate immune response. There are 13 TLRs identified in mice. Among them, TLR 2, 3, 4, 8, and 9 are expressed in the CNS, and their contribution to various phenomena is suggested, including neurite outgrowth, NPC proliferation, structural plasticity, cognition, anxiety, sensory, and motor behaviors, as discussed in Kioussis and Pachnis (2009), Okun et al. (2011), and Khariv et al. (2013). For example, TLR3 is highly expressed during early developmental stages, its activation reduces embryonic NPC proliferation in the subventricular zone and adult NPC proliferation in the dentate gyrus, and it inhibits neural outgrowth. TLR3 signaling has great impact on cognition; TLR3 deficiency causes improved spatial working memory and contextual fear memory, impaired amygdala-dependent cued fear memory and anxiety. However, the endogenous ligands that activate TLRs under physiological conditions, and whether they affect the development of neural circuits and/or cause more acute effects on synaptic plasticity remain unknown.

Pentraxins

The pentraxins (PTX) are an evolutionarily conserved family of proteins characterized by a pentraxin protein domain. Some of them, such as C-reactive protein (CRP) and PTX3, are involved in acute immunological responses. It is well known that CRP is a binding partner of C1q and may be involved in synaptic pruning through C1q, and PTX3 can modulate phagocytic activity of microglia and promote functional synapse formation (Jeon et al., 2010; Fossati et al., 2019). Moreover, neural pentraxin 1 (NPTX1) and 2 (NPTX2) mediate synaptic refinement in the developing visual system (Bjartmar et al., 2006) and NPTX2 and neuronal pentraxin receptor (NPTXR) are required for GluA4 expression within parvalbumin-positive fast-spiking interneurons. In $Nptx2^{-/-}Nptxr^{-/-}$ mice, GluA4 is markedly reduced, with consequent reductions in AMPA receptor function in the parvalbumin-positive interneurons, which compromise circuit recruitment of these interneurons, leading to deficits in network rhythmogenesis and behavior (Pelkey et al., 2015).

FUNCTION OF MAJOR NEURONAL MOLECULES IN THE IMMUNE SYSTEM

In contrast to the molecules that were originally discovered in the immune system and later found to have functions in the nervous system, several molecules, such as Protocadherin (Pcdh), and the Eph/Ephrin and Semaphorin families, were first reported in the nervous system. However, they are also regarded as immune-modulatory molecules. Pcdh18 is an activation marker of $CD8^{+}$ memory T cells that can function as an inhibitory signaling receptor and restrict the effector phase (Vazquez-Cintron et al., 2012). Ephrin-B1(Efnb1) is a specific marker for germinal center (GC) and memory precursor B cells (Laidlaw et al., 2017), and Efnb1 repulsively inhibits GC recruitment and retention of follicular T helper (Tfh) cells. This repulsion requires forward signaling through Eph-B6 on Tfh. Efnb1 also promotes GC Tfh production of IL-21 through forward signaling via Eph-B4 (Lu et al., 2017). Semaphorins, major axon guidance molecules, are also involved in the various phases of physiological and pathological immune responses associated with rheumatoid arthritis, systemic lupus erythematosus, systemic sclerosis and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. For example, Sema3A synthesized by activated dendritic cells and T cells downregulates T cell proliferation, activates macrophages, and is also involved in DC transmigration across the lymphatics. Sema4A is related to T cell priming and Th1/Th2 regulation and maintenance of Treg stability (Kumanogoh and Kikutani, 2013; Nishide and Kumanogoh, 2018).

PERSPECTIVE ON FUTURE DIRECTIONS

As discussed above, the CNS and immune system share many common characteristics; however, some clear fundamental differences do exist, especially with regard to the manner of target molecule/cell recognition. Immune cells can dynamically move around to search for targets and can clonally expand in cell number. The primary means of communication between immune cells include direct contacts with nearby cells that are attracted by chemokines or via secretory molecules. In the nervous system, mature neurons themselves do not proliferate or actively migrate; therefore, where a neuron is located during development is critically important. Moreover, the specific order of signaling between cells in a neural network is essential. For example, when an excitatory neuron A directly projects to neuron B, neuron A would activate neuron B. In contrast, if neuron A indirectly communicates with neuron B through an inhibitory neuron C (i.e., A→C→B), neuron B would be suppressed by the activation of neuron A. The formation of these specific neuronal connections could, if not exclusively, be accomplished by specific and diverse cell adhesion molecules, such as Dscam (Schmucker and Chen, 2009) and clustered Pcdh (Yagi, 2008). For example, the Drosophila *Dscam* gene could theoretically generate 38,016 isoforms (19,008 for the extracellular

domain) by alternative splicing. Interestingly, the Dscam protein is also detected in Drosophila immune-competent cells (hemolymph) and is believed to be involved in bacterial binding followed by phagocytosis, suggesting that this molecular diversity may provide a highly diverse innate immune system in insects (Watson et al., 2005). In these examples, extraordinary diversity and specificity, shared by the nervous system and immune system, may be established based on a common molecular machinery or operational modes between these two systems. From this perspective, to understand the mechanisms of development of the complex brain network, significantly more effort should be directed at uncovering why some molecules or cells that are known to work in acquired immunity are expressed/exist in the developing nervous system.

AUTHOR CONTRIBUTIONS

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REVIEW
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The Moderating Effects of Sex on Consequences of Childhood Maltreatment: From Clinical Studies to Animal Models

*Jordon D. White and Arie Kaffman**

Department of Psychiatry, Yale School of Medicine, Yale University, New Haven, CT, United States

Stress has pronounced effects on the brain, and thus behavioral outputs. This is particularly true when the stress occurs during vulnerable points in development. A review of the clinical literature regarding the moderating effects of sex on psychopathology in individuals exposed to childhood maltreatment (CM) is complicated by a host of variables that are difficult to quantify and control in clinical settings. As a result, the precise role of sex in moderating the consequences of CM remains elusive. In this review, we explore the rationale for studying this important question and their implications for treatment. We examine this issue using the threat/deprivation conceptual framework and highlight a growing body of work demonstrating important sex differences in human studies and in animal models of early life stress (ELS). The challenges and obstacles for effectively studying this question are reviewed and are followed by recommendations on how to move forward at the clinical and preclinical settings. We hope that this review will help inspire additional studies on this important topic.

Keywords: sex, childhood maltreatment, early life stress, animal models, limited bedding nesting, maternal separation, deprivation, threat

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Ashok K. Shetty,
Texas A&M College of Medicine,
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Sraboni Chaudhury,
University of Michigan, United States
Meg Dennison,
Consultant, Melbourne, Australia

***Correspondence:**
Arie Kaffman
arie.kaffman@yale.edu

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INTRODUCTION

Childhood maltreatment (CM) is a heterogenous group of childhood adversities (i.e., subtypes) that include, physical abuse, physical neglect, sexual abuse, emotional abuse, emotional neglect, erratic parenting and severe bullying by peers. Exposure to CM is associated with enormous clinical and economic burden as CM exposure accounts for roughly 50% of all childhood psychiatric disorders in the United States (Green et al., 2010). CM increases the risk for multiple psychopathologies, including depression, anxiety, substance abuse, psychosis, and PTSD (Anda et al., 2006; Kaffman and Meaney, 2007; Nemeroff, 2016; Teicher and Samson, 2016). CM also increases the risk for several medical conditions, such as cardiovascular disease, arthritis, metabolic syndrome, cancer, and generally reduced life expectancy (Kaffman and Meaney, 2007; Teicher and Samson, 2016). Interventions that improve quality of parental care in high-risk children lead to robust and sustained improvement in several behavioral and cognitive outcomes (Olds et al., 1998, 2004a,b; Zeanah et al., 2009; Humphreys et al., 2015), supporting a causal relationship between CM and the presence of behavioral abnormalities later in life. Indeed, CM is now recognized as a significant risk factor for abnormal brain development in industrialized countries (Kaffman and Meaney, 2007; Garner et al., 2012; Nemeroff, 2016; Teicher and Samson, 2016) with an estimated cost of $500 billion annually in the United States alone (Fang et al., 2012).

One of the most robust findings across the CM literature is its additive effect, where the risk for developing a broad range of psychological and medical conditions increases linearly with exposure to a greater number of adversities (Kessler et al., 1997; Anda et al., 2006; Chen et al., 2010; Evans et al., 2013). This dose-dependent effect has led to the development of diagnostic tools that calculate a cumulative-risk score as a way to quantify exposure to CM (Evans et al., 2013). The cumulative model has been expanded by McLaughlin, Sheridan and Lambert who proposed that a two-dimensional "*Threat/Deprivation*" system would better characterize and quantify CM exposure (McLaughlin et al., 2014; McLaughlin and Sheridan, 2016). This model maps adversity along a "*threat*" scale on the *X*-axis and a "*deprivation*" scale on the *Y* axis. Threatening adversities trigger fear of physical harm/death and include experiences that range from physical and sexual abuse to exposure to domestic and neighborhood violence. Deprivation on the other hand is characterized by an early environment that is devoid of appropriate stimulation and parental care and include subtypes such as physical and emotional neglect or severe poverty (see **Figure 1A**). The authors argue that deprivation and threat lead to different developmental outcomes and psychopathologies. Moreover, they proposed that mapping CM along these two dimensions helps resolve the complexity and heterogeneity of CM allowing for better predicted outcomes when compared to single dimension scale used in the cumulative-risk approach (McLaughlin et al., 2014; McLaughlin and Sheridan, 2016). In this review, we use the *Threat/Deprivation* conceptual model to examine whether CM affects males and females differently in clinical and preclinical studies.

Although a large body of work has shown that CM affects male and females differently, very few findings have been replicated across studies and little information is currently available about the mechanisms by which sex moderates the outcomes of CM (see **Supplementary Table S1** for a list of key studies that have examined the effects of sex on psychopathology). This review extends previous discussion on this topic (Gobinath et al., 2014; Bale and Epperson, 2015; Cameron et al., 2017) in three important ways. First, we examine the rationale for studying sex as an important moderator of the consequences of CM and how sex can affect response to treatment (section "Sex as an Important Moderator of Consequences of CM"), an issue that has not received adequate attention to date. Second, we utilize the *Threat/Deprivation* conceptual model to review clinical and preclinical studies that have examined the issue of sex (section "Modereating Effects of Sex in Clinical and Preclinical Studies"). Third, we outline challenges and obstacles that hinder progress and make specific recommendations on how to move forward (section "Challenges and Recommendations").

SEX AS AN IMPORTANT MODERATOR OF CONSEQUENCES OF CM

Male and female brains, physiology, and immune systems differ in many ways (Gillies and McArthur, 2010; McCarthy, 2015; McCarthy et al., 2015). These differences reflect distinct and specialized roles that males and females play in ensuring reproductive success (Cahill, 2006). In mammals, these differences are first established by increased levels of testosterone during a critical period of development in males. Testosterone is aromatized locally and converted to estrogen leading to several structural and functional differences in the brains of males and females. For example, the anteroventral periventricular nucleus (AVPV) is larger in females, and this sexual dimorphism is considered responsible for establishing a pulsatile pattern of GnRH release in males and a cyclical pattern in females that drives ovulation. These structural differences are established during the postnatal period by a wave of apoptosis in GABAergic neurons in the male AVPV. Structural and functional sexual dimorphic variations that emerge early in development are maintained and extended by different levels of sex hormones produced during reproductive age (i.e., estrogens and progesterone in females and testosterone in males). For excellent reviews on this topic see Gillies and McArthur (2010), McCarthy (2015), McCarthy et al. (2015).

These structural and hormonal alterations are responsible for important differences in the way males and females respond to injury, stress, and medications (Gillies and McArthur, 2010). For example, both male and female mice develop chronic neuropathic pain in response to spared nerve injury. However, the mechanisms responsible for this hypersensitivity to pain differs; it is mediated by the brain's endogenous immune cells (i.e., microglia), in males but not in females (Sorge et al., 2015). Sex differences in response to environmental insults are well documented in neonates. For example, exposure to hypoxic-ischemic injury in pre-term and full-term babies causes significantly more neurologic damage and long-term disabilities in males compared to females. These sex differences are seen in both humans and rodents and are thought to be mediated by higher rates of apoptosis in male neural stem cells (Hill and Fitch, 2012). Recent genomic work found little overlap in genes that are differentially expressed in men and women diagnosed with major depression across multiple brain regions, including the medial prefrontal cortex (mPFC). Similar sex-specific genomic changes were seen in mice exposed to chronic stress, suggesting that these sex-specific changes are evolutionary conserved across mammalian species (Labonte et al., 2017). Network analysis identified the phosphatase Dusp6 as a central hub in depressed women and overexpression of the transcription factor Emx1 as a central hub in depressed men. Knockdown of Dusp6 in the mPFC combined with subthreshold stress induced a depression-like phenotype in female but not male mice. In contrast, the overexpression of Emx1 induced depression-like behavior in males only. Interestingly, down regulation of Dusp6 in females and upregulation of Emx1 in males led to similar increase in spontaneous firing of glutamatergic neurons in the mPFC (Labonte et al., 2017). Together, these findings suggest that different mechanisms converge in males and females to produce major depression and that some but not all interventions will have sex-specific effects when treating depression. These findings highlight the importance of studying disease mechanism in both sexes as it has critical implications for treatment.

Although many studies show that CM causes different outcomes in males and females, very few of these findings have

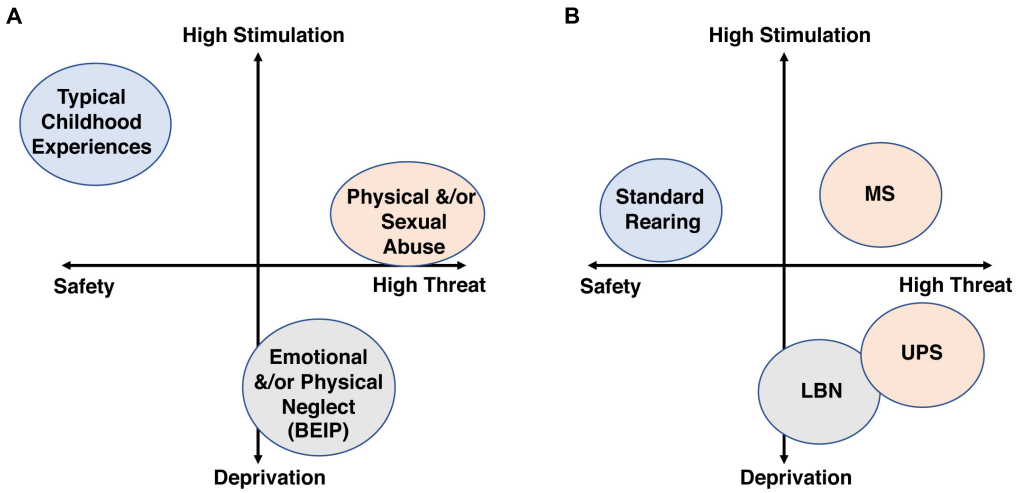

FIGURE 1 | The threat/deprivation conceptualization is shown for human subtypes of CM **(A)** and rodent models of ELS **(B)**.

been replicated across studies and there is little information about the mechanisms underlying the moderating effects of sex on CM outcomes (see **Supplementary Table S1** and section "Modereating Effects of Sex in Clinical and Preclinical Studies" below). Moreover, as demonstrated above, even in the presence of similar clinical presentation, the mechanisms responsible for these outcomes might be different in males and females, requiring sex-specific interventions. This is an important consideration given the enormous clinical and economic burden associated with CM.

MODEREATING EFFECTS OF SEX IN CLINICAL AND PRECLINICAL STUDIES

Sex as a Moderator of Psychopathology

Over the past 30 years, more than 50 studies, including several systematic reviews and meta-analyses have examined the moderating effects of sex on the psychological consequences of CM in humans (Jumper, 1995; Rind and Tromovitch, 1997; Rind et al., 1998; Paolucci et al., 2001; Gershon et al., 2008; Chen et al., 2010). While some studies found that females are more sensitive to CM (McGee et al., 1997; MacMillan et al., 2001; Lansford et al., 2002; Banyard et al., 2004; Fletcher, 2009; Herringa et al., 2013b), others maintain that males are more sensitive (Hibbard et al., 1990; Garnefski and Diekstra, 1997; McGloin and Widom, 2001; De Bellis and Keshavan, 2003; Bergen et al., 2004; Zeanah et al., 2009; Coohey, 2010; Crozier et al., 2014). A third group of studies proposed a more nuanced and complex relationship between sex and CM, suggesting that the outcome depends on the type of maltreatment, genetic vulnerability, the specific circuit involved, and the developmental stage when the outcomes are assessed (Darves-Bornoz et al., 1998; Gershon et al., 2008; Keyes et al., 2012; Bale and Epperson, 2015; Humphreys et al., 2015; Teicher and Samson, 2016; Gauthier-Duchesne et al., 2017). See also **Supplementary Table S1**.

Of the systematic reviews, one conducted by Gershon et al. (2008) is particularly helpful as it focuses on more than 30 studies with sufficient power to formally assess sex by ELS interactions in both adulthood and adolescence. Of the 14 studies conducted in adulthood, 50% found no sex differences, 29% reported worse outcomes in females, while 21% found worse clinical outcomes in males. In contrast, of the 19 studies conducted in adolescents, 58% found worse outcomes in males, 30% reported no differences, 5% found mixed effects with females more severely affected in some domains and males more affected in others, and only 5% noted worse outcomes in females. No significant sex by CM interactions were reported by 3 other large meta-analyses (Jumper, 1995; Paolucci et al., 2001; Chen et al., 2010); although, these analyses did not separately assess outcomes in adults and adolescents (**Supplementary Table S1**).

The assertion that male adolescents are more symptomatic across multiple psychopathologies was challenged by a recent study examining the effects of childhood sexual abuse in a large cohort of adolescents (Gauthier-Duchesne et al., 2017). Their findings indicate a mixed effect, with females being more likely to develop PTSD, males being more likely to develop externalizing disorders, and no sex difference in the vulnerability to internalizing disorders. These findings suggest a more complex moderating effect of sex, but further supports the notion that male and female adolescents respond differently to consequences of sexual abuse. Important sex differences were also demonstrated by Keyes et al. (2012) using a large sample of adults (n = 34,653, 52% men) in which physical abuse caused a significant increase in externalizing disorders in males while increasing the rate of internalizing disorders in females (Keyes et al., 2012). Interestingly, in contrast to the work conducted in adolescents, exposure to sexual abuse increased the risk for internalizing and externalizing disorders in both male and female adults (**Supplementary Table S1**). These findings raise the question as to why physical, but not sexual abuse, is associated with different symptomatology in males and

females and highlight the complexity by which sex interacts with different forms of CM. Moreover, as discussed above, one should not assume that comparable levels of symptomatology reflect similar developmental changes or response to treatment in males and females.

The Bucharest Early Intervention Project (BEIP) as a Model of Deprivation

The BEIP provides a unique opportunity to examine the moderating effects of sex on a relatively well characterized and homogeneous cohort of children that were exposed to high levels of deprivation, with relatively low-level exposure to threat with respect to the *Threat/Deprivation* model (**Figure 1A**). In this project, Romanian children, orphaned at birth, were placed in government-run institutions. These orphanages were understaffed and caregivers were insufficiently trained and lacked the necessary resources to provide adequate sensory, emotional and cognitive stimulation for these children (Bos et al., 2011). During the first stage of the project, a large cohort of toddlers (n = 104, mean ages 21 months), institutionalized for 6–31 months, was characterized and compared to non-institutionalized age- matched controls (n = 66) for developmental milestones. Institutionalized toddlers were physically smaller, showed cognitive delays, and had higher levels of behavioral and emotional problems compared to non-institutionalized controls (Smyke et al., 2007). Importantly, male and female toddlers were similarly, affected at this age (Smyke et al., 2007). Further, male and female institutionalized toddlers showed multiple EEG differences compared to non-institutionalized controls which suggested altered neurodevelopment (Marshall et al., 2004). Together, these findings indicate that early deprivation leads to similar physical, emotional, and cognitive deficits across male and female toddlers.

After the initial characterization, the institutionalized toddlers either stayed at the institution or were adopted into middle-class, Romanian families (n = 68 children in each group). The chronically institutionalized group (CIG) and the institutionalized and then adopted group (IAG) were followed over time and compared to an age-matched, never-institutionalized group (NIG). This randomized clinical trial-like setup was used to assess the long-term effects of early deprivation/neglect and adoption on the emotional and cognitive development in a fairly large group of children.

The second assessment was conducted when the children were roughly 4.5 years old; at this time, there was a significant interaction between sex and history of institutionalization (CIG and AIG children grouped together). This interaction was driven by increased internalizing, externalizing and ADHD disorders in institutionalized boys compared to institutionalized girls (Zeanah et al., 2009). Unfortunately, no formal assessment for an interaction between sex and history of institutionalization is available for the third assessment conducted at ages 11–15 (Humphreys et al., 2015). While institutionalized females (CIG and AIG grouped together), but not males, showed higher levels of internalizing symptoms compared to controls, the rates of externalizing disorders and ADHD were similarly, elevated in institutionalized males and females. Interestingly, adoption only reduced levels of externalizing symptoms in boys, and adoption had no effect on the rate of internalizing disorders or ADHD (Humphreys et al., 2015).

In summary, the initial assessment at 2 years of age found no sex differences, the second assessment at age 4.5 found increased psychopathology in males, and the third assessment at ages 11–15 found an increased sensitivity to internalizing disorders in females and equal sensitivity between males and females to externalizing disorders and ADHD. It is unclear if these outcomes reflect true changes in the moderating effects of sex over time, or if the different assessment tools and analyses used at each time point are contributing to these reported differences (**Supplementary Table S1**). These variable outcomes highlight the difficulties of assessing the moderating effects of sex even in a fairly large and homogeneous group of maltreated children.

CM by Sex Interaction: Lessons From Imaging Techniques

Structural MRI

The use of objective, measurable outcomes such as imaging, EEG, neurocognitive testing, and peripheral biomarkers have provided some of the most robust findings on the moderating effects of sex on the consequences of CM. The best example of this is the consistently documented reduced hippocampal volume in adults exposed to CM (Teicher and Samson, 2016). Both men and women show reduced hippocampal volume, but the effect size in men is significantly more pronounced (Teicher and Samson, 2016). These findings provide some of the most compelling evidence for the existence of sex differences among the long-term consequences of CM. Since reduced hippocampal volume is more consistently found in adults with CM, it would be interesting to determine if these structural changes correspond with more pronounced deficits in hippocampal-mediated tasks in adult males as well. Moreover, most of the structural MRI studies to date have focused on hippocampal changes in individuals exposed to high threat subtypes of CM (Teicher and Samson, 2013) and there is a need to clarify how high levels of deprivation experiences may affect hippocampal volume in males and females.

Reduced corpus callosum size is another consistent finding associated with a history of CM, with male adolescents being more affected than age-matched females (Teicher and Samson, 2016). This is consistent with a meta-analysis by Gershon et al. (2008) and other imaging studies (Herringa et al., 2013a; Crozier et al., 2014; Colich et al., 2017) indicating important sex differences in adolescents exposed to CM.

Task-Mediated fMRI

Individuals with a history of CM show increased amygdala activation in response to fearful or angry faces (Teicher and Samson, 2016). This result has been replicated in many clinical studies, with similar results also being reported in rodents (**Supplementary Table S2**). Since the amygdala plays an important role in detecting and responding to threat, this finding provides a possible explanation for the increased anxiety seen in individuals with a history of CM (Anda et al., 2006;

Chen et al., 2010; Nemeroff, 2016; Teicher and Samson, 2016). Exposure to CM increased amygdala activation in children, adolescents and adults, suggesting that inappropriate parental care during a critical period of development, alters the amygdala's response to threat in a manner that persists into adulthood, reviewed in VanTieghem and Tottenham (2018). This assertion is supported by work showing that the presence of maternal cues reduces amygdala activation in normally developing young children, but not in adolescents (Gee et al., 2014). This phenomenon is called "maternal buffering" and is associated with increased negative connectivity between the prefrontal cortex (PFC) and the amygdala in young children, but not in adolescents, indicating that top-down suppression of amygdala activation becomes independent of maternal cues during adolescence, further supporting the idea of a critical period in development.

Therefore, CM may disrupt the normal maturation of connectivity between the PFC and the amygdala during childhood, leading to abnormal amygdala activation and emotional dysregulation throughout life (Herringa, 2017; VanTieghem and Tottenham, 2018). This assertion is supported by work showing that normally developing children (ages 6–10) displayed positive connectivity between the PFC and the amygdala in response to fearful faces, whereas age-matched children raised in an orphanage showed negative connectivity between the PFC and the amygdala (Gee et al., 2013). These findings led the authors to suggest that early parental deprivation leads to precocious maturation of amygdala-PFC connections which serve to help these children negotiate unfavorable environments (Gee et al., 2013). However, the negative connectivity seen in parentally deprived children was associated with elevated amygdala activation and high levels of anxiety (Gee et al., 2013), a pattern that is qualitatively different than the negative connectivity seen in typically reared adolescents. Thus, rather than precocious maturation along normal developmental trajectory, parental deprivation seems to impair the normal maturation of top-down inhibitory tone between the PFC and the amygdala. As discussed further below, additional work is needed to clarify whether parental neglect/deprivation leads to similar alterations in fronto-limbic connectivity compared to individuals exposed to the high threat form of CM.

To our knowledge, only two studies have used task-mediated fMRI to test the effects of CM and sex on fronto-limbic connectivity (Crozier et al., 2014; Colich et al., 2017). Crozier et al. (2014) used the emotional odd-ball task to assess non-emotional and emotional responses in a group of CM children with a history of physical abuse and neglect (n = 29, 55% males, ages 8–16). All CM subjects had positive forensic investigation with the Department of Children and Families (DCF) and were assessed using the Kiddie Schedule for Affective Disorders and Schizophrenia-Present and Lifetime Version (Kaufman et al., 1997). The CM group was compared to an age and sex matched control group (n = 45, 42% males) and was found to have lower socioeconomic status, lower IQ, and higher rates of both internalizing and externalizing symptoms (Crozier et al., 2014). No sex differences or interactions between CM and sex were found for IQ, internalizing, externalizing symptoms or performance in the task. However, there were many significant interactions between CM and sex in BOLD signal. For example, in response to fearful faces CM females showed reduced activation of the dorsal medial PFC (dmPFC) while CM males showed increased activation in this region. Additionally, CM males showed increased activation over both CM females and controls in both the calcarine region and the left hippocampus. This is the first study to demonstrate extensive differences in how CM affects the way in which male and female adolescents process fearful faces and it is consistent with a growing body of work showing significant sex differences in the sequela of CM among adolescents (Gershon et al., 2008).

Colich et al. (2017) used the Traumatic Event Screening Inventory for children (TESI-R), to characterize a broad range of adversities (e.g., childhood abuse, neglect, moving homes, and witnessing injury) in a large cohort of children (ages 9–13, males n = 59, females n = 78). These authors used the implicit emotional-regulation fMRI task and analyzed the effect of sex based on Tanner stage, to control for the earlier sexual maturity in females. In their cohort, higher levels of CM were associated with increased internalizing symptoms in females but not in males after controlling for age. Three brain regions (left vlPFC, right dlPFC/vlPFC, and intracalcarine cortex) showed significant CM X sex interactions, with increased activation in females, but not males. Activation of these three regions in females and the intra-calcarine cortex in males was correlated with internalizing symptoms. Further, higher levels of CM were associated with a stronger negative correlation between the PFC and the amygdala, a finding that was seen in both males and females. However, there was no correlation between the strength of these connections and internalizing symptoms (Colich et al., 2017). Although this study found CM X sex differences in emotional processing, there was little overlap with the findings reported by Crozier et al. (2014). Most notably Crozier et al. (2014) found increased PFC activation in CM males and hypoactivation in females, while (Colich et al., 2017) found increased activation in the PFC of females and little change in males. These differences may be a result of the different tasks utilized (e.g., the emotional odd-ball task vs. the implicit emotional-regulation task) or the subtype of CM experienced; most notably, higher levels of threat are reported in the Crozier cohort. Despite these disparities, both studies highlight important sex differences in how the PFC processes threating faces in maltreated male and female adolescents.

Fronto-Limbic Connectivity Using rsfMRI and Tractography

Unlike task mediated fMRI, in which brain connectivity may change depending on the nature of the task, functional or structural connectivity obtained using resting state fMRI (rsfMRI) or diffusion tensor imaging (DTI), do not involve an explicit cognitive task, allowing for a more direct comparison between studies. Jalbrzikowski et al. (2017) have used rsfMRI and DTI to characterize the effects of age and sex on amygdala-PFC connectivity in a large cohort of typically developing adolescents (n = 246, ages 10–25, 49% females). Using a longitudinal

approach, they found an age-dependent reduction in amygdala-PFC connectivity in this cohort. They replicated these findings using an independent cohort and further substantiated these findings using structural tractography. This refinement process was associated with reduced internalizing symptoms during adolescence with similar outcomes seen in males and females (Jalbrzikowski et al., 2017). This is one of the most rigorous studies to examine how fronto-limbic connectivity matures in typically developing males and females, providing a solid ground to investigate how different types of CM alter this pattern of connectivity.

Several studies have examined the effects of early adversity on amygdala-PFC connectivity using rsfMRI (**Supplementary Table S2**). Some studies reported reduced connectivity (Herringa et al., 2013b; Birn et al., 2014; Wang et al., 2014), others found no change (Burghy et al., 2012; van der Werff et al., 2013), while others noted increased connectivity (Cisler et al., 2013; Philip et al., 2013; Dean et al., 2014; Nicholson et al., 2015). These conflicting findings are likely due to differences in the composition and severity of CM, the age and sex of the subjects, and additional comorbidities, such as a history of substance abuse and/or depression. For an extensive review on this issue see Herringa (2017), Johnson et al. (2018).

Most studies published to date lack sufficient power to test for CM by sex interactions on fronto-limbic connectivity. The only exception is work by Herringa et al. (2013a) which examined the relationship between CM, levels of internalizing symptoms and resting state connectivity in a cohort of adolescents ($n = 64$, ages 18 ± 0.19, 46% females). CM was assessed using the Childhood Trauma Questionnaire and ranged from 25 to 40, indicating a low to moderate severity. Higher levels of CM, were correlated with higher levels of internalizing symptoms, with females having higher overall levels of internalizing symptoms compared to males (Herringa et al., 2013a). Maltreated females showed reduced functional connectivity between the right amygdala and the vmPFC, an effect not seen in CM males. In contrast, reduced connectivity between the left hippocampus and the vmPFC was seen in both males and females exposed to CM (Herringa et al., 2013a). The authors proposed that this "double hit" in females (e.g., reduced connectivity between the mPFC-amygdala and the mPFC-hippocampus) versus a "single hit" in males (e.g., reduced connectivity between the mPFC-hippocampus), may explain the higher symptomatology seen in females exposed to CM. Since the majority of studies did not find higher levels of internalizing symptoms in maltreated adolescent females (Gershon et al., 2008), additional work is needed to confirm these findings and to clarify whether differences in connectivity are due to sex differences and/or the severity of the internalizing symptoms. Nevertheless, this work is consistent with other studies discussed above showing important sex differences between maltreated male and female adolescents.

Modeling Early Life Stress in Rodents

We use the term early life stress (ELS) to describe work in rodents that attempts to model aspects of CM, focusing on paradigms that use postnatal stress in order to mimic childhood adversity. Rodents exposed to ELS show many of the developmental and behavioral changes reported in humans, suggesting that work in rodents can clarify important details about how different types of ELS alter neurodevelopment and behavior in males and females (see **Supplementary Table S2**). Although aspects of the moderating effects of sex on ELS have been reviewed by others in recent years (Gobinath et al., 2014; Lucassen et al., 2017; Walker et al., 2017), the rapid progress in this area warranted an updated reexamination of the issue. Moreover, we examine the preclinical work in regards to the deprivation/threat model and have attempted to link important clinical findings with preclinical studies. This is especially relevant for a growing body of work in rodents that has used human imaging tools such as rsfMRI and high-resolution diffusion MRI (dMRI) to examine the effects of ELS on fronto-limbic connectivity in rodents.

Due to a large number of inconsistent findings across the ELS literature, we searched for paradigms that provided distinct features within the deprivation/threat model and also produced reproducible outcomes in male and female rodents across different labs. Unfortunately, the licking and grooming paradigm developed by Meaney et al. (2002) did not provided enough examples of consistent findings across labs to be included in the current review. Thus, we focused on the low bedding/nesting (LBN) paradigm, a model with a relatively high deprivation score and moderate levels of threat, and the maternal separation (MS) paradigm, a model of moderate/low levels of deprivation and high threat level (**Figure 1B**). Despite a wealth of literature on these two paradigms from a variety of groups, much of the work was done only in males, with very few studies that document reproducible outcomes in both sexes. As a result, we present several reproducible and clinically relevant findings described only in males, in order to highlight the pressing need to further explore the consequences of these paradigms in females.

Limited Bedding/Nesting Paradigms

The LBN paradigm was originally developed by Tallie Baram's lab in an attempt to model chronic postnatal stress due to impoverished nesting condition and fragmented/erratic maternal care (Walker et al., 2017). In the original, and most commonly implemented version, the dam's access to nesting materials is severely reduced from PND2 to PND9 and pups are raised on an elevated mesh platform, while the control condition receives standard amounts of bedding and nesting material. The paucity of nesting material models an impoverished, sub-standard rearing condition that leads to fragmented maternal care, characterized by rapid transition in and out of the nest (Rice et al., 2008; Heun-Johnson and Levitt, 2016; Molet et al., 2016a). The exact reason for this fragmented maternal care is unclear, but it might reflect a compensatory foraging mechanism aimed at improving nesting conditions. Rodent pups are fully dependent on the dam for survival (Kuhn and Schanberg, 1998; Kaffman and Meaney, 2007). Therefore the erratic but constant maternal presence in the LBN paradigm is likely to induce a less threatening rearing environment compared to the removal of pups from the nest in the absences of any maternal cues used in the MS paradigm (**Figure 1B**). Moreover, the limited availability of soft nesting material deprives LBN pups of important sensory/tactile cues during a critical period

(Kaffman and Meaney, 2007), as opposed to the ample bedding and nesting material provided in the MS paradigm.

Since newly born pups are unable to regulate their body temperature (Lagerspetz, 1962), LBN pups are also likely to experience mild but chronic hypothermia. Mild hypothermia is a form of deprivation that likely plays an important role in mediating several key developmental abnormalities seen in LBN pups, i.e., stunted growth and elevated corticosterone levels (Lagerspetz, 1962; Anisman et al., 1998; Walker et al., 2017). The observation that female pups appear to be more resilient to the effects of hypothermia (Harshaw and Alberts, 2012) may account for some of the sex-specific outcomes reported in this paradigm; including hippocampal dependent cognitive deficits, altered adult neurogenesis, and changes in reward sensitivity and response to threat (see below for more details).

LBN Causes Similar Reduction in Body Weight in Male and Females

One of the most robust findings across the LBN literature is a reduction in body weight, an effect found in both rats and mice, with similar pattern in both sexes. This reduced body weight has been reported to persist into adulthood by some (Maniam et al., 2015a,b; Bath et al., 2016, 2017; Goodwill et al., 2018; Johnson et al., 2018), while others have noted only transient reductions at PND9 that are restored by weaning (Brunson et al., 2005; Rice et al., 2008; Kanatsou et al., 2015; Naninck et al., 2015; Arp et al., 2016; Fuentes et al., 2018). This effect is also seen in studies conducted in humans, where delayed (Smyke et al., 2007) and even stunted growth is seen in individuals exposed to severe CM (Grantham-McGregor et al., 2007). While male and female rodents show similar reductions in weight (Naninck et al., 2015; Arp et al., 2016; Moussaoui et al., 2016; Bath et al., 2017; Goodwill et al., 2018; Johnson et al., 2018), important sex-differences are already present during the early postnatal period (see section "Sex as an Important Moderator of Consequences of CM" above) raising the possibility that somewhat different mechanisms drive the slower growth in male and female pups exposed to LBN.

LBN Leads to More Significant Hippocampal Deficits in Male Offspring

Abnormal hippocampal function among LBN-reared offspring is consistently reported. The initial reports indicated that hippocampal impairment associated with LBN emerges only in middle-aged or aged animals (Brunson et al., 2005), and while this is also seen in more contemporary studies (Naninck et al., 2015), recent studies have found deficits much earlier when utilizing the novel object location task (NOLT) instead of novel object recognition or the Morris water maze (Molet et al., 2016b; Bath et al., 2017). For example, deficits in NOLT are seen in male and female LBN-reared mice as early as PND21 (Bath et al., 2017) and in LBN-reared male rats at 2 months (Molet et al., 2016b). These deficits are more significant in males compared to females (Naninck et al., 2015; Bath et al., 2017). Naninck et al. (2015) provided evidence that these sex-specific effects were due to reduced neural stem cell survival in the dentate gyrus of adult male, but not female LBN offspring. These findings are consistent with human literature showing greater reduction in hippocampal size in males exposed to ELS. While reduced volume in the dorsal hippocampus of male LBN offspring has also been found using high resolution MRI (Molet et al., 2016b), females were not included in this study. Thus, it is unclear whether this model recapitulates all sex differences reported in human imaging studies.

LBN Increases the Susceptibility to Secondary Stress in Male Offspring

Most studies found no effect of LBN on anxiety in male and female offspring (Brunson et al., 2005; Rice et al., 2008; Naninck et al., 2015; Molet et al., 2016a; Bath et al., 2017; Goodwill et al., 2018; Manzano-Nieves et al., 2018). Two reports found increased anxiety following LBN (Molle et al., 2012; Guadagno et al., 2018) and two others noted mixed effects (Wang et al., 2012; Johnson et al., 2018). The absence of robust changes in anxiety might be due to the short developmental window in which the rodents are exposed to LBN or the relatively mild nature of the stressor. Indeed, van der Kooij et al. (2015) found that exposure to LBN between PND10 and PND17, but not PND2-9, leads to increased anxiety in adult male mice. These findings underscore the important role that timing of exposure plays in modifying developmental and behavioral outcomes later in life.

Work from several groups suggests that a "second hit" might be necessary to unmask underlying changes in anxiety in LBN offspring. For example, adding only 6 episodes of unpredictable maternal separation (UPS) at PND14, 16, 17, 21, 22, and 25 to pups raised under LBN conditions followed by nest disruption leads to a robust increase in anxiety that is not seen in LBN mice that were not separated. Importantly, exposure to UPS increased anxiety in adult male but not female littermates (Johnson et al., 2018). Newborn pups depend on their mother for survival (Kuhn and Schanberg, 1998; Kaffman and Meaney, 2007). Therefore, the UPS paradigm exposes LBN pups to increased levels of threat (**Figure 1B**), which appears to unmask important sex-differences in anxiety-like behavior. Additionally, single-housing adult LBN animals may also unmask important differences in anxiety and response to threat, as adult LBN-reared male rats that were single-housed showed increased anxiety and greater dendritic arborization in BLA neurons compared to LBN females or controls (Guadagno et al., 2018). Additionally, Arp et al. (2016) found that single housed LBN male mice displayed high levels of freezing during the safety period (tone-off) that was not seen in LBN females or CTL mice.

Depression-Like Behaviors in LBN Offspring

Inconsistent findings have been reported for the effects of LBN on depression-like behaviors. For example, studies have found increased helpless behavior in the forced swim test (Cui et al., 2006; Raineki et al., 2012) and reduced sucrose preference (Molet et al., 2016a; Bolton et al., 2017) in males exposed to LBN, but provided no information on female behavior. In contrast, three studies found no effect of LBN on helpless behavior in males (Molet et al., 2016a; Bolton et al., 2018; Goodwill et al., 2018). Bolton et al. (2018), showed that reducing CRF expression in the central amygdala of LBN males reverses anhedonia like behavior, providing a possible mechanism to explain the

depressive phenotype documented in LBN males. The only study that examined the effects LBN on depression-like behaviors in both males and females, found increased depression-like behavior in female mice and not male littermates (Goodwill et al., 2018). The Goodwill et al. (2018) work is particularly interesting as, in addition to using standard assays of depression-like behaviors like sucrose preference and forced swim test, home-cage behavior, i.e., locomotor activity and self-grooming, was assessed over 5 days. Such prolonged and unbiased assessment of behavior can identify robust behavioral changes in domains such as self-care and energy levels that map well onto clinical presentation of depression. It is currently unclear if the different outcomes with regard to the effects of LBN on sucrose preference in males are due to differences between C57 mice (Goodwill et al., 2018) and Sprague Dawley rats (Molet et al., 2016a; Bolton et al., 2018) or some other methodological differences between these studies. Further work in both male and female LBN offspring is needed to determine whether the LBN-induced depression phenotype is truly sex-specific and what mechanisms, other than CRF expression may be mediating these changes.

LBN Increases CRF Levels and Alters Amygdala Connectivity in Males

Work from several groups provided compelling evidence that LBN increase CRF levels in the hippocampus and that this prolonged exposure to high levels of CRF reduces spine density, dendritic arborization, and contributes to hippocampal-dependent cognitive deficits (Chen et al., 2013). In addition, abnormal expression of CRF in the central nucleus of the amygdala appears to induce an anhedonia-like state in LBN male rats (Bolton et al., 2018). Unfortunately, this work was done exclusively in males and it is currently unclear whether similar alterations in CRF are also seen in females and whether elevated levels of CRF cause similar outcomes in males and females. This is one of many examples, some of which are outlined below, in which outcomes in females have not been explored.

Recent advances in imaging has allowed the use of rsfMRI and DTI in rodents to assess the effect of LBN on functional and structural connectivity in fronto-limbic circuits that include the amygdala, prefrontal cortex, and the hippocampus. Such studies allow for direct comparison between humans and rodents and can help clarify whether variations of ELS cause different alterations in fronto-limbic connectivity and whether sex modulates these effects. Using rsfMRI, Johnson et al. (2018) showed that exposure to UPS, a modified version of the LBN paradigm described in section "LBN Increases the Susceptibility to Secondary Stress in Male Offspring," leads to increased connectivity between the amygdala and the prefrontal cortex, as well as between the amygdala and the hippocampus in adult male mice. The strength of these connections was highly correlated with anxiety-like behavior providing a possible explanation for the increased anxiety seen in UPS male mice compared to control reared males. In the aforementioned study, UPS did not increase anxiety-like behavior in females, so it would be interesting to know whether similar changes in connectivity are seen in UPS females as well. Similarly, UPS, but not LBN leads to robust increase in anxiety-like behavior (Johnson et al., 2018) raising the question as to whether the higher levels of threat associated with the UPS paradigm (**Figure 1B**) induce a different pattern of fronto-limbic connectivity when compared to LBN.

Direct comparisons between the effects of LBN and UPS on fronto-limbic connectivity have not been reported yet, but three papers have examined the effects of LBN on fronto-limbic connectivity in male rats (Yan et al., 2017; Bolton et al., 2018; Guadagno et al., 2018). Bolton et al. (2018), found increased structural connectivity between the amygdala and the mPFC in LBN-reared males (Bolton et al., 2018). Yan et al. (2017), used an abbreviated scarcity model in which the limited bedding occurred from PND8-12 to assess the effects of LBN and age (PND45 vs. 60) on functional connectivity between the amygdala and the PFC. Although they did not find a robust LBN effect, there was an age-dependent increase in functional connectivity in control rats that was not seen in LBN rats. This change in trajectory was due to a relatively high connectivity in LBN male adolescents that plateaued in adulthood. These results suggest that LBN causes precocious maturation of fronto-limbic connections in males (Yan et al., 2017), an effect consistent with findings reported in parentally deprived children (see Gee et al., 2013 and section "Task-Mediated fMRI"). Guadagno et al. (2018), used rsMRI to assess functional connectivity between the anterior and posterior BLA and the PFC in PND18 and PND74 rats. They found reduced connectivity between the right anterior BLA and the PFC in both ages but mixed effects, i.e., increased or decreased connectivity between the left anterior BLA and the posterior BLA connections with the PFC. In summary, although differences in connectivity between the amygdala and the PFC were found in LBN-reared adult rats (**Supplementary Table S2**), no consistent pattern emerged in males, and to our knowledge, no group has yet studied this issue in females.

In summary, LBN represents an ELS model with relatively high deprivation score and moderate levels of threat that is associated with important sex-specific effects on hippocampal function and distinct threat/deprivation profile and long-term consequences when compared to the MS paradigm (**Figure 1B**, and see also section "Maternal Separation Paradigms" below).

Maternal Separation Paradigms

Despite the fact that maternal separation (MS), maternal deprivation (MD) and brief maternal separation (BMS, also known as handling) are different postnatal stress paradigms that lead to different outcomes, they are commonly lumped together and discussed interchangeably. Several reviews have previously detailed the rationale for developing MS, BMS, and MD and highlighted important differences in their ability to modulate neurodevelopment, physiology, and behavior (Lehmann and Feldon, 2000; Meaney, 2001; Pryce and Feldon, 2003; Schmidt et al., 2011; Tractenberg et al., 2016).

The term MS is used here to describe a group of procedures in which pups are separated for 1–6 h daily during the first 2–3 weeks of life. Such prolonged separation represents a significant threat to rodent pups that are fully dependent on the dam for survival (Kuhn and Schanberg, 1998; Kaffman and Meaney, 2007). Compared to the LBN paradigm, MS pups experience higher levels of stimulation (**Figure 1B**) due to exposure to a

novel environment, and, in many cases, to increased levels of maternal care after reunification with the dam (Reeb-Sutherland and Tang, 2012; Gobinath et al., 2014; Couto-Pereira et al., 2016).

Maternal separation paradigms allow for high level of flexibility in modifying the complexity of the early life stressor, but this added flexibility is responsible for the development of numerous variations, lack of standardization, and difficulties reproducing developmental outcomes (Lehmann and Feldon, 2000; Tractenberg et al., 2016; Murthy and Gould, 2018). Additionally, even when the paradigm is consistent, strain effects are often present (Millstein et al., 2006; Mehta and Schmauss, 2011; Tractenberg et al., 2016). To identify robust and reproducible outcomes we extracted outcomes specifically associated with MS from two systematic reviews (Loi et al., 2015; Tractenberg et al., 2016) and one meta-analysis (Chen and Jackson, 2016).

The work by Loi et al. (2015) is particularly germane as it specifically explores the effects of different ELS paradigms (e.g., MD, MS, BMS, and LBN) on behavior in male and female rodents. When examining these paradigms together, they note a trend for increased vulnerability in males compared to females in tests for social behavior, cognition, and depression-like behaviors. Yet, when only looking at MS studies utilizing both sexes, Loi et al., 2015 did not find a significant effect of MS on anxiety, depression, or hippocampal-dependent function, in either sex. The few studies that reported a significant main effect of MS, did not present a clear outcome with regard to the moderating effects of sex on MS. The vast majority (i.e., 87%) of the MS studies cited by Loi et al. (2015) used rats with only three studies (13%) conducted in mice. The effects of MS, MD and BDS on behavior in the mouse was systematically reviewed by Tractenberg et al. (2016) and revealed a more consistent pattern. Specifically, when focusing on MS studies, there is a trend for increased depression and anxiety-like behavior across studies, but most studies only used males, and several of the studies that used both males and females did not formally assess for an interaction, making it difficult to determine how sex interacts with MS in the mouse.

Chen and Jackson (2016) conducted the only meta-analysis looking at the effects of MS, MD and BMS on pain sensitivity (Chen and Jackson, 2016). They found a significant reduction in pain sensitivity in rodents exposed to ELS that was due to the effects of BMS and not MS on pain sensitivity. In fact, MS studies found an opposite trend for increased pain sensitivity that did not reach statistical significance. Sex emerged as a significant factor in studies involving MS but not BMS, with males showing greater sensitivity compared to females. MS studies in mice (CD1) showed greater effect size compared to studies conducted in rats consistent with the notion that mice are more sensitive to the consequences of MS. This study demonstrates the utility of meta-analysis to quantify an overall effect size for the different paradigms, identify publication bias, and to reveal a significant effect of sex.

MS Alters DNA Methylation and Dopaminergic Development in Males

The challenges of producing consistent behavioral outcomes using the MS paradigm is likely responsible for the paucity of studies describing reproducible cellular and molecular changes in offspring exposed to MS. Nevertheless, two important exceptions are worth noting. First, several groups have found increased levels of DNA methyltransferases (DNMTs), including DNMT1, in the brain of adult male offspring exposed to MS (Anier et al., 2014; Boku et al., 2014; Todkar et al., 2015; Ignacio et al., 2017; Park et al., 2018). This is an important observation because DNMT1 plays a critical role in maintaining DNA methylation and transcription across a large number of promoters in neural stem cells, neurons and glia that regulate circuit development, neuroplasticity, and complex behavior (Guo et al., 2011; Heyward and Sweatt, 2015). This type of epigenetic regulation is now recognized as an important mechanism by which early life stress causes long term changes in gene expression in both animals and humans (Kaffman and Meaney, 2007). Exactly how an increase in DNMTs affects circuits that regulate complex behaviors in adult offspring exposed to MS is not fully elucidated, but work by Boku et al. (2014) has shown that elevated levels of DNMT1 in neural stem cells causes hypermethylation of the retinoic acid receptor (RARα) promoter. This increase in DNA methylation reduced RARα expression and impaired NSC differentiation into neuronal pathway *in vitro* (Boku et al., 2014). DNA methylation also plays a critical role in establishing sex-specific differences early in development (McCarthy, 2016), providing a possible mechanism by which MS may alter developmental trajectory in males and females. As seen with other examples noted above, none of the studies examined the effect of MS on DNMTs expression in females, an issue we suspect will provide important details about whether sex modulates the functional consequences of MS.

Secondly, work by Pena et al. (2017) found that exposing pups to MS from P10-20 causes latent vulnerability to depression that is not seen when pups are separated from P2-12. These different outcomes are due to the ability of MS from P10-20, but not from P2-12, to transiently reduce the expression of the transcription factor OTX2 in the ventral tegmental area (VTA) of male mice. Reduced OTX2 levels during this critical period of development impairs dopaminergic innervation and increases vulnerability to additional stress in adulthood (Pena et al., 2017). It is important to note that the MS paradigm used by Pena et al. (2017) also utilized low amounts of bedding commonly used in the LBN paradigm (i.e., increased levels of deprivation) and required exposure to additional trauma in adulthood in order to induce a depression-like phenotype. Additional studies are needed to clarify whether MS from P10-20 also reduces expression of OTX2 in females and whether OTX2 plays a similar role in dopaminergic development in both sexes.

CHALLENGES AND RECOMMENDATIONS

As discussed above, many questions are yet to be clarified about the moderating effects of sex on consequences of CM and outcomes of ELS in animal models. In this section, we highlight key obstacles for effectively studying this question and

make several recommendations about how to overcome these challenges. We first discuss these issues in clinical setting and then examine them in preclinical studies.

Challenges in Clinical Setting

The conflicting clinical results are not surprising given the number of variables involved and their complex interaction. For instance, different subtypes of CM (e.g., physical abuse vs. emotional neglect) cause somewhat different neurodevelopmental and behavioral outcomes (Keyes et al., 2012; McLaughlin et al., 2014; Teicher and Samson, 2016). These different developmental trajectories are further modified by the timing in which the trauma occurred (Bale and Epperson, 2015; Teicher and Samson, 2016) and the genetic vulnerability of the individual (Caspi et al., 2002, 2003; Klengel et al., 2013). Perhaps most relevant to this review, is work indicating that different forms of CM interact differently with sex (see **Supplementary Table S1** and section "Sex as a Moderator of Psychopathology"). Moreover, pure forms of CM are rarely encountered, with most cases of CM characterized by a combination of several subtypes of maltreatment (Kessler et al., 1997; Anda et al., 2006; Keyes et al., 2012). Co-occurring types of CM interact with one another in a manner that is not easy to quantify, but affects the risk for psychopathology (Fergusson et al., 1996; Kessler et al., 1997; Anda et al., 2006).

Sex also appears to moderate the prevalence and the nature of certain forms of CM. Specifically, while men experience lower prevalence of sexual abuse (Cutler and Nolen-Hoeksema, 1991; Coohey, 2010; Gauthier-Duchesne et al., 2017), young males are more likely to experience severe and frequent sexual abuse perpetuated by adolescent males while females are more likely to be abused by adult males (Gauthier-Duchesne et al., 2017). Work by MacMillan et al. (2001) provides a good example of how these differences may influence the interpretation of data with regard to the moderating effects of sex on psychopathology (**Supplementary Table S1**). For instance, adult women exposed to childhood physical abuse, and to a lesser extent sexual abuse, were more likely than males exposed to the same CM to meet criteria for either depression, substance use disorder, or antisocial behavior (MacMillan et al., 2001). Importantly, 33% of the physically abused women in this study were also sexually abused while only 11% of the men that were physically abused reported sexual abuse (MacMillan et al., 2001). These differences raise the possibility that the increased vulnerability seen in women may be due to more severe trauma and not actual sex differences.

One of the most important contributing factors to the confusion is the lack of a unifying method for characterizing CM. Different diagnostic tools are used to assess CM (**Supplementary Table S1**), making it practically impossible to compare outcomes across studies or to conduct meaningful meta-analyses. Moreover, most scales use the cumulative-risk model and there is a need to develop tools that diagnose CM along the threat/deprivation dimensions. These critical issues have not received enough attention and we hope that this review will help galvanize an effort to implement a uniformly accepted scale in future studies (see also section "Recommendations for Clinical Work" below).

Another issue that complicates the analysis and interpretation of the data is the use of an appropriate comparison group (Banyard et al., 2004). This is important, as the rates of internalizing disorders are almost twice as high in females (Kilpatrick et al., 2003; Altemus, 2006; Gobinath et al., 2014). This female-specific effect raises the question of whether a direct comparison between males and females is even appropriate. For example, Gold et al. (1999), found no difference in the rates of depression and anxiety between adult men and women exposed to childhood sexual abuse. However, when the rates were normalized to the rates seen in the same sex, non-abused general population, men were found to have higher rates of normalized internalizing disorders compared to women (Gold et al., 1999). While intriguing, these findings were not replicated by Banyard et al. (2004) who reported higher rates of internalizing symptoms in women exposed to sexual abuse, but no sex differences when the rates were normalized to the non-abused same sex general population. Another unresolved methodological question is how to address differences in sexual maturation between males and females. Females enter puberty roughly 18 months before males, suggesting that a comparison should be made based on Tanner phase, and not age *per se* (Colich et al., 2017). This issue is further complicated by extensive work showing that CM accelerates entry into puberty in females, with less clear data available on this issue in males (Cowan and Richardson, 2018).

Cultural norms regarding issues of masculinity, femininity and sexual orientation also influence the moderating effects of sex on the consequences of the traumatic experience (Maikovich-Fong and Jaffee, 2010; Gauthier-Duchesne et al., 2017). These cultural expectations may cause reporting biases that affect rates of psychopathology between males and females (Coohey, 2010). Moreover, it may be more culturally acceptable for boys to act aggressively compared to girls, leading to higher levels of externalizing disorders in boys (Coohey, 2010; Gauthier-Duchesne et al., 2017). For a comprehensive discussion of this important issue see (Rutter et al., 2003).

Recommendations for Clinical Work

We start by highlighting the need to increase awareness that sex differences matter in terms of the developmental consequences of CM and patient response to treatment. This includes the realization that similar presentation does not necessarily mean similar mechanism, and the interaction between threat, deprivation and sex are likely to be complex and circuit specific. Perhaps the most important and necessary change is the implementation of a uniformly accepted scale to assess CM. Such a scale should be guided by the threat/deprivation conceptual model to better map the complexity and heterogeneity of the CM experiences. This is not a call for the elimination of all other scales, but rather an effort to include one common scale that will allow for better comparisons between studies and help conduct meaningful meta-analyses. In addition, given the broad range of psychopathologies seen after exposure to CM, it would be helpful to include a measurement of global psychopathology in the form of the *p* factor in both males and females. For a detailed review on the p factor and its relationship to CM see Caspi et al. (2014), Ronald (2019). Additional studies using objective measurable

outcomes such as imaging, neurocognitive testing, and peripheral markers should provide important details about how different types of CM alter specific circuits in males and females. Such studies should be adequately powered to detect sex differences and will help resolve important discrepancies in imaging studies described above.

Challenges Faced by Preclinical Studies

A major issue in the preclinical literature is the paucity of studies that have examined outcomes of ELS in both males and females (Loi et al., 2015; Tractenberg et al., 2016). The large historical bias in male-exclusive studies, i.e., 5 to 1, in neuroscience and biomedical research (Beery and Zucker, 2011) led to the 2015 implementation of an NIH initiative emphasizing the importance of sex as a biological variable (Lee, 2018). While the percentage of studies using both males and female rodents has drastically increased from 17 to 38%, very few of those studies (15–25%) utilized sex as an experimental variable of interest (Beery and Zucker, 2011; Will et al., 2017). Moreover, methodological issues related to statistical analyses and reporting bias have also contributed to the large number of inconsistent findings in the preclinical ELS literature. For example, formal assessment of general linear modeling (GLM) assumptions, e.g., normal distribution and/or equal variance across groups, are often lacking, sample sizes are frequently low without proper justification or power analysis (Button et al., 2013; Dumas-Mallet et al., 2017; Smith, 2017), and the inadequate analysis of "nested" data (Aarts et al., 2014) can all lead to an increased rate of false-positive reporting (Colquhoun, 2014). This is particularly relevant for ELS studies where both fixed (rearing condition) and random (dam) effects are present. In other words, the behavior of each pup is nested within the dam (or litter), thus yielding clustered observations that cannot be considered fully independent, making the traditional use of GLM problematic. This tendency toward underpowered studies and inadequate data analysis may mask individual litters driving effects, making both within group and between group replication more difficult.

Beyond issues related to analysis, it can be difficult to compare findings within the same paradigm, as the specifics of the stress timing, testing age, animal species or strain can directly alter results. For instance, in a systematic review conducted by Tractenberg et al. (2016), MS paradigms were found to be highly varied in terms of separation length, animal strain utilized, and biological and behavioral phenotypes. Variability in methodology is further complicated by inadequate reporting practices. For instance, according to Tractenberg et al. (2016), only half of the 96 studies included in their systematic review met 75% of the criteria for guidelines on reporting animal research, and only three studies had a quality score above 90% (Tractenberg et al., 2016). Additionally, some reports demonstrate little to no strain variation (Millstein and Holmes, 2007), while others show resiliency to MS paradigms in certain strains, e.g., C57bl/6 (Own and Patel, 2013). This inconsistency suggests a need for more in depth methodological reporting or more standardized paradigms between research groups.

An important statistical tool to address conflicting results is to conduct systematic reviews followed by meta-analyses. This approach has been used frequently in clinical studies, but is rarely used to resolve inconsistent findings in preclinical studies in general, and to an even less extent in the ELS literature. In fact, while we are aware of only one meta-analysis that has conducted this kind of analysis using rodent models of ELS (Chen and Jackson, 2016), this type of approach can be very effective in addressing the relative vulnerabilities of males and females to different paradigms of ELS and in identifying important moderators and publication biases.

Finally, preclinical studies would also benefit from using the threat/deprivation conceptual model to study consequences of ELS (**Figure 1B**). In this regard, one of the most important caveats is that the "standard rearing" condition provides fairly low levels of stimulation that does not adequately reflect the complexity seen in nature or the levels of stimulation seen in children exposed to normal rearing conditions.

Recommendations for Preclinical Studies

There is a desperate need for additional work directly exploring outcomes and underlying mechanisms in both males and females exposed to forms of ELS (see sections "Modeling early life stress in rodents" and "Maternal Separation Paradigms"). The use of human imaging modalities such as rsfMRI and dMRI provide a particularly promising area of translational research that can help clarify how sex moderates the effects of deprivation and threat on many aspects of brain development. Such imaging findings should be coupled with genomic, retrograde tracing, optogenetic tools, and behavioral assays to rigorously clarify how these structural and functional changes alter complex behavior in males and females. To improve the clinical relevance of these ELS models, additional enrichment/stimulation during early development in the control group is warranted, and could unmask small, but consistent outcomes that have otherwise been overlooked. Finally, effort should be made to improve the standardization and reporting of rearing conditions and appropriate sample sizes and statistical tools, i.e., hierarchical linear modeling (Woltman et al., 2012; Aarts et al., 2014) should be utilized; see Naninck et al. (2015) for an example of this strategy used in ELS research. Finally, conducting more meta-analyses using animal models of ELS could prove helpful in identifying subtle-sex differences in behavioral and developmental outcomes and help correct for publication bias.

CONCLUSION

Important sex differences are present early in development affecting the way males and females respond to environmental challenges early in life. Despite the large number of inconsistent clinical and preclinical findings, a growing body of work has identified important differences in the way sex moderates outcomes of CM. These sex differences will likely have important treatment implications, and, therefore deserve additional research effort to elucidate them. However, such

effort would need to address key obstacles at both the clinical and preclinical levels. The most important suggested changes include the development of a uniformly accepted method of characterizing CM and the use of advanced human imaging tools in preclinical studies.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to reviewing the literature, conceptualizing the questions, writing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

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Neuronal Cell-Intrinsic Defects in Mouse Models of Down Syndrome

Alessandra Maria Adelaide Chiotto[1,2], Martina Migliorero[1], Gianmarco Pallavicini[1,2], Federico Tommaso Bianchi[1], Marta Gai[2], Ferdinando Di Cunto[1,3] and Gaia Elena Berto[1,3]**

[1] Neuroscience Institute Cavalieri Ottolenghi, University of Turin, Turin, Italy, [2] Department of Molecular Biotechnology and Health Sciences, University of Turin, Turin, Italy, [3] Department of Neuroscience, University of Turin, Turin, Italy

Down Syndrome (DS) is the most common genetic disorder associated with intellectual disability (ID). Excitatory neurons of DS patients and mouse models show decreased size of dendritic field and reduction of spine density. Whether these defects are caused by cell autonomous alterations or by abnormal multicellular circuitry is still unknown. In this work, we explored this issue by culturing cortical neurons obtained from two mouse models of DS: the widely used Ts65Dn and the less characterized Ts2Cje. We observed that, in the *in vitro* conditions, axon specification and elongation, as well as dendritogenesis, take place without evident abnormalities, indicating that the initial phases of neuronal differentiation do not suffer from the presence of an imbalanced genetic dosage. Conversely, our analysis highlighted differences between trisomic and euploid neurons in terms of reduction of spine density, in accordance with *in vivo* data obtained by other groups, proposing the presence of a cell-intrinsic malfunction. This work suggests that the characteristic morphological defects of DS neurons are likely to be caused by the possible combination of cell-intrinsic defects together with cell-extrinsic cues. Additionally, our data support the possibility of using the more sustainable line Ts2Cje as a standard model for the study of DS.

Keywords: Down syndrome, neural differentiation, Ts65Dn, Ts2Cje, dendritic spines

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Benedikt Berninger, King's College London, United Kingdom

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Aditi Deshpande, University of California, San Francisco, United States
Laura Andreae, King's College London, United Kingdom

***Correspondence:**
Ferdinando Di Cunto
ferdinando.dicunto@unito.it
Gaia Elena Berto
gaiaelena.berto@gmail.com

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INTRODUCTION

Trisomy for human chromosome 21 (HSA21) causes Down syndrome (DS) in one every 800 live births (de Graaf et al., 2017), making it the most common genetic cause of developmental delay and intellectual disability (ID). DS is characterized by several phenotypes affecting many organ systems, including CNS abnormalities that lead to cognitive and motor impairment, congenital heart defects, megakaryocytic leukemia and early onset Alzheimer's disease (AD) (Haydar and Reeves, 2012; Hibaoui et al., 2014; Hartley et al., 2015). Thanks to the constant improvement of medical care and to increased access to it, a vast majority of these problems can be now addressed medically (e.g., megakaryocytic leukemia) or surgically (e.g., congenital heart defects). However, despite the presence of several medical trials, cognitive impairment remains a limiting factor in DS patients, by reducing the accomplishment of personal and social goals.

A number of studies performed on patients and animal models demonstrated that the brain structures more affected in DS are the hippocampus, the cerebellum and the cerebral neocortex (Dierssen et al., 2009; Rueda et al., 2012). MRI studies revealed that neuro-anatomic abnormalities in the cerebral cortex are correlated with the cognitive profile of DS patients (Raz et al., 1995;

Pinter et al., 2001; Lott, 2012). However, the alterations that characterize neocortical structure and its development in DS patients are less studied than the abnormalities of other districts. Qualitative and quantitative defects, such as reduction of dendritic arborizations, decreased synaptic contacts and altered information processing, have been documented in DS patients cortical neurons (Wisniewski et al., 1984; Golden and Hyman, 1994).

Cortical alterations similar to those found in patients have also been described in Ts65Dn mice (Dierssen et al., 2003) the most commonly used and best characterized rodent model of DS (Davisson et al., 1993). Ts65Dn mice possess an extra chromosome, containing approximately two-thirds of HSA21 orthologous genes. Recently, Ts[Rb (12.17^{16})]2Cje (Ts2Cje) mice have been established, after a fortuitous translocation of the Ts65Dn extra chromosome to chromosome 12 (Villar et al., 2005). Ts2Cje possess the same amount of triplicated DNA sequence as Ts65Dn, but the stable rearrangement spares fertility in males and increases the frequency of transmission of the segmental trisomy through the female germline. These differences make Ts2Cje mice a much easier model to study, as compared to Ts65Dn animals.

Since the presence of an extra, freely segregating chromosome may contribute to DS phenotypes, Ts65Dn mice may represent a more faithful model. However, it is noteworthy that possessing an entire extra HSA21 is not necessary for DS-related ID, since a subset of DS patients show partial trisomy, associated with chromosome 21 translocation and fusion events. Ts2Cje mice have not been characterized as deeply as the Ts65Dn line, but they show some of the DS-relevant phenotypes previously found in Ts65Dn mice, such as structural dendritic spine abnormalities, ventriculomegaly and altered neurogenesis (Villar et al., 2005; Ishihara et al., 2010; Raveau et al., 2017). Considering the difficulties of breeding the Ts65Dn line, a deeper characterization of Ts2Cje mice could provide valuable insight for further establishing this more tractable model. In addition, in both models, it is not well understood whether cortical neuron abnormalities are primary and cell-autonomous or the result of altered dynamics of neurogenesis.

To address these issues, we cultured cortical neurons from newborn Ts65Dn and Ts2Cje mice and evaluated their ability to differentiate in *in vitro* conditions.

Our data indicate that, in both mouse models, axonogenesis and dendritogenesis are unaffected, while dendritic spines are both reduced and immature, suggesting that only the latter phenotypes are a cell-autonomous consequence of the genetic imbalance.

MATERIALS AND METHODS

Mice

Ts65Dn and Ts2Cje lines were bred accordingly to Jackson's Laboratories directions, conforming to the Italian laws on animal experimentation and under the supervision of the veterinary service of our animal facility. Mice were genotyped with PCR using primers spanning the translocation site.

Neuronal Primary Cell Culture and Transfection

Mouse cortical neurons were isolated from Ts65Dn and Ts2Cje pups and euploid litters on the day of birth (P0) as previously described (Beaudoin et al., 2012). Briefly, PCR was performed on a small amount of tissue obtained from the tail and mice with the same genotype were then processed as a single individual. Brains from both euploid and trisomic mice were extracted from the skull, meninges were removed, the two hemispheres were separated, hippocampus removed, cortices were isolated and transferred into 1 ml of pre-warmed 2,5% trypsin (Sigma) for 15 min at 37°C. Cortices were then washed five times with HBSS (Thermo Fisher), DNAseI (Promega) was added to the last wash and incubated at 37°C for 10 min. Subsequently, cells were carefully disaggregated with a P1000 sterile filtered tip eight to ten times, counted and plated in Mem Horse medium (MEM 1×, 10% horse serum, 2 Mm L-glutamine) on poly-L-lysine (Sigma, 1 mg/ml.) pre-coated coverslips with a density of 32,500 cells/cm^2. After 4 h, medium was changed into Neurobasal (Thermo Fisher) supplemented with 2% B27 (Thermo Fisher) and 2 mM L-glutamine (Gibco). Fresh supplemented Neurobasal was added to cultures every 4 days after the removal of half of the medium.

To highlight neuronal morphology for dendritogenesis and dendritic spines analysis, pEGFP-C1 plasmid (Clontech) was transfected using Lipofectamine LTX (Thermo Fisher) according to manufacturer's indications.

Immunofluorescence, Image Acquisition, and Analysis

Neurons were fixed with 4% paraformaldehyde in PBS for 10 min, quenched with 50 mM NH_4Cl for 15 min, permeabilized with 0.1% Triton X-100/PBS for 5 min. Non-specific sites were blocked with 5% BSA/PBS for 30 min. Immunofluorescence (IF) was performed using the anti-GFP antibodies (Rabbit polyclonal AB290, 1:1000, Abcam), followed by incubation with appropriate Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Polymeric F-actin was detected with Tritc or Fitc phalloidin (Sigma). Interneurons were identified with GAD67 staining (mouse monoclonal, 1:100, Abcam). Axons were stained with anti neurofilament H (mouse monoclonal SMI 32, 1:200, Biolegend) and pre-synaptic sites were stained with Bassoon (mouse monoclonal, 1:200, Stressgene).

Images were acquired with ViCo (Nikon) fluorescent microscope or with SP5 Leica confocal microscope. All analyses were performed with FiJi software (Schindelin et al., 2012). Traces of neurites were obtained using the NeuronJ plugin for FiJi. In brief, Z-stacks of GFP transfected neurons were projected on one plane ("maximum projection") and traces were manually drawn with a line. Concentric circles were centered on cell soma and the number of intersections was counted manually. Total dendritic length was measured with FiJi "segmented line" tool.

Dendritic spines were counted manually on 10 μm dendritic segments, 20 μm far from cell soma. At least two segments per cell were analyzed.

RESULTS

Neuronal Polarity Is Not Altered in Ts65Dn and Ts2Cje Mouse Cortical Neurons

To evaluate whether the cortical defects described in DS patients and in mouse models could be attributed to cell intrinsic defects, we resorted to the use of *in vitro* primary cultures, obtained from post-natal 0 (P0) pups. Neuronal cultures were prepared from both Ts65Dn and Ts2Cje mice (Villar et al., 2005). In both cases, euploid littermates were used as matched controls. Cultures were composed by a majority of excitatory neurons. Indeed, they contained approximately 20% inhibitory neurons, with no significant differences between genotypes (**Supplementary Figure 1A**). Moreover, we only analyzed cultures containing less than 20% glial cells (**Supplementary Figure 1C**)(see methods for technical details about the procedure). We evaluated all the main stages of neuronal development (Banker and Goslin, 1988): axonogenesis (days *in vitro*∼DIV-3), dendritogenesis (∼DIV7) and synaptic maturation (∼DIV14) (**Figure 1A**). Three days after plating, we counted the percentage of cells in stage II (multipolar cells, with equally long neurites) and in stage III (polarized SMI^+ cells, **Supplementary Figure 1B**) (Banker and Goslin, 1988): we did not observe any difference in the distribution of cells between the two stages in the two analyzed strains (**Figures 1B,C,F**). It has previously been reported that the number of projections is higher and axonal length is increased in cultured hippocampal neurons obtained from Ts65Dn and in cortical neurons obtained from patients' samples (Sosa et al., 2014). To evaluate whether cortical neurons from Ts65Dn and Ts2Cje would behave the same, we counted the number of primary neurites emerging directly from the cell soma and the length of the axon after 3 days in culture. In both models, we did not appreciate any alteration in the number of primary projections (**Figures 1D,G**). In addition, although in both genotypes the average axonal length tended to a slight increase, the differences from controls were not statistically significant (**Figures 1E,H**). Together, these data indicate that cortical neurons in primary culture, obtained from post-natal brain of two different mouse models of DS, show a normal pattern of neuritogenesis, with no significant alterations in the stage progression or in the axonal outgrowth.

Dendritic Arborization Is Unaffected in Ts65Dn and Ts2Cje Cortical Neurons

During neuronal differentiation, the establishment of neuronal polarity and axon sprouting are followed by rapid growth of the minor neurites into dendrites. *In vitro*, this step takes place around DIV7 (Takano et al., 2015). Previous *in vivo* work has reported that Ts65Dn layer III pyramidal neurons display simplified branching pattern and shorter dendritic length (Dierssen et al., 2003), while no data are currently available for Ts2Cje. We thus evaluated the differentiation potential of Ts65Dn and Ts2Cje cortical neurons, by analyzing their capability to form dendrites after 7 days *in vitro*. To clearly highlight the dendritic tree, neurons were transfected at DIV5 with a GFP expressing plasmid. Sholl analysis (Sholl, 1953) was performed on GFP-positive cells (**Figures 2A,B**) counting the number of intersections up to a radius of 60 μm from cell center. We did not analyze larger radii, because of the high number of breaks taking place at the tiny tip of dendrites. Interestingly, this analysis did not reveal significant changes in the number of intersections (**Figures 2C,F**). Even the cumulative number of intersections (**Figures 2D,G**), as well as the total dendritic length (calculated inside the Sholl' area) (**Figures 2E,H**) were not different from controls in both genotypes.

Considering the previous work showing that Ts65Dn cortical neurons display dendritic alterations *in vivo* (Dierssen et al., 2003), we next asked whether these defects may depend on the inability of neurons to properly maintain, rather than establish, the structure of dendritic fields. Indeed, around DIV7-9, differentiating mouse neurons display a dynamic phase characterized by branching instability, with continuous progression and retraction of the neurites. This is followed, around DIV10-15, by a stabilization phase, leading to the final dendritic configuration (Baj et al., 2014). To evaluate whether the stabilization phase is affected in trisomic mice, we performed Sholl analysis also in mature Ts65Dn and Ts2Cje neurons, at DIV14. As expected, the number of intersections is increased at this time, as compared to DIV7. However, even at this stage, no differences were detected between trisomic and euploid neurons (**Figures 3A,B**). These data suggest that cortical neurons of trisomic mice, grown in *in vitro* conditions, possess a similar intrinsic capability to generate dendrites and to establish a dendritic arbor as euploid controls.

Dendritic Spines of Trisomic Neurons in Primary Culture Show Immature Morphology and Reduced Density

We next wondered whether Ts65Dn and Ts2Cje neurons in culture show alterations of dendritic spines. Indeed, *in vivo* studies have reported that Ts65Dn neurons exhibit a reduced number of spines, which are further characterized by immature shape and function (Dierssen et al., 2003; Belichenko et al., 2004). To further investigate this topic, we transfected at DIV5 Ts65Dn, Ts2Cje and matched control neurons, with a GFP expressing plasmid. We then quantified the density of dendritic spines at DIV14. Spines were grouped in four classes, as previously described with morphological parameters (Harris et al., 1992): filopodia (without a visible head), stubby (no distinguishable neck), long neck and mushroom (defined as active contacts, **Figure 4G**). Concerning Ts65Dn, we observed a slight decrease of the long neck spines and a significant decrease of mushroom spines. No dramatic changes were seen in stubby and filopodial spines (**Figures 4A,B**). The overall spine density (spines/10 μm segment) was also decreased in Ts65Dn, compared to euploid cells (**Figure 4E**). In Ts2Cje neurons, we observed a similar

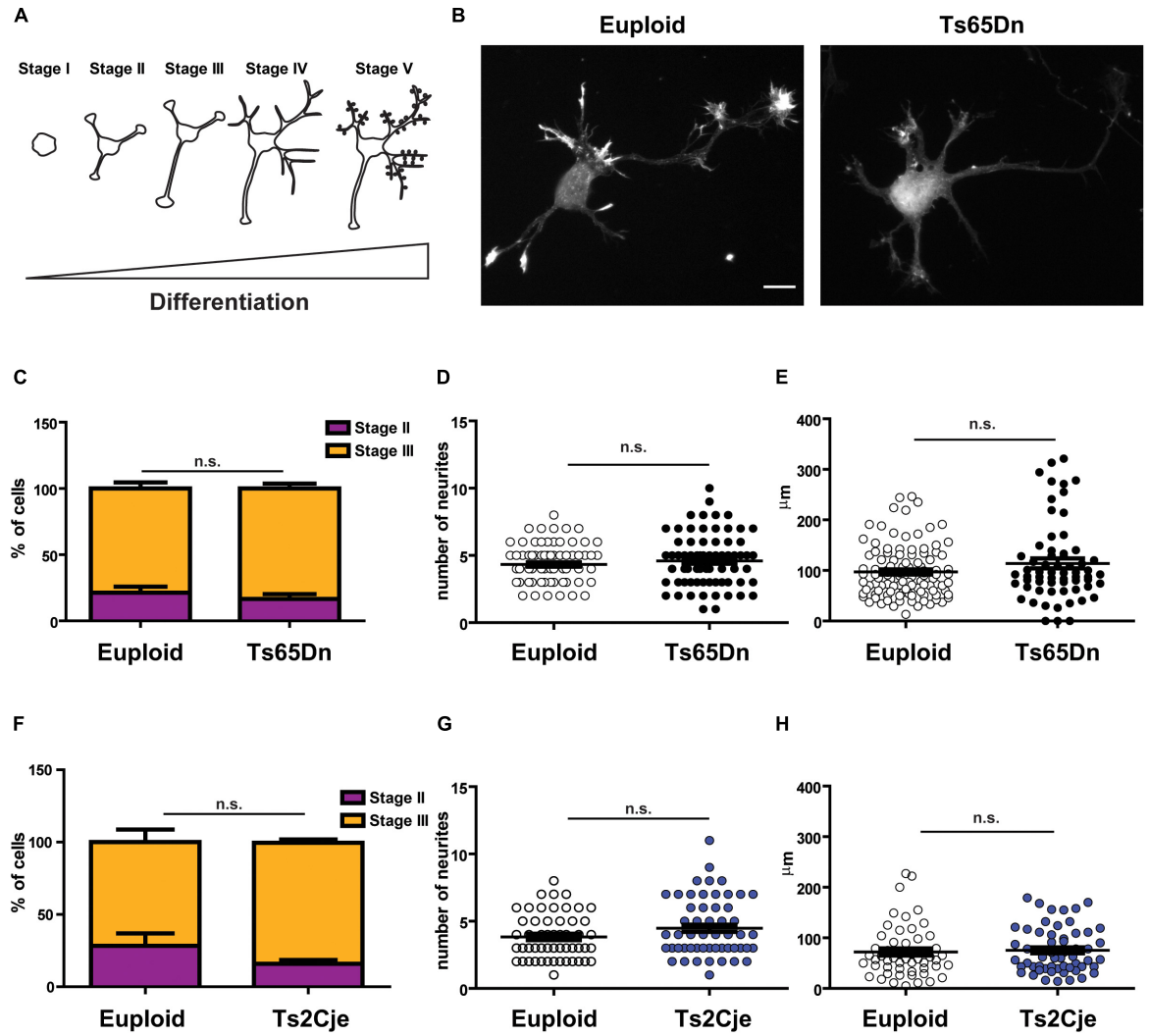

FIGURE 1 | Neuronal polarity is not altered in Ts65Dn and Ts2Cje mouse cortical neurons. **(A)** Schematic representation of neuronal differentiation. We observed that during the first 3 days in culture, neurons generate and specify the axon (stages I to III). Next, around day in culture (DIV) 7, cells start to elongate dendrites (stage IV) and after 2 weeks they begin to be synaptically active (stage V). **(B)** Euploid (left panel) and Ts65Dn (right panel) cortical neurons at DIV3, stained for filamentous actin (phalloidin). Cells appear properly differentiated and show no evidence of delay or maturation alteration. A similar pattern was observed in Ts2Cje cells. **(C,F)** Percentage of cells in stage II or stage III after 3DIV (**C:** euploid in stage II $n = 44$, stage III $n = 144$, from 5 mice; Ts65Dn in stage II $n = 18$, stage III $n = 94$, from 4 mice. **F:** euploid in stage II $n = 15$, stage III $n = 38$, from 4 mice; Ts2Cje in stage II $n = 10$, stage III $n = 51$, from 4 mice). Euploid vs. Ts65Dn $p = 0.32$ (stage II) and $p = 0.65$ (stage III); euploid vs. Ts2Cje $p = 0.28$ (stage II) and $p = 0.25$ (stage III). **(D,G)** Total number of primary neurites emerging directly from cell soma (**D:** 78 cells from 5 mice for euploid, 75 cells from 4 mice for Ts65Dn; 1G: 53 cells from 6 mice for euploid, 61 cells from 6 mice for Ts2Cje). Euploid vs. Ts65Dn $p = 0.32$; euploid vs. Ts2Cje $p = 0.07$. **(E,H)** Axon length after 3 DIV. All the evaluated parameters showed no significant differences in the two trisomic models, when compared to the matched euploid controls (**E:** 105 cells from 5 mice for euploid, 63 cells from 4 mice for Ts65Dn; **H:** 53 cells from 6 mice for euploid, 57 cells from 6 mice for Ts2Cje). Euploid vs. Ts65Dn $p = 0.09$; euploid vs. Ts2Cje $p = 0.71$. Statistics: Unpaired two tailed Student's *t*-test. $P > 0.05$ was considered not significant. Error bars represent SEM. Scale bar = 10 µm.

alteration of spine phenotype as the one observed in Ts65Dn. In Ts2Cje the difference between control and trisomic neurons was significant for all morphological classes, with the only exception of stubby spines (**Figures 4C,D**). In addition, also in Ts2Cje, spine density was significantly reduced with respect to controls (**Figure 4F**).

Taken together, these data indicate that Ts65Dn and Ts2Cje neurons have an intrinsically compromised capability to form and mature dendritic spines.

DISCUSSION

Down syndrome is a genetic disorder characterized by a large cohort of symptoms that greatly vary in both penetrance and severity. Nevertheless, all DS patients share the common hallmark of ID (Chapman and Hesketh, 2000). Much information is available about the abnormalities existing in hippocampus and cerebellum (Dierssen et al., 2009; Rueda et al., 2012). Much less is known about the alterations produced

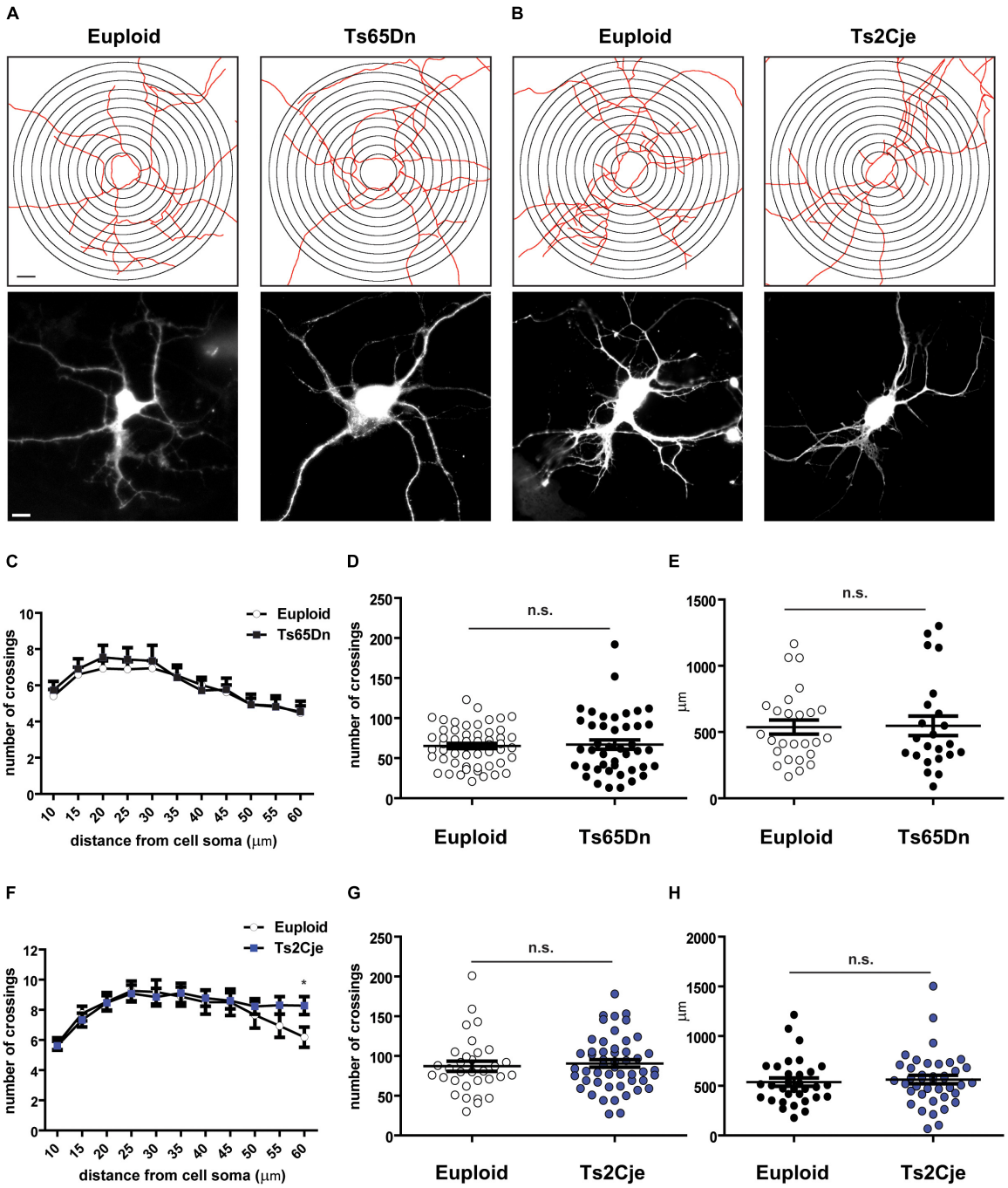

FIGURE 2 | Dendritic arborization is unaffected in Ts65Dn and Ts2Cje cortical neurons. **(A,B)** Skeletonization with Sholl analysis target superimposition (upper panels) and GFP fluorescent images (lower panels) of DIV7 cortical neurons. **(C,F)** Sholl analysis of cells images processed as in panels **(A,B)** (**C:** 55 cells from 8 mice for euploid, 43 cells from 5 mice for Ts65Dn; **F:** 32 cells from 4 mice for euploid, 50 cells from 6 mice for Ts2Cje). In panel **F**, $^*p = 0.03$. **(D,G)** Total number of crossings counted in panels **(C,F)**, respectively. Euploid vs. Ts65Dn $p = 0.78$; Euploid vs. Ts2Cje $p = 0.65$. **(E,H)** Total dendritic length measured within the Sholl area (**E:** 26 cells from 8 mice for euploid, 23 cells from 5 mice for Ts65Dn; **H:** 32 cells from 4 mice for euploid, 40 cells from 6 mice for Ts2Cje). Euploid vs. Ts65Dn $p = 0.90$; euploid vs. Ts2Cje $p = 0.60$. Statistics: unpaired two tailed Student's *t*-test. $P > 0.05$ was considered not significant. Error bars represent SEM. Scale bar is 10 μm.

by HSA21 trisomy on cortical circuits, which are likely to significantly contribute to ID (Raz et al., 1995; Pinter et al., 2001; Lott, 2012). In this work, we investigated more in depth the possible cellular basis of cortical structure alterations that characterize DS brain. We evaluated the ability of cortical neurons obtained from Ts65Dn and Ts2Cje to complete the differentiation program within the minimal environment of 2D culture condition. In both models, we could not detect

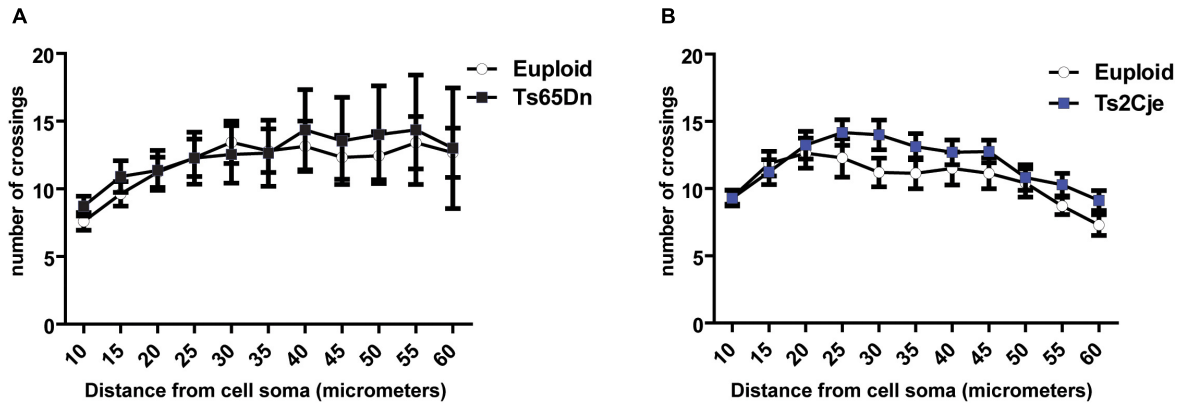

FIGURE 3 | Dendritic arborization is not altered in Ts65Dn and Ts2Cje neurons at later stages of maturation. **(A,B)** Sholl analysis of DIV14 GFP + neurons from both Ts65Dn and Ts2Cje (and age matched controls). The number of intersections between euploid and trisomic neurons is comparable also at this time point (**A:** 27 cells from 3 mice for euploid, 11 cells from 3 mice for Ts65Dn; **B:** 15 cells from 3 mice for euploid, 16 cells from 3 mice for Ts2Cje). Euploid vs. Ts65Dn $p \geq 0.5$ for all the points; euploid vs. Ts2Cje $p \geq 0.5$ for all the points. Statistics: unpaired two tailed Student's *t*-test. Error bars represent SEM. $P > 0.05$ was considered not significant.

significant impairment or delay in establishment of neuronal polarity, axon outgrowth and dendritogenesis. In particular, the normal axonal development is in accordance with data obtained with transplantation of human DS neurons, differentiated from induced pluripotent stem cells, into adult mice brain (Real et al., 2018). In this study, chronic *in vivo* imaging revealed that DS neurons had a normal pattern of axon development, with rates of both axon growth and retraction similar to those of control neurons. On the contrary, our data are in partial contrast with previous work, in which an increased length of the axon in Ts65Dn cultures was described (Sosa et al., 2014). However, the two experimental conditions differ for cellular type (cortical vs. hippocampal neurons), culturing substrate (poly-L-lysine vs. laminin) and timing of axonal length measurement (72 h vs. 24 h).

In the case of dendritogenesis, we were not able to detect significant differences between the two genotypes and their matched littermate controls in dendritic tree development, complexity and total length.

Data about dendritic arborization in DS mouse models and humans are quite heterogeneous. For instance, in one study (Haas et al., 2013) dendritic fields in Ts1Rhr and Tc1 mice were relatively normal. In contrast, studies performed on both DS patients (Becker et al., 1991) and Ts65Dn mice (Dierssen et al., 2003) reported a simplification of dendrites in cortical neurons. However, age specific differences could exist. Analyses performed in infants with DS (< 6 months of age) indicated a higher number of intersections, particularly evident in cortical layer III cells (Becker et al., 1991). Later on, already after 6 months of age, the reverse situation was found, with reduced dendritic arborization in DS individuals respect to healthy age matched controls (Becker et al., 1991). These results indicate that in DS there is a dramatic cessation of the neuronal growth soon after birth, with dendritic shortening and atrophy. One possible explanation to reconcile these data is that trisomic 3D environment could be characterized by abnormally low concentrations of trophic factors, or abnormally high concentrations of inhibitory cues that fluctuate during time, differentially affecting dendrites throughout the different phases of their development. In particular, the discrepancy between *in vitro* and *in vivo* data could imply that factors operating in the 3D environment of developing brain, but not in 2D cultures, may be specifically altered by HSA21 trisomy. These factors could consist of missing close contacts between pyramidal neurons and other cell types, such interneurons, astrocytes or other glial cells. The study of trisomic neurons developing within brain organoids (Faundez et al., 2018) may represent a very interesting possibility to further address this phenomenon and to unravel its molecular details.

In contrast with axonogenesis and dendritogenesis, analysis of the late stages of *in vitro* differentiation showed in both models a significant reduction of mature dendritic spines. This result is consistent with the *in vivo* data, supporting the notion that the synaptic defects that characterize DS are at least in part due to cell-autonomous mechanisms, together with additional cell-extrinsic factors that may exacerbate the phenomenon.

From a genetic point of view, the most obvious candidates for such a synaptic effect are the HSA21-located APP and DYRK1A genes. APP is localized to both pre- and post-synaptic boutons, playing a role in the stabilization of the whole synapse. Imaging analyses performed in APP knockout mice showed that layer V cortical neurons exhibited alterations in spine turnover, a reduction in the number of thin spines and an increase in the fraction of mushroom ones (Zou et al., 2016). Another candidate is DYRK1A, because transgenic mice overexpressing this protein show reduced spine density and increased frequency of filopodial spines, similar to DS individuals and mouse models (Martinez de Lagran et al., 2012).

Importantly, our work demonstrated that the spine phenotype observed in the fully described Ts65Dn could be reproduced also in the novel and less characterized Ts2Cje mouse model. For this reason and together with the consistent increase in fertility

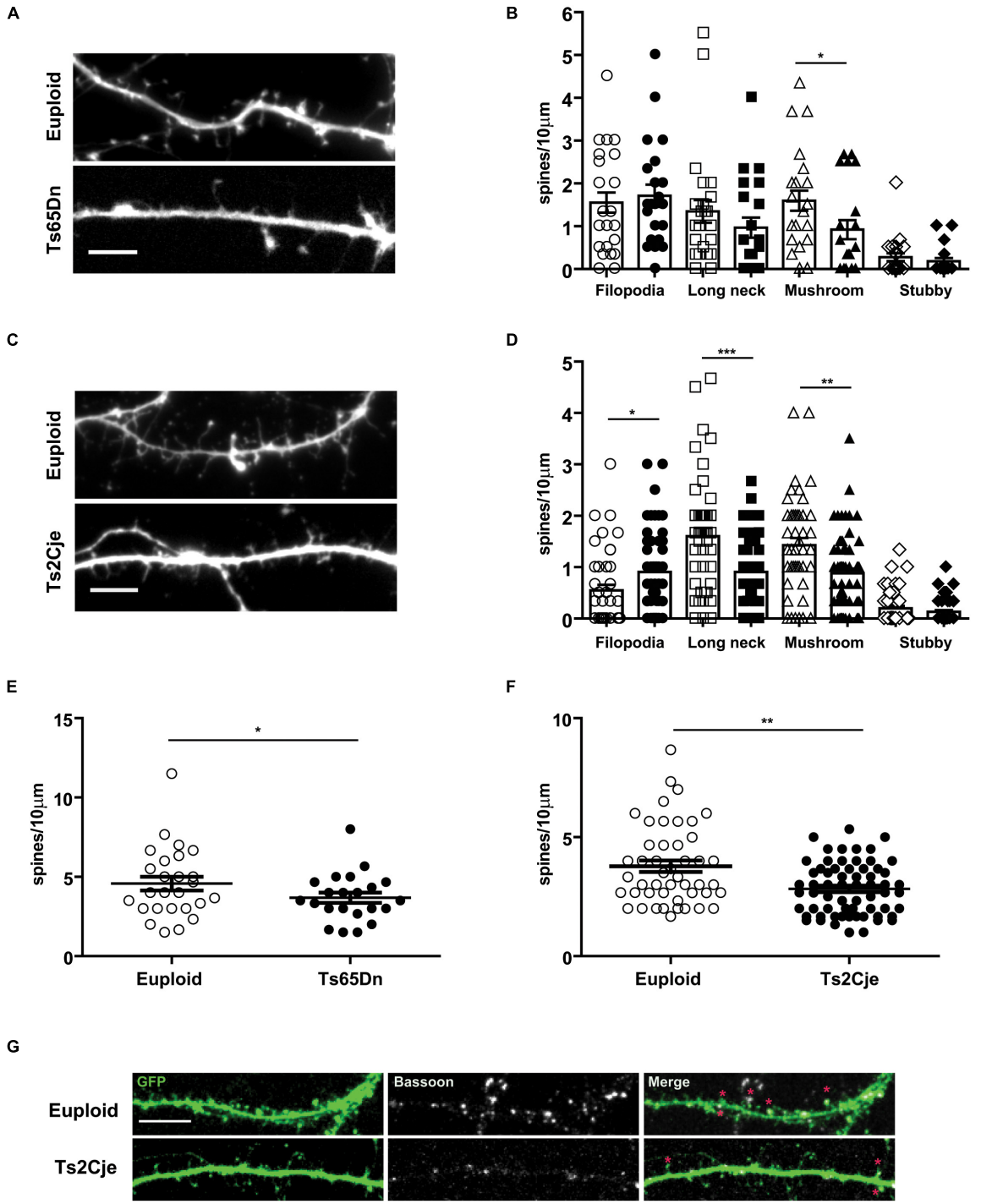

FIGURE 4 | Spine morphology is altered in Ts65Dn and Ts2Cje cortical neurons. **(A,C)** High magnification images of dendrites of euploid and trisomic DIV14 cortical neurons (respectively, Ts65Dn and Ts2Cje); scale bar 5 μm. **(B,D)** Quantification of different classes of spines calculated as the density of spines on ten micrometers. Based upon morphological parameters, spines were classified as filopodia, long neck, mushroom, stubby. In Ts65Dn and Ts2Cje the number of long neck and mushroom spines was decreased. The amount of filopodia spines was significantly higher only in Ts2Cje. White symbols represent euploid mice, black ones Ts65Dn in panel **(B)** and Ts2Cje in panel **(D)**. Bars represent mean. Error bar is SEM. **(E,F)** Quantification of total number of spines calculated as the density of spines on ten micrometers segments. In trisomic conditions the total number of spines is decreased. (**B–E**: 26 cells from 3 mice for euploid, 22 cells from 3 mice for Ts65Dn. **D–F**: 47 cells from 5 mice from euploid, 64 cells from 6 mice from Ts2Cje). **B**-Euploid vs. Ts65Dn (each group p-value in the same order represented in the graph): $p = 0.62$, $p = 0.22$, $^{*}p = 0.03$, $p = 0.26$. **D**-Euploid vs. Ts2Cje: $^{*}p = 0.013$, $^{***}p = 0.0004$, $^{**}p = 0.0018$, $p = 0.41$. **E:** $^{*}p = 0.04$. **F:** $^{**}p = 0.0024$. Statistics: Two-tailed Mann–Whitney test. Error bars represent SEM. $P > 0.05$ was considered not significant. **(G)** Representative images of spine contacts. Stars indicate GFP (filling post-synaptic protrusions) and Bassoon (pre-synaptic marker) positive excitatory spines in the two genotypes. Error bars represent SEM.

of both males and females of this line, as compared to Ts65Dn, our results suggest that 2D neuronal cultures of Ts2Cje mice could provide an efficient model not only to identify the genetic factors by which gene dosage imbalance leads to altered synaptic development, but also to screen for pharmacological compounds capable of reverting the phenotype. On this basis, we propose that Ts2Cje should be taken into stronger consideration as a standard model for the study of DS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Italian Ministry of Health, Istituto Superiore di Sanità. The protocol was approved by the Italian Ministry of Health, Istituto Superiore di Sanità.

AUTHOR CONTRIBUTIONS

AC, FD, and GB: conceptualization and experimental design. GB: experiments supervision. AC, MM, GP, FB, and MG: experiments execution. AC, GB, and FD: data analysis. AC and GB: writing – original draft preparation. FD, GB, and AC: writing – review and editing. FD: funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.01081/full#supplementary-material

FIGURE S1 | (A) Percentage of Gad67 positive cells in Euploid and Ts65Dn DIV3 cultures. For euploid 59 cells from 2 mice, for Ts65Dn 67 cells from 3 mice. **(B)** SMI staining of axon at DIV3. Scale bar is 10 μm. **(C)** Brightfield images of DIV5 neurons. Scale bar is 50 μm. Error bars represent SEM. Unpaired two tailed Student's *t*-test: $p = 0.71$ was considered not significant.

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REVIEW
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Complement System in Brain Architecture and Neurodevelopmental Disorders

*Juliana Magdalon[1,2], Fernanda Mansur[1], André Luiz Teles e Silva[1,3], Vitor Abreu de Goes[1,2], Orly Reiner[4] and Andréa Laurato Sertié[1]**

[1] Center for Experimental Research, Hospital Israelita Albert Einstein, São Paulo, Brazil, [2] School of Medicine, Faculdade Israelita de Ciências da Saúde Albert Einstein, São Paulo, Brazil, [3] Department of Genetics and Evolutionary Biology, University of São Paulo, São Paulo, Brazil, [4] Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

Current evidence indicates that certain immune molecules such as components of the complement system are directly involved in neurobiological processes related to brain development, including neurogenesis, neuronal migration, synaptic remodeling, and response to prenatal or early postnatal brain insults. Consequently, complement system dysfunction has been increasingly implicated in disorders of neurodevelopmental origin, such as schizophrenia, autism spectrum disorder (ASD) and Rett syndrome. However, the mechanistic evidence for a causal relationship between impaired complement regulation and these disorders varies depending on the disease involved. Also, it is still unclear to what extent altered complement expression plays a role in these disorders through inflammation-independent or -dependent mechanisms. Furthermore, pathogenic mutations in specific complement components have been implicated in the etiology of 3MC syndrome, a rare autosomal recessive developmental disorder. The aims of this review are to discuss the current knowledge on the roles of the complement system in sculpting brain architecture and function during normal development as well as after specific inflammatory insults, such as maternal immune activation (MIA) during pregnancy, and to evaluate the existing evidence associating aberrant complement with developmental brain disorders.

Keywords: complement system, neural progenitor proliferation, neurogenesis, neuronal migration, synapse refinement, neurodevelopmental disorders

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Alfonso Represa, INSERM U901 Institut de Neurobiologie de la Méditerranée, France

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Bryan Paul Morgan, Cardiff University, United Kingdom

****Correspondence:***
Andréa Laurato Sertié
andrea.sertie@einstein.br

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INTRODUCTION

The complement system has been increasingly implicated in multiple physiological and homeostatic functions, including development and maintenance of the central nervous system (CNS) (Orsini et al., 2014; Coulthard et al., 2018b), in addition to its well documented roles in immune surveillance and host defense against pathogens and injured cells (Kolev et al., 2014). Virtually all complement components can be locally produced in the brain, where they play critical roles in almost every aspect of normal brain development, including neurogenesis (Rahpeymai et al., 2006; Coulthard et al., 2017; Gorelik et al., 2017a,b), neuronal migration (Gorelik et al., 2017a,b), and synaptic refinement (Schafer et al., 2012; Stephan et al., 2012). Moreover, the complement system plays an important role in the maintenance of uninjured

brain homeostasis, protecting from infection and inflammation, eliminating damaged cells and supporting regeneration (Alawieh et al., 2015; Hammad et al., 2018). However, in the injured, aged or diseased CNS, the synthesis of components of the complement pathway markedly increases and contributes to local inflammation and tissue damage, which may lead to blood brain barrier injury (Orsini et al., 2014; Hammad et al., 2018). Consequently, the brain parenchyma can be invaded by a number of peripheral blood-derived inflammatory cells and molecules, including complement proteins, which amplify local damage and brain malfunction (Brennan et al., 2012).

Given the multi-faceted functions of complement in CNS development, dysfunction of specific components of the complement system has been increasingly linked to developmental brain disorders, including 3MC syndrome, a rare autosomal recessive disorder with facial dysmorphism, growth deficiency and cognitive deficit (Sirmaci et al., 2010; Rooryck et al., 2011; Munye et al., 2017), as well as to more prevalent and genetically complex disorders, such as schizophrenia (Sekar et al., 2016) and autism spectrum disorder (ASD) (Warren et al., 1991; Fagan et al., 2017). However, despite recent advances, several aspects of the involvement of the complement system in the pathogenesis of these complex neurodevelopmental disorders are still unclear. It is not yet fully established, for example, whether aberrant complement expression produced locally during brain development plays an etiological role in these disorders independently of any role in inflammation, or whether aberrant complement activation both systemically and in the CNS, as a result of inflammatory insults during prenatal or early postnatal neurodevelopment, also plays a part in the pathophysiology of these disorders as a secondary event.

In support of the first possibility are, as abovementioned, recent findings reporting multiple emerging novel non-inflammatory roles of complement in every stage of brain development (Coulthard et al., 2018b), as well as data from genetic studies showing an association between risk variants in complement genes and neurodevelopmental disorders (Warren et al., 1991; Odell et al., 2005; Sekar et al., 2016). In addition, there are studies suggesting that the neuropathological and behavioral phenotypes in genetically modified mouse models with aberrant complement expression in the brain parallel known features of these human developmental brain disorders (Chu et al., 2010; Perez-Alcazar et al., 2014; Sekar et al., 2016; Comer et al., 2019).

In support of the latter possibility are data from different studies describing the presence of activated astrocytes and microglia in brains as well as the presence of altered expression of immune molecules, including complement components, in peripheral blood and/or cerebrospinal fluid obtained from individuals with schizophrenia and ASD (Mayilyan et al., 2006; Corbett et al., 2007; Morgan et al., 2010; Ashwood et al., 2011; Ishii et al., 2018). In addition, recent studies using animal models have suggested a role for the complement system in brain and behavioral abnormalities in offspring associated with prenatal maternal immune activation (MIA) (Pedroni et al., 2014; McDonald et al., 2015). In humans, severe maternal infections during pregnancy have been highlighted as a potential risk factor for these neurodevelopmental disorders (Patterson, 2009, 2011; Malkova et al., 2012; Knuesel et al., 2014; Coiro et al., 2015). One possibility is that immune molecules released by the maternal immune response can cross the placenta and enter the fetal brain, where they contribute to the pathological and behavioral changes. The ongoing immune dysregulation in the brain and the peripheral immune system of individuals with these neurodevelopmental diseases suggest that MIA or other inflammatory insults during prenatal or early postnatal development may induce an irregular immune phenotype that persists into adulthood.

This review aims to highlight the importance of the complement system in regulating the development of the healthy and diseased brain. First, we provide an overview of the well-known concepts of complement system activation in the immune system context. Then, we discuss recent progress in understanding the roles of the complement system in important physiological processes of normal brain development, as well as initial findings suggesting a potential role for complement in neuropathological and behavioral abnormalities in MIA offspring. Finally, we evaluate the current evidence for the involvement of the complement system dysfunction in disorders that trace their origin to abnormal brain development, including schizophrenia, ASD, Rett syndrome, and 3MC syndrome.

COMPLEMENT SYSTEM ACTIVATION: A BACKGROUND

The complement cascade is composed of several soluble and membrane-bound proteins that are mainly secreted by the liver, but also by leukocytes, adipocytes, cells in the CNS (such as neurons, astrocytes, and microglia), among others (Veerhuis et al., 2011; Bajic et al., 2015). Complement is activated through the classical, lectin and alternative pathways, which are initiated by different stimuli and result in the generation of: (1) opsonins (such as C3b and C4b), which recognize and bind to target cells to facilitate their removal by phagocytic cells that express complement receptors (such as complement receptor type 1, CR1); (2) anaphylatoxin proteins (such as C3a and C5a), which are proinflammatory peptides that interact with and activate immune cells through interaction with their receptors (C3a receptor, C3aR, and C5a receptor, C5aR); (3) terminal membrane attack complex (MAC), which are pores that disrupt lipid bilayers and lyses targeted (opsonized) pathogens or self-damaged cells (Ricklin et al., 2010; Bajic et al., 2015; **Figure 1**).

The classical pathway is initiated by C1 complex activation, which consists of a recognition molecule C1q and two copies of each of the homologous C1r and C1s serine proteases. Initially, C1q binds to one of its ligands, such as antibodies, C-reactive protein and some structures on invading microorganisms or apoptotic cells, which triggers a conformational change within the C1 complex resulting in the activation of C1r, which subsequently cleaves and activates C1s (Gaboriaud et al., 2004). The activated C1s then cleaves the complement protein C4 into the fragments C4a and C4b. C4b may then opsonize the activator and facilitates phagocytosis. Additionally, C4b binds to the complement protein

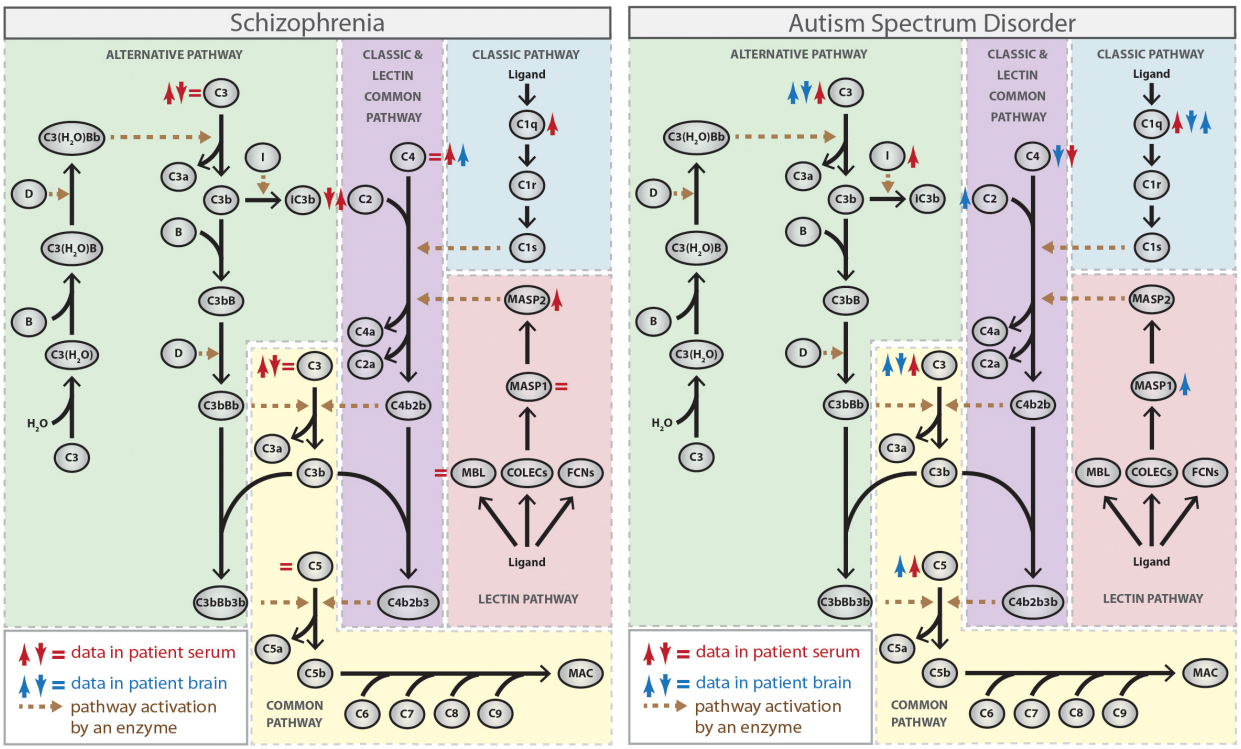

FIGURE 1 | Complement activation pathways and the activity or expression of their individual components (either protein or mRNA) in schizophrenia and autism spectrum disorder (ASD). ↑ red, increased protein activity or expression in blood from patients; ↓ red, decreased protein activity or expression in blood from patients; ↑ blue, increased RNA expression in brain tissues from patients; ↓ blue, decreased RNA expression in brain tissues from patients (Spivak et al., 1989, 1993; Warren et al., 1994; Wong et al., 1996; Maes et al., 1997; Shcherbakova et al., 1999; Hakobyan et al., 2005; Mayilyan et al., 2006; Corbett et al., 2007; Boyajyan et al., 2010; Momeni et al., 2012; Nardone et al., 2014; Li et al., 2016; Sekar et al., 2016; Fagan et al., 2017; Shen et al., 2018). The Figure does not distinguish between strong and weak evidence.

C2, which is subsequently cleaved by C1s into the fragments C2a and C2b. C2a remains attached to C4b and forms the C3 convertase C4b2b (previously termed C4b2a) of the classical pathway, which cleaves the complement protein C3 into the fragments C3a and C3b, triggering the final common part of the complement cascade (Muller-Eberhard et al., 1967; Dunkelberger and Song, 2010; **Figure 1**).

The lectin pathway is functionally similar to the classical pathway and also leads to formation of the C3 convertase C4b2b. However, the lectin pathway is initiated by binding of collectins [mannose-binding lectin (MBL), collectin-10 (COLEC10), and collectin-11 (COLEC11)] or ficolins (ficolin-1, ficolin-2, and ficolin-3) to sugar moieties or certain acetyl groups present on the surface of a large variety of pathogens, which leads to activation of the MBL-associated serine proteases (MASP) 1 and 2, structurally and functionally similar to C1r and C1s (Garred et al., 2016). While both enzymes cleave C2, only MASP2 cleaves C4, generating the C3 convertase C4b2b in a reaction analogous to the classical pathway (Chen and Wallis, 2004; Heja et al., 2012; **Figure 1**).

In contrast to the other two pathways, the alternative pathway does not depend on the recognition of exogenous materials and is constitutively active at low levels through a process called "tickover." This pathway is activated by spontaneous hydrolysis of plasma C3 to form $C3(H_2O)$, which binds to factor B. Factor D cleaves factor B to form Ba and Bb, which then generates $C3(H_2O)Bb$, the initial alternative pathway C3 convertase that can cleave C3 into C3a and C3b (Bexborn et al., 2008; Lachmann, 2018). Subsequently, C3b generated from any of the pathways can bind to factor B, which is cleaved by factor D, generating C3bBb, the main C3 convertase of the alternative pathway. C3bBb produces more C3b molecules, promoting an amplification loop for the entire system. Also, factor I cleaves C3b to iC3b, allowing iC3b to interact with leukocyte complement receptors (CR3 and CR4) and trigger the inflammatory response (Lachmann, 2009; **Figure 1**).

Therefore, the terminal phase of the complement cascade is similar for the classical, lectin, and alternative pathways and leads to the generation of C3 convertases. The incorporation of C3b into the C3 convertases generates C5 convertases (C4b2b3b for the classical and lectin pathways, and C3bBbC3b for the alternative pathway) that cleave C5 into C5a, which binds to its receptors on phagocytic cells, and C5b, which associates sequentially to other complement proteins (C6, C7, C8, and C9) forming the MAC and leading to cell lysis (Muller-Eberhard, 1985; Bajic et al., 2015; **Figure 1**).

It should be noted that the activity of the complement system is tightly regulated to protect host cells from indiscriminate

attack and limit the deposition of complement molecules on pathogen and host surfaces. Among the complement regulators is SERPING1 (or C1-inhibitor), which binds to and inactivates C1r, C1s, and MASP-1/2 proteases, thus leading to inhibition of the classical and lectin pathways (Ricklin et al., 2010). Other regulators include: factor H, which acts as a cofactor for complement factor I (CFI) in the C3b cleavage and also favors the dissociation of factor Bb from C3b (Lachmann, 2009); CSMD1, which acts as a cofactor to CFI-mediated degradation of C4b and C3b and also inhibits MAC assembly by preventing binding of C7 to the C5b-6 complex (Escudero-Esparza et al., 2013); and CD59, which inhibits MAC formation by preventing binding of C9 to the C5b-8 complex (Farkas et al., 2002).

COMPLEMENT SYSTEM IN THE DEVELOPMENT OF HEALTHY AND DISEASED BRAIN

Complement System in Neurogenesis

Besides playing a critical role during embryogenesis, the generation of new neurons is sustained throughout adulthood in the mammalian brain through the proliferation and differentiation of neural progenitor cells (NPC) present in neurogenic niches, mainly the subventricular zone of lateral ventricles and the subgranular zone of the hippocampal dentate gyrus (Fuentealba et al., 2012). Studies using animal and *in vitro* cell models have shown important roles for specific complement components in the regulation of neurogenesis both in the embryonic and adult brain under normal physiological conditions.

It has recently been shown that mouse embryos deficient for *C3*, *Masp2* or treated with C3aR antagonist exhibit increased proliferation of NPC in the brain ventricular or subventricular zones, suggesting that these complement components inhibit NPC proliferation at early stages of cortical development (Gorelik et al., 2017a; Coulthard et al., 2018a). It is noteworthy, however, that an opposite trend was observed for *C3aR* knockout mouse embryos, that display decreased proliferation of NPC within the ventricular zone (Coulthard et al., 2018a; **Table 1**). This discrepancy between the use of C3aR pharmacological blocker and *C3aR* knockout may be attributed in part to combinatorial modulation of other signaling pathways in the absence of C3aR during the entire developmental period (Coulthard et al., 2018a). In the context of adult mouse brain, previous studies have shown that young adult mice lacking *C3*, *C3aR* or treated with C3aR antagonist exhibit reduced neurogenesis from NPC in the neurogenic niches, possibly due to impaired NPC differentiation rather than decreased proliferation of these cells (Rahpeymai et al., 2006). These findings were further corroborated by an *in vitro* study using NPC isolated from adult mouse brain showing that C3a stimulates their neuronal differentiation without altering their survival and proliferation (Shinjyo et al., 2009; **Table 1**). Consistent with the findings that C3a/C3aR signaling regulates neurogenesis, adult *C3aR* knockout mice show deficits in memory (Coulthard et al., 2018a).

Constitutive deficiency of *C5aR* and acute pharmacological blockade of C5aR during neurogenesis also caused opposing phenotypes of NPC proliferation. While the use of C5aR antagonist inhibits NPC proliferation in the ventricular zone of mouse embryos and lead to brain microstructural alterations and behavioral deficits (such as heightened anxiety, impaired coordination, and short-term memory) later in life (Coulthard et al., 2017), *C5aR* knockout mice exhibit increased proliferation of NPC within the ventricular zone (Coulthard et al., 2018a; **Table 1**). In addition, while in the postnatal rat cerebellar cortex a C5aR agonist was shown to stimulate proliferation of immature granule neurons, which suggests a role for the C5a-C5aR axis in the cerebellar histogenesis (Benard et al., 2008), C5a-C5aR1 signaling seems not to be involved in NPC proliferation and differentiation in the neurogenic niches of the adult brain (Bogestal et al., 2007; Shinjyo et al., 2009; **Table 1**).

Interestingly, it has recently been shown that mouse embryos deficient in the *Serping1* gene, a known inhibitor of the classical and lectin pathways of the complement system, display decreased proliferation of both ventricular zone (radial) and intermediate (basal) progenitors during development of the cortex, suggesting that SERPING1 stimulates proliferation of NPC at early stages of cortical development (Gorelik et al., 2017b; **Table 1**). However, it is still unknown whether this function of SERPING1 is either dependent or independent on downstream activation of the complement system.

Together, the abovementioned studies suggest a role mostly for the anaphylatoxins in NPC proliferation and differentiation in the absence of other factors of the canonical pathogen-initiated complement activation routes. Also, these studies suggest that the spatiotemporal expression pattern of these complement components in different subsets of NPC seems to determine their role in progenitor neurogenesis.

Complement System in Neuronal Migration

Neuronal migration is an essential phenomenon for proper brain formation and establishment of neural circuit since most neurons must move from their birth position to their final location in the brain. During development, excitatory neurons arising from the proliferative neuroepithelium surface (the ventricular zone) exhibit mainly radial migration, in which early postmitotic neurons migrate along the processes of radial glial progenitors to their correct laminar position within the cortical plate. Inhibitory neurons are born in the ganglionic eminences and exhibit, initially, tangential migration, in which nascent neurons move in trajectories that are parallel to the ventricular surface (Marin et al., 2010). There is mounting evidence showing that the complement system plays an important role in the radial migration of pyramidal neurons during normal brain development.

A recent study has uncovered a direct role for the lectin arm of the complement system in radial neuronal

TABLE 1 | Summary of the phenotypes observed after disturbances in the expression of individual components of the complement pathway.

Functional alteration	Pathway	Model	NPC proliferation	NPC differentiation	Neuronal migration	Brain wiring	References
C1q knockout	Cl	Mouse embryo			=		Gorelik et al., 2017a
		Postnatal mouse				↑	Comer et al., 2019, Bialas and Stevens, 2013
C1s knockdown	Cl	Mouse embryo			↓		Gorelik et al., 2017a
Masp1 knockout	L	Mouse embryo			↓		Gorelik et al., 2017a
Masp1 knockdown	L	Mouse embryo	=		↓		Gorelik et al., 2017a
		Zebrafish embryo			↓*		Rooryck et al., 2011
Masp2 knockout	L	Mouse embryo			↓		Gorelik et al., 2017a
Masp2 knockdown	L	Mouse embryo	↑		↓		Gorelik et al., 2017a
Colec11 knockdown	L	Zebrafish embryo			↓*		Rooryck et al., 2011
C4 knockout	Cl and L	Postnatal mouse				↑	Sekar et al., 2016
C4 overexpression	Cl and L	Postnatal mouse				↓	Perez-Alcazar et al., 2014
C3 knockout	C	Mouse embryo	↑		↓		Gorelik et al., 2017a
		Postnatal mouse				↑	Schafer et al., 2012; Bialas and Stevens, 2013
		Adult mouse		↓			Rahpeymai et al., 2006
C3 knockdown	C	Mouse embryo	↑		↓		Gorelik et al., 2017a
C3a antibody	C	*Xenopus* embryo			↓*		Gorelik et al., 2018
		Xenopus NCC *in vitro*			↓*		Gorelik et al., 2018
C3aR knockout	C	Mouse embryo	↓				Shinjyo et al., 2009
		Adult mouse		↓			Rahpeymai et al., 2006
C3aR knockdown	C	*Xenopus* embryo			↓*		Gorelik et al., 2018
		Xenopus NCC *in vitro*			↓*		Gorelik et al., 2018
C3aR antagonist	C	Mouse embryo	↑				Shinjyo et al., 2009
		Adult mouse		↓			Rahpeymai et al., 2006
		Postnatal rat granule cell *in vitro*			=		Shinjyo et al., 2009
C3aR agonist	C	Mouse embryo	↓				Shinjyo et al., 2009
		Mouse embryo NPC *in vitro*	↓				Shinjyo et al., 2009
		Postnatal rat granule cell *in vitro*	=		↑		Shinjyo et al., 2009
		Adult mouse NPC *in vitro*		↑	=		Benard et al., 2008
CR3 knockout	C	Postnatal mouse				↑	Schafer et al., 2012
C5aR knockout	C	Adult mouse		=			Marin et al., 2010
C5aR antagonist	C	Mouse embryo	↓				Coulthard et al., 2017
		Postnatal rat cerebellum	=				Shinjyo et al., 2009
		Postnatal rat granule cell *in vitro*	=				Shinjyo et al., 2009
C5aR agonist	C	Mouse embryo	↑				Coulthard et al., 2017
		Human and mouse NPC *in vitro*	↑				Coulthard et al., 2017
		Postnatal rat cerebellum	↑				Shinjyo et al., 2009
		Postnatal rat granule cell *in vitro*	↑		=		Shinjyo et al., 2009
		Adult mouse NPC *in vitro*		=	=		Benard et al., 2008
Serping1 knockout	Ci	Mouse embryo	↓		↓		Gorelik et al., 2017b
Serping1 knockdown	Ci	Mouse embryo	↓		↓		Gorelik et al., 2017b

*Cl, Classical pathway; L, Lectin pathway; C, Common final pathway; Ci, Complement inhibitor;=, No difference; *, Neural crest cells.*

migration in the developing cerebral cortex. *C3*-, *Masp1*- or *Masp2*-deficient mice exhibit impairments in radial migration resulting in improper positioning of neurons and disorganized cortical layers (Gorelik et al., 2017a). Importantly, the migration deficits observed in *C3*- or *Masp2*-deficient mice were partially rescued by addition of polypeptides that mimic C3 cleavage products, C3aR agonist or a dual C3aR/C5aR agonist, suggesting that activation of the lectin cascade leading to C3 cleavage, C3a and C5a generation and activation of both C3aR and C5aR are necessary for proper radial neuronal migration and cortical development (Gorelik et al., 2017a; **Table 1**).

It is also noteworthy that components of the lectin complement pathway, including MASP1 and CL-K1 (encoded by the *Colec11* gene) proteins, as well as the C3a-C3aR axis, were shown to behave as early guidance cues to direct the migration of neural crest cells during embryonic vertebrate development, since deficiency of these components in zebrafish or *Xenopus* causes craniofacial abnormalities (Rooryck et al., 2011) or disorganized collective cell migration (Carmona-Fontaine et al., 2011; **Table 1**).

Abnormal radial neuronal migration was also observed in *Serping1* knockout and knockdown mice but, unexpectedly, deficiency of SERPING1 resulted in a small but significant decrease in C3b levels, suggesting that the complement pathways are not being activated (Gorelik et al., 2017b). Moreover, addition of C3 mimicry cleavage products, or addition of a dual C3aR/C5aR agonist, but not a C3a peptide or a specific C3aR agonist, significantly improved impaired neuronal migration (**Table 1**). These findings corroborate a role for C3 cleavage and downstream complement activation at the level of C5a in controlling normal migration of newly born neurons to the cortical plate (Gorelik et al., 2017b). Interestingly, it has been recently shown that *C3* knockout mouse embryos display reduced activity of the small GTPase Rac1 and reduced phosphorylation of Cofilin, a cytoskeleton protein, in migrating neurons entering the cortical plate. This suggests that Rac1 may be one of the key downstream mediators of the complement activity to control cytoskeletal remodeling required for proper neuronal migration in the developing brain (Gorelik et al., 2018).

In agreement with the results obtained with mouse embryos, previous studies have shown that adult mice deficient for C3a/C3aR signaling show impaired migration of both neuroblasts and newly formed neurons from the brain neurogenic niches (Rahpeymai et al., 2006), and that C3a stimulates migration of *in vitro* mouse adult brain NPC in response to low concentrations of the chemokine stromal cell-derived factor 1 alpha (Shinjyo et al., 2009). Also, the C3a-C3aR signaling was shown to regulate migration of immature granule neurons in the postnatal rat cerebellar cortex, suggesting that this signaling also plays a role in the cerebellum ontogenesis (Benard et al., 2008; **Table 1**).

Taken together, the existing findings indicate that functional activation of the lectin complement pathway and consequent production of anaphylatoxins are important for proper neuronal migration and correct positioning of neurons during brain development.

Complement System in Brain Wiring

Formation of precise neural circuitry during development is essential for proper functions of the CNS. Synaptic contacts are generated in excess during early phases of development, and postnatally unnecessary synapses are eliminated while functionally important synapses are strengthened to construct appropriate neural circuits (Rakic et al., 1986; Riccomagno and Kolodkin, 2015). Much of the current understanding about the mechanisms underlying synapse refinement during CNS development has been studied using the mouse retinogeniculate pathway. Early in development, axons from retinal ganglion cells form transient connections with neurons of dorsal lateral geniculate nucleolus (dLGN) of the thalamus and, during early post-natal development, these connections are sculpted through the pruning of redundant synapses (Hooks and Chen, 2006). Important studies have revealed that mice deficient for complement *C1q*, *C3*, *C4* or microglia-specific *CR3* exhibit impaired elimination of retinogeniculate synapses and defects in eye-specific segregation (Stevens et al., 2007; Schafer et al., 2012; Sekar et al., 2016), suggesting that the classical complement system plays a crucial role in synapse pruning during development (**Table 1**). Mechanistically, it has been proposed that transforming growth factor (TGF)-β released by retinal astrocytes induces the expression of C1q in retinal ganglion cells, which is transported from cell bodies along axons to the dLGN, where it is released to bind weak synapses (Bialas and Stevens, 2013). Similar to the immune system, the binding of C1q and formation of C1 complex results in activation of the classical complement pathway, cleavage of C4, formation of C3 convertase and then production of C3a and C3b/iC3b fragments. Activated C3 (iC3b) binds to CR3 in microglia ultimately promoting the engulfment of overlapping and weaker synapses (Schafer et al., 2012).

It is noteworthy that, in accordance with the abovementioned data using the mouse retinogeniculate pathway, *C1q*- or *C3*-deficient mice exhibit increased number of excitatory synapses in the cortex and hippocampus, respectively (Chu et al., 2010; Perez-Alcazar et al., 2014), epilepsy (Chu et al., 2010) or abnormal hippocampus-dependent learning (Perez-Alcazar et al., 2014; **Table 1**). Also, *C3* knockdown specifically in the prefrontal cortex lead to repetitive behavior and impaired social interaction in mice, possibly due to reduced synaptic pruning (Fagan et al., 2017). In addition, a study using cortical wild type neurons co-cultured with astrocytes derived from *IkBα* knockout mice, which overexpress the transcription factor NFkB and consequently complement C3, has shown that C3 released by astrocytes acts through neuronal C3aR reducing excitatory synaptic density and dendritic length and complexity (Lian et al., 2015). Moreover, it was shown that overexpression of *C4* in mouse prefrontal cortex leads to dendritic spine dysgenesis, reduced connectivity in cortical neurons, enhanced microglia-mediated engulfment of synapses and deficits in social interactions (Comer et al., 2019; **Table 1**).

Although the mechanisms that drives the opsonization of weaker synapses are not yet fully understood, a recent study using whole cortical tissue from newborn and adult mice has shown that C1q is predominantly localized to the presynaptic region of the labeled synapses, and that C1q-tagged synapses display downregulation of proteins associated with synaptic transmission and increased expression of apoptotic markers, suggesting that weaker synapses induce apoptotic-like mechanisms that attracts C1q and then triggers synaptic elimination by microglia (Gyorffy et al., 2018).

Altogether, the findings abovementioned suggest that microglia- and astroglia-mediated complement-dependent synaptic refinement occurs in different regions of the developing

CNS, and that alterations in this process affect behavior in animal models.

Complement System in Brain Pathology Associated With Prenatal Maternal Immune Activation

The developing brain is particularly vulnerable to environmental insults, such as ischemic and inflammatory insults, that can cause injury and long-term neurodevelopmental abnormalities manifesting as cognitive difficulties or behavioral problems (Meyer et al., 2006; Bilbo and Schwarz, 2009). Although the link between environmental risk factors, the immune response, and neurological dysfunction is not completely clear at present, accumulating evidence suggests that MIA via infection during pregnancy alters brain development and increases the risk for neurodevelopmental disorders in the offspring (Patterson, 2009, 2011; Malkova et al., 2012; Coiro et al., 2015). In this regard, some studies using animal models have reported a role for the complement system in brain abnormalities in the offspring following MIA.

Using a mouse model of inflammation-induced preterm birth and brain injury, a study has shown that treatment of pregnant mice with lipopolysaccharide (LPS) induced an increase in the levels of C5a in both the amniotic fluid and the fetal brain, as well as cortical abnormalities in the preterm fetuses, characterized by decreased expression of neuronal markers and increased cell death (Pedroni et al., 2014). Interestingly, these fetal cortical brain alterations associated with LPS-induced preterm birth were not observed in fetuses deficient for *C5aR* or born from mice treated with anti-C5 antibody (Pedroni et al., 2014). Furthermore, the neurotoxic effect of C5a was confirmed *in vitro* by treating isolated fetal cortical neurons with this anaphylatoxin, which inhibited the growth of neurites and increased cell death, phenotypes that were blocked by C5aR antagonist (Pedroni et al., 2014). Similar results were observed in another study using a mouse model of malaria in pregnancy, in which the offspring from infected mice has shown impaired learning and memory and depressive-like behavior compared to non-infected controls. The neurocognitive impairments observed in the malaria-exposed offspring were rescued by deletion of *C5aR* in the fetuses or by treating infected pregnant mice with anti-C5 antibody (McDonald et al., 2015). Together, these findings suggest a new role for the anaphylatoxin C5a in the cortical brain damages and behavioral disturbances observed in fetuses exposed to prenatal inflammation.

More recent studies have shown that MIA induced by synthetic dsRNA (polyI:C) in pregnant rodents, which acts to initiate an inflammatory response similar to that caused by viral infection, caused long-term increase in C1q (Han et al., 2017) and *C4* (Duchatel et al., 2018) expression in the cortex of their offspring. The involvement of C1q and C4 in synaptic pruning (Stevens et al., 2007; Schafer et al., 2012; Sekar et al., 2016) suggests that these complement molecules may be involved in MIA-associated brain abnormalities in the offspring, but additional studies are necessary to confirm this possibility.

COMPLEMENT SYSTEM DYSREGULATION IN NEURODEVELOPMENTAL DISORDERS

Complement in Schizophrenia

Schizophrenia (MIM 181500) is a chronic and disabling mental disorder that affects about 1% of the world population (Hafner and der Heiden, 1997; McGrath et al., 2008). The symptoms of schizophrenia include psychosis and deficits in cognition and social interaction, which most commonly emerge in late adolescence or early adulthood (Howes and Murray, 2014). Neuropathological findings in individuals with schizophrenia include abnormal cortical organization possibly due to altered neuronal migration (Akbarian et al., 1996; Arnold et al., 1997; Shenton et al., 2001) and reduced cortical gray matter thickness (Selemon and Goldman-Rakic, 1999; Cannon et al., 2002, 2015) and diminished synaptic density possibly due to excessive pruning of cortical synapses, thus producing hypoconnectivity of prefrontal cortex (Glantz and Lewis, 2000; Glausier and Lewis, 2013). Although the exact mechanisms underlying schizophrenia are not yet fully understood, both complex genetic and environmental risk factors have been implicated in its pathogenesis. Importantly, accumulating evidence suggests that complement dysregulation is among the risk factors for the disorder.

The earliest studies of the complement system in schizophrenia have focused on the complement hemolytic activity to measure both the overall function of the classical pathway and the function of its specific components in blood. Although the results from the different studies were diverse with some studies reporting either a decrease (Spivak et al., 1989, 1993) or no difference (Sasaki et al., 1994) in complement total hemolytic activity in patients compared to control individuals, the majority of data, mainly concerning individual complement components (such as C1, C2, C3, and C4), points toward higher activity of the classical pathway in patients (Maes et al., 1997; Shcherbakova et al., 1999; Hakobyan et al., 2005; Mayilyan et al., 2006; **Figure 1**). Accordingly, increased levels of C1q attached to circulating immune complexes (C1q-CIC) and increased expression of CR1 on blood cells, which can bind to C1q-CIC and mediate clearance of immune-complexes, were found in patients with schizophrenia (Arakelyan et al., 2011; **Figure 1**). Also, increased circulating C1q levels were observed in mothers of infants who later developed schizophrenia, which suggest that exposure of the fetal brain to maternally derived C1q might be a contributory factor for the disease (Severance et al., 2014), as suggested by rodent models of MIA. In addition, it is noteworthy that decreased expression of *CSMD1*, which codes for an inhibitor of the classical complement pathway, was observed in serum from patients with schizophrenia (Liu et al., 2019).

Curiously, a negative correlation was recently found between superior frontal cortical thickness and the expression levels of *C5* and *SERPING1* genes in peripheral blood mononuclear cells from a sample of adult Swedish twins enriched for schizophrenia patients, who are known to show reduced cortical gray matter

thickness (Allswede et al., 2018). Whereas C5 is an activator of the complement system, *SERPING1* codes for a protease involved in the inhibition of the classical and lectin complement cascades, and its increased expression could reflect a compensatory mechanism for higher complement activity. Although this finding cannot establish causality and further studies are clearly needed, it is tempting to speculate that the relationship between enhanced expression of these specific complement components in the blood and cortical thickness may represent a trace of earlier immune dysregulation during development.

While several lines of evidence support the involvement of the classical complement pathway in schizophrenia susceptibility, only a few studies have addressed specifically the role of the lectin and alternative pathways in the disease. Data on the involvement of the lectin cascade show higher MBL-bound MASP2 activity in serum from individuals with schizophrenia, suggesting increased activity of this pathway in the disorder (Mayilyan et al., 2006; **Figure 1**). On the other hand, conflicting results exist regarding the involvement of the alternative cascade, because a hemolysis-based assay indicated an upregulation of the alternative pathway (Boyajyan et al., 2010), while an ELISA-based assay identified suppression of the same cascade in the peripheral blood from patients with schizophrenia (Li et al., 2012). Therefore, further studies are required to confirm whether dysfunction of lectin and alternative complement cascades contribute somehow to schizophrenia.

As mentioned above, all of the complement pathways converge at the point of C3 and data related to C3 levels in blood from schizophrenia patients are also controversial. Some studies found no alteration (Spivak et al., 1993) or increased levels of C3 in patients compared to controls (Maes et al., 1997; Shcherbakova et al., 1999; Hakobyan et al., 2005; Boyajyan et al., 2010), while others found a decrease and suggest a negative correlation between the serum levels of C3 and the severity of the symptoms (Wong et al., 1996; Li et al., 2016; **Figure 1**). The reasons for these differences are unknown, but may include either population-specific genetics and environmental risk factors or the stage of illness (acute vs. chronic). Thus, additional studies using larger groups are needed to elucidate the precise nature of the relationship between C3 levels and schizophrenia susceptibility.

Genetic studies evaluating the association between complement gene polymorphisms and schizophrenia have also been conducted. Initial studies have yielded unreliable or conflicting results due to lack of replication or small sample sizes (Rudduck et al., 1985; Fananas et al., 1992; Wang et al., 1992; Schroers et al., 1997; Mayilyan et al., 2008; Zakharyan et al., 2011). However, more recent large-scale genome-wide association studies evaluating very large sample collections for hundreds of thousands of single-nucleotide polymorphisms (SNPs) have shown variants significantly associated with higher risk of schizophrenia at *CSMD1* (Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium, 2011) and *C4* genes (Sekar et al., 2016). *CSMD1* is highly expressed in the CNS (Kraus et al., 2006) and its schizophrenia risk allele was associated with poor performance on neuropsychological measures of general cognitive ability and memory function (Donohoe et al., 2013; Koiliari et al., 2014). C4 is encoded by two different genes, *C4A* and *C4B*, which vary in structure and copy number leading to a wide range of expression levels of each isotype. Interestingly, the strongest association with schizophrenia was found with alleles that increase expression of *C4A* (Sekar et al., 2016). Accordingly, higher *C4A* expression was observed in brain samples from patients with schizophrenia compared to controls (Sekar et al., 2016) and a positive correlation was found between the copy numbers of *C4* and neuropil contraction in different brain regions in patients (Prasad et al., 2018; **Figure 1**), which strongly suggest that increased C4 levels constitute a risk factor for schizophrenia.

Whereas the abnormal complement expression in the peripheral blood of both patients with schizophrenia and their mothers suggests that complement dysfunction as part of standard immune pathways may contribute to schizophrenia in a subset of patients, findings from several of the abovementioned studies also suggest that the clinical and neuropathological phenotypes of schizophrenia may also be, at least in part, due to dysfunction of locally synthesized complement in the brain during specific periods of neural development. The strong association between increased *C4A* expression with schizophrenia (Sekar et al., 2016), the involvement of classical complement cascade in synapse elimination (Stevens et al., 2007; Schafer et al., 2012; Bialas and Stevens, 2013; Sekar et al., 2016; Comer et al., 2019), and the decreased brain connectivity and sociability in mice overexpressing *C4* in the prefrontal cortex (Comer et al., 2019) strongly suggest that enhanced complement-mediated synaptic pruning contributes directly to reduction in cortical gray matter thickness and to schizophrenia pathogenesis. On the other hand, while it is highly attractive to speculate that complement-mediated dysregulation in neuronal migration may contribute to the pathogenesis of schizophrenia, further detailed studies are still required to directly establish a causal link.

Complement in Autism Spectrum Disorder and in Rett Syndrome

Autism spectrum disorder (MIM 209850) comprises a heterogeneous group of early onset neurodevelopmental diseases characterized by impairments in social-communicative skills and repetitive behaviors (APA, 2013) that affects at least 1.5% of the population worldwide (Christensen et al., 2016). The most consistent neuropathological findings in patients with ASD include increased cortical surface area during early childhood (Miles et al., 2000; Hazlett et al., 2011) and reduced number of Purkinje cells in the cerebellum (Allen, 2005) possibly due to abnormal progenitor cell neurogenesis, altered cortical organization (presence of heterotopias and more frequent and narrower minicolumns) suggestive of abnormal neuronal migration (Casanova et al., 2002; Whitney et al., 2008; Stoner et al., 2014), and increased cortical dendritic spine densities possibly due to defective synapse elimination during brain development (Hutsler and Zhang, 2010; Tang et al., 2014). Recent molecular genetic studies have identified a specific cause for ASD in almost 30% of the cases, while in the remaining cases the underlying pathogenic mechanisms may involve complex genetic and environmental risk factors (Bourgeron, 2015).

Preliminary evidence suggests a possible role for the complement system in the pathogenesis of ASD.

Genetic association studies have reported that the complement *C4B* gene null allele has increased frequency in patients with ASD compared to control individuals (Warren et al., 1991; Odell et al., 2005; Mostafa and Shehab, 2010) and, accordingly, a significant decrease in the plasma levels of C4B protein was observed in ASD patients (Warren et al., 1994; **Figure 1**). On the other hand, proteomic analyses have suggested that the levels of other complement system proteins, such as C1q, C3, and C5, are elevated in plasma from patients with ASD (Corbett et al., 2007; Shen et al., 2018; **Figure 1**). In addition, a significantly increased activity of CFI, a negative regulatory component of the alternative pathway responsible for cleavage and inactivation of C3b and C4b, has been observed in plasma from ASD patients (Momeni et al., 2012).

The expression of complement system components in brain tissues from patients with ASD was also investigated and some conflicting results were obtained. While a genome-wide DNA methylation profiling of prefrontal cortex (Brodmann areas BA10 and BA24) has shown hypomethylation and, consequently, overexpression of *C1q*, *C3,* and *CR3* genes in the ASD brains (Nardone et al., 2014), a more recent study found decreased mRNA levels of *C1q*, *C3,* and *C4*, and increased mRNA levels of *C2*, *C5,* and *MASP1* in the middle frontal gyrus from patients compared to controls (Fagan et al., 2017; **Figure 1**). The discrepancies obtained in the expression of *C1q* and *C3* genes in brain tissues from patients with ASD could be due to the analysis of different brain regions and/or analysis of different subgroups of patients combined with small sample sizes and need to be clarified in further studies.

Collectively, the evidence regarding association between complement dysfunction and ASD is far weaker than the evidence of complement dysregulation in schizophrenia susceptibility, and a recent large-scale genome-wide association study did not report common variants in complement genes significantly associated with ASD (Grove et al., 2019). However, the altered complement system expression in peripheral blood and in brain from patients with ASD might suggest that an aberrant activity of this system may somehow contribute to ASD. Based on decreased expression of some complement components in post-mortem brain of ASD patients (Fagan et al., 2017), the increased number of dendritic spines and glutamate synapses in ASD brains (Hutsler and Zhang, 2010; Tang et al., 2014) as well as in *C1q*- and *C3*-deficient mouse brains (Chu et al., 2010; Perez-Alcazar et al., 2014), the reduced elimination of retinogeniculate synapses in mice lacking *C4* (Sekar et al., 2016) and the ASD-like phenotypes observed in *C3*-deficient mice (Perez-Alcazar et al., 2014; Fagan et al., 2017), it is tempting to speculate that diminished complement-mediated synaptic pruning, among other known mechanisms (Tang et al., 2014), may contribute to the cortical hyperconnectivity and behavioral phenotypes in ASD. Nevertheless, additional studies are clearly necessary to further explore the possible role of complement dysregulation in key aspects of ASD neuropathology.

Finally, it is noteworthy that, as occurs with schizophrenia and ASD, immune dysregulation may also contribute to some of the neuropathological features of Rett syndrome (RTT; MIM 312750) (Cortelazzo et al., 2014; De Felice et al., 2014; O'Driscoll et al., 2015), an X-linked progressive neurodevelopmental disorder primarily affecting girls at a frequency of 1:10,000 live female births. Although RTT is not classified as an ASD and is recognized as a distinct pathological entity (APA, 2013) (DSM-5), RTT may show overlapping symptoms with ASD, and is characterized by typical early development until age 6–18 months followed by a rapid deceleration in growth associated with progressive loss of acquired motor and language skills, severe cognitive impairment, intractable seizures, spasticity and stereotypic hand movements (Chahrour and Zoghbi, 2007). More than 95% of classic RTT cases are caused by sporadic loss-of-function mutations in the gene encoding MECP2 (Amir et al., 1999), a transcriptional regulator of gene expression that acts through epigenetic mechanisms on chromatin structure (Nan et al., 1998; Bienvenu and Chelly, 2006). Interestingly, a proteomic study has shown that the levels of several proteins involved in the immune system are altered in plasma from patients with RTT, including overexpression of complement factor B of the alternative complement pathway (Cortelazzo et al., 2014). Also, a recent genome-wide transcriptome (RNA-seq) analysis of post-mortem brain samples (from both frontal and temporal cortex) from young RTT patients showed that all three genes encoding complement C1q complex (*C1QA*, *C1QB*, *C1QC*) were downregulated in RTT human brains as they are in *Mecp2* knockout mice (Lin et al., 2016), which suggest that the expression of these genes is regulated by MECP2. Although the involvement of C1q in RTT pathogenesis has not yet been clarified and additional studies are required, the role of C1q in regulating dendritic spine density (Chu et al., 2010; Perez-Alcazar et al., 2014) may contribute somehow to RTT.

Complement in 3MC Syndrome

3MC syndrome (MIM 257920; 265050; 248340) comprises a rare developmental disorder that unifies four autosomal recessive diseases with overlapping features previously known as Mingarelli, Malpeuch, Michels, and Carnevale syndromes (Titomanlio et al., 2005). This syndrome is characterized by a spectrum of developmental anomalies that include facial dysmorphism, cleft lip and/or palate, postnatal growth deficiency, learning disability and hearing impairment. Less often, individuals with 3MC syndrome may also show craniosynostosis, genital, limb, and vesicorenal anomalies (Titomanlio et al., 2005; Leal et al., 2008).

Several different potentially deleterious mutations in genes of the lectin arm of the complement system - such as *MASP1*, *COLEC10,* and *COLEC11* genes – have been described in patients with 3MC syndrome (Sirmaci et al., 2010; Rooryck et al., 2011; Atik et al., 2015; Urquhart et al., 2016; Gardner et al., 2017; Munye et al., 2017; Graul-Neumann et al., 2018; Basdemirci et al., 2019). It has recently been shown that disease-associated mutations in *COLEC10* and *COLEC11* inhibit either production or secretion of encoded proteins in mammalian cells (Rooryck et al., 2011; Venkatraman Girija et al., 2015), which may explain the undetectable levels of CL-K1 in the serum of affected individuals (Rooryck et al., 2011).

However, it is noteworthy that although at least some of these mutations impair the normal function of the encoded proteins, normal levels of downstream reaction cascade components (C2, C3, and C4) were found in serum from patients, suggesting a possible direct role of these lectin cascade proteins in 3MC syndrome pathogenesis independently of standard lectin pathway activation (Rooryck et al., 2011).

Studies using zebrafish (Rooryck et al., 2011) and an *in vitro* cell model (Munye et al., 2017) have shown that these components of the lectin pathway act as chemoattractants to guide cell migration and suggest that the craniofacial abnormalities in 3MC syndrome are the result of deficient migration of neural crest cells during development (Rooryck et al., 2011). Also, the involvement of components of the lectin pathway in regulating neuronal migration in the developing cerebral cortex (Gorelik et al., 2017a) also suggests that the cognitive impairment observed in 3MC patients may be explained in part by deficits in neuronal migration. Further studies, however, are necessary to further understand the mechanisms by which dysfunctional *MASP1*, *COLEC10,* and *COLEC11* genes may lead to 3MC syndrome.

CONCLUDING REMARKS

Here, we have highlighted the emerging functions of the complement system as a key regulator of normal CNS development. Components of lectin and terminal complement pathways, mainly the C3a and C5a anaphylatoxins, have been shown to regulate neural progenitor cell proliferation, neurogenesis and neuronal migration. Components of classical and terminal complement pathways, such as C1q, C3, and C4, have been shown to tag weaker synapses for removal by microglia, which sculpts brain connectivity. Consequently, alterations in the expression of complement components in the brain may lead to long-lasting changes in brain development and function. The severe autosomal recessive 3MC syndrome originates from rare pathogenic mutations in genes of the lectin pathway that regulate cell migration during embryonic development. In both schizophrenia and ASD, evidence is growing that abnormal complement signaling owing to genetic mutations or as a result of inflammatory insults during prenatal or early postnatal development may lead to changes in brain connectivity and may contribute to disease pathophysiology. Although research into the molecular mechanisms downstream from complement components has just begun to be explored, the progress in this field holds tremendous promise not only for increasing our understanding of neurodevelopment, but also for elucidating and potentially even treating neurodevelopmental disorders.

AUTHOR CONTRIBUTIONS

All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Glial Cell-Axonal Growth Cone Interactions in Neurodevelopment and Regeneration

Michael J. Rigby[1,2,3], *Timothy M. Gomez*[2,4] *and Luigi Puglielli*[1,2,3,5]*

[1] *Department of Medicine, University of Wisconsin-Madison, Madison, WI, United States,* [2] *Neuroscience Training Program, University of Wisconsin-Madison, Madison, WI, United States,* [3] *Waisman Center, University of Wisconsin-Madison, Madison, WI, United States,* [4] *Department of Neuroscience, University of Wisconsin-Madison, Madison, WI, United States,* [5] *Geriatric Research Education Clinical Center, Veterans Affairs Medical Center, Madison, WI, United States*

The developing nervous system is a complex yet organized system of neurons, glial support cells, and extracellular matrix that arranges into an elegant, highly structured network. The extracellular and intracellular events that guide axons to their target locations have been well characterized in many regions of the developing nervous system. However, despite extensive work, we have a poor understanding of how axonal growth cones interact with surrounding glial cells to regulate network assembly. Glia-to-growth cone communication is either direct through cellular contacts or indirect through modulation of the local microenvironment via the secretion of factors or signaling molecules. Microglia, oligodendrocytes, astrocytes, Schwann cells, neural progenitor cells, and olfactory ensheathing cells have all been demonstrated to directly impact axon growth and guidance. Expanding our understanding of how different glial cell types directly interact with growing axons throughout neurodevelopment will inform basic and clinical neuroscientists. For example, identifying the key cellular players beyond the axonal growth cone itself may provide translational clues to develop therapeutic interventions to modulate neuron growth during development or regeneration following injury. This review will provide an overview of the current knowledge about glial involvement in development of the nervous system, specifically focusing on how glia directly interact with growing and maturing axons to influence neuronal connectivity. This focus will be applied to the clinically-relevant field of regeneration following spinal cord injury, highlighting how a better understanding of the roles of glia in neurodevelopment can inform strategies to improve axon regeneration after injury.

Keywords: glia, growth cone, axon, neurodevelopment, cell-cell interaction, spinal cord injury

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Brian Key, University of Queensland, Australia

***Correspondence:**
Luigi Puglielli
lp1@medicine.wisc.edu

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INTRODUCTION

The developing nervous system is a complex *milieu* of neurons, glial support cells, extracellular matrix, and budding vasculature that elegantly organizes into a highly stereotyped structure. Combinatorial actions of many well-characterized extracellular and intracellular events guide axons to their target locations, which is heavily influenced by tissue mechanics, bound and soluble secreted chemical factors, and cell-cell interactions. The interactions between axonal growth cones and surrounding cells within the developing nervous system is an important component of

neurodevelopmental biology but is often not well characterized due to the challenges with observing these transient cellular interactions *in vivo*.

Glial cells in the developing nervous system have many supportive physiological functions, like maintaining solute and nutrient homeostasis, as well as migratory and synaptic development and maintenance roles, such as assisting neurons in reaching their target locations (Chotard and Salecker, 2004; Chao et al., 2009). Glial cells regulate axon growth cone pathfinding, such as acting as guidepost cells along migratory routes creating cellular boundaries as "no-go" zones; they can also assist in proper targeting of axon terminals by serving as transient synaptic partners. Glia have the ability to either directly interact with growing axons through cell adhesion or indirectly by secreting factors that modulate the local microenvironment to promote or inhibit axon growth. Many glial subtypes have been demonstrated to directly impact axon growth and guidance including microglia (Reemst et al., 2016), oligodendrocytes (Chen et al., 2002a; Gang et al., 2015), astrocytes (Cavalcante et al., 2002; Liu R. et al., 2015), Schwann cells (Thompson and Buettner, 2006; De Luca et al., 2015), neural progenitor cells (Merianda et al., 2017), and olfactory ensheathing cells (Windus et al., 2010), and they can either promote or inhibit growth depending on the circumstance. Expanding the knowledge of how different supporting cell types may directly or indirectly interact with growing axons will offer a deeper understanding of the intercellular crosstalk occurring in neurodevelopment, as well as provide clinically useful information, such as identifying potential drugs to modulate neuron regeneration (De Luca et al., 2015; Gang et al., 2015).

This review will provide a brief overview of the current knowledge about glial cell contributions to axon growth and guidance, specifically focusing on how glial cells directly interact with growing axons to influence neuronal connectivity. A selection of examples where glial cell-axonal growth cone interactions are shown to play a crucial role during neurodevelopment will be critically discussed. This basic science background is then linked with translational work targeting glia to promote regeneration following spinal cord injury, highlighting how a better understanding of the role of glia in neurodevelopment can inform strategies to improve axon regeneration after injury.

NEURON AND GLIA DEVELOPMENT: RIGHT PLACE AT THE RIGHT TIME

Glial cell development in the central nervous system (CNS) and peripheral nervous system (PNS) often occurs alongside neuron development and maturation as many glial subtypes originate from a common precursor stem cell (Cameron and Rakic, 1991; Lee et al., 2000; Pinto and Gotz, 2007; Grabel, 2012). Therefore, many glial cell subtypes are present at the right time and place to directly impact axon growth and guidance.

Microglia are the only glial cell to enter the CNS from the periphery, doing so well before CNS-resident glia differentiate (Reemst et al., 2016). Unlike other glial cell types, microglia possess a unique origin, the yolk sac (Samokhvalov et al., 2007; Prinz and Priller, 2014), which in mice can be detected as early as embryonic day 7.5 (E7.5) (Kierdorf et al., 2013) and invade the CNS beginning around E8-9 using specific matrix metalloproteinases (Alliot et al., 1999; Kierdorf et al., 2013). Colonization of the brain has been observed to occur in two waves with the first being at E8-9 and second at E14-16; both of these events are independent of the vascular system as the cells enter via the meninges or from the ventricles, thus invading the brain parenchyma from both superficial and deep layers (Reemst et al., 2016). In contrast to the adult brain, microglia in the embryonic brain tend to cluster near developing axons (Reemst et al., 2016), such as around the axonal tracts of the subpallium at E14.5 (Squarzoni et al., 2014) and corpus callosum at E15.5-17.5 (Pont-Lezica et al., 2014). This pattern continues in postnatal development with microglia associating with subcerebral, callosal, and hippocampal perforant path-projecting axons (Dalmau et al., 1998; Rochefort et al., 2002; Ishii et al., 2013). The close association between microglia and developing white matter tracts suggests a role in axon growth, guidance, and/or survival during CNS development, which is further strengthened by the accumulating data from studying CNS injury and regeneration.

Astrocytes and oligodendrocytes, the macroglia in the CNS, originate from a common precursor, the radial glial (RG) cell (Cameron and Rakic, 1991; Lee et al., 2000; Grabel, 2012). RG cells appear around E9-10 in mice marking the beginning of neurogenesis, followed by gliogenesis. Derived from neuroepithelial cells, RG cells span the neural tube in the brain and spinal cord with their apical endfeet on the ventricular surface and a single radial process that contacts the basal pial surface. This dynamic cell type undergoes a series of symmetric or asymmetric divisions that either self-renew or begin producing committed postmitotic neurons or glial daughter cells (Huttner and Kosodo, 2005). In the cortex, postmitotic cells migrate toward the pial surface along the radial process to complete differentiation at the appropriate layer (Grabel, 2012), and this glial-guided neural migration is dependent on gap junction adhesions (Elias et al., 2007). Oligodendrocyte precursor cells (OPCs) develop primarily in the ventral neural tube, migrate laterally and dorsally to their proper locations, and continue to differentiate and change morphologically to begin the myelination process (Lee et al., 2000). In the adult brain, oligodendrocytes can also be derived from parenchymal oligodendrocyte progenitor cells as well as adult neural precursor cells from the subventricular zone following a demyelinating disease (Xing et al., 2014). Astrocytes develop later than oligodendrocytes and are primarily born in the dorsal neural tube; they populate developing white and gray matter and serve a myriad of functions including maintenance of solute homeostasis, axon guidance, and synaptic formation (Mason et al., 1988; Giaume and Venance, 1998; Bacci et al., 1999; Cavalcante et al., 2002; Liu R. et al., 2015). Additionally, astrocytes are classified into two subtypes: (1) fibrous astrocytes within white matter and (2) protoplasmic astrocytes within gray matter (Kimelberg, 2010); recent studies have demonstrated differences in their propensity to promote neurite growth (Liu

R. et al., 2015), which is discussed in greater detail below. It is important to note that there are well-documented differences in radial glial development in the cortex versus spinal cord. For example, radial glia are vital in regulating vascular patterning within the spinal cord (Matsuoka et al., 2016), and astrocytes derived from regionally-distinct sites exhibit unique molecular signatures (Bachoo et al., 2004; Yoon et al., 2017; Bradley et al., 2019). What remains to be fully elucidated is how knowledge of these differences can be utilized for region-specific intervention, such as aiding in regeneration in the cortex versus spinal cord.

In the PNS, glial cells are derived from neural crest cells that differentiate while migrating to their final destination (Jessen and Mirsky, 2005). These migrating neural crest cells form Schwann cell precursors (SCPs) and then immature Schwann cells that begin to associate with axons; an additional branching lineage includes the formation of satellite cells that eventually associate with peripheral ganglia (Le Douarin and Ziller, 1993). The eventual fate of immature Schwann cells is determined by the type of axons they associate with, directing them to become non-myelinating or myelinating Schwann cells. Interestingly, even though SCPs are present during times of perfuse axon extension and development, they are not required for the axons to reach their target location (Grim et al., 1992; Sepp et al., 2001). Nonetheless, several studies have shown Schwann cells can impact axon outgrowth and guidance, which is especially relevant in PNS injury and repair (Thompson and Buettner, 2006; De Luca et al., 2015).

Finally, olfactory ensheathing cells (OECs) are a unique population of Schwann cells that facilitate the replenishment of olfactory neurons (Farbman and Squinto, 1985; Chuah and Au, 1991; Barnett and Riddell, 2004; Windus et al., 2010). This unusual PNS-CNS connection involves the invasion of peripheral olfactory receptor neurons, which originate from the basal stem cells of the olfactory epithelium, into the cribriform plate and olfactory bulb to form synapses with second-order neurons in the glomerular layer (Barnett and Riddell, 2004). OECs are derived from precursor cells within the olfactory epithelium and closely associate with growing axons (Chuah and Au, 1991). Interestingly, the olfactory receptor neurons are continually turning over so new olfactory receptor neurons must be replenished throughout life. The OEC Schwann cells provide permissive substrata for the migration of new olfactory receptor neurons into the olfactory epithelium where new synapses form throughout life (discussed further below).

GLIAL CELL-AXONAL GROWTH CONE INTERACTIONS: A SELECTION OF EXAMPLES

Some of the earliest work studying the cellular events that underlie neurodevelopment established the importance of glia in the growth and guidance of migrating neurons and axons. For example, the first axons to cross the corpus callosum in the developing mouse brain cross a cellular "sling" made up of primitive glial cells suspended below the longitudinal cerebral fissure, which disappears after birth (Silver et al., 1982). These commissural axons also avoid regions containing glial cells, such as the "glial wedge" that express inhibitory axon guidance cues (Shu and Richards, 2001). On the other hand, migrating granule cells in the developing mouse cerebellum follow along vertically oriented Bergmann fibers arising from Golgi epithelial cells, a protoplasmic astrocyte (Rakic, 1971). During *Drosophila* early embryogenesis, three classes of glial cells form an organized pattern at each body segment before axon outgrowth occurs, and these cells enwrap the axon tracts as they migrate (Jacobs and Goodman, 1989). Importantly, loss of peripheral glia in *Drosophila* results in sensory axon stalling and pathfinding defects as they migrate toward the CNS, as well as early migration defects in pioneer motor axons as they cross the CNS/PNS transition zone (Sepp et al., 2001). Although these initial studies relied heavily on fixed sample imaging that provided authors only a static view of specific time points, they provided much of the foundational observations to influence future studies examining the dynamic interface between glia and growing axons. A focused view on specific glial subtypes will be discussed citing important events in specific regions of the CNS and PNS during development (see **Figure 1** for a summary).

Astrocyte-Axonal Growth Cone Interactions

Astrocytes can form a variety of cellular processes that directly interact with growing axons. *In vitro*, both astrocytes and granule neurons form plasma membrane "microspikes" that continually protrude and retract; it is only after contact is made between the astrocyte and granule neuron that the granule neuron microspikes are stabilized, promoting the formation of a neurite that grows over the cell body of the astrocyte (Mason et al., 1988). Using electron microscopy (EM), small, adherent junctions were observed at contact points between the astrocytes and granule neurons. Functional studies demonstrated that these interactions depend on neural cell adhesion molecule (NCAM), N-cadherin, and integrin β1, as blocking the astrocyte-neuron interactions via antisera against these proteins resulted in reduction or elimination of neurite outgrowth onto cultured astrocytes (Keilhauer et al., 1985; Neugebauer et al., 1988; Tomaselli et al., 1988). Interestingly, integrin β1 antisera only blocked E8 but not E14 chick ciliary ganglion neuron outgrowth on rat cortical astrocytes (Tomaselli et al., 1988), unlike chick retinal neurons, which were not affected by age (Neugebauer et al., 1988). Additionally, blocking NCAM also had neuron-type dependent effects: NCAM antisera impacted cerebellar neuron-astrocyte interactions (Keilhauer et al., 1985) and E11 chick retinal neurons interactions with rat cortical astrocytes (Neugebauer et al., 1988), but not chick ciliary ganglion neuron interactions with rat cortical astrocytes (Tomaselli et al., 1988) nor E8 chick retinal neuron interactions with rat cortical astrocytes (Neugebauer et al., 1988). Finally, inhibition of N-cadherin consistently blocked astrocyte-neuron interactions regardless of neuronal type and developmental time point (Keilhauer et al., 1985; Neugebauer et al., 1988; Tomaselli et al., 1988). Together

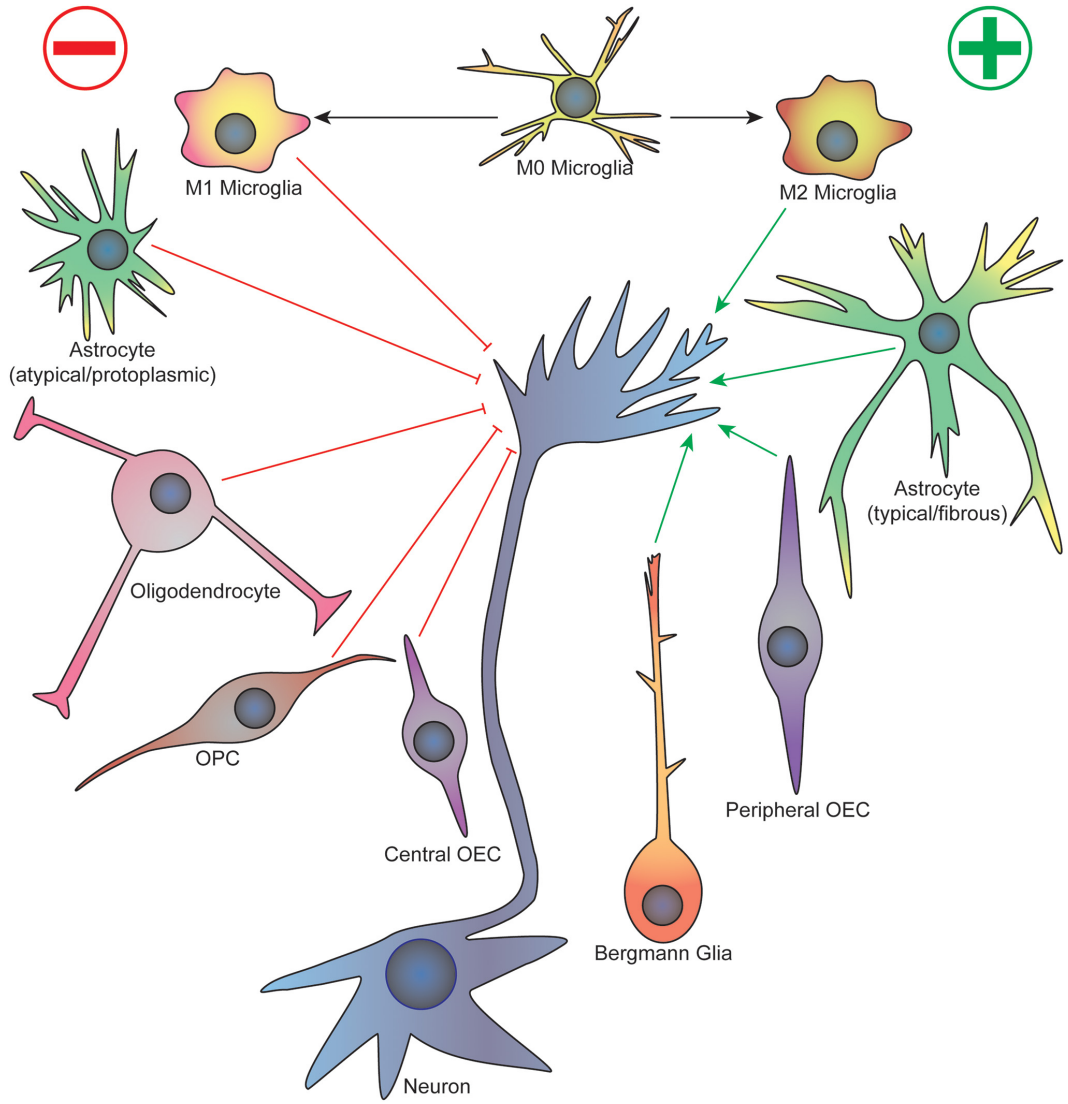

FIGURE 1 | Summary of glial cell-axonal growth cone interactions during neurodevelopment and regeneration. Green arrows represent attractive guidance cues while red represent repellent. See text for description. OPC, oligodendrocyte precursor cell; OEC, olfactory ensheathing cell.

these results suggest a heterogeneity in the molecules used for neuron-glia interactions, with either a neuron type-specific or time-dependent switch over critical time periods.

While heterogeneity in neuronal populations is expected, distinct astrocyte populations also appear to have differential effects on axon development. Liu R. et al. (2015) characterized the development of two astrocyte subpopulations termed "typical" and "atypical" that spontaneously develop when rat primary cortical glial cells were grown *in vitro*. The typical astrocytes were the majority (∼70%) of the astrocytes in the culture, which exhibited a variety of shapes and arrangements, and often co-localized with oligodendrocytes. The atypical astrocytes exhibited a spindle shape with a high cell density arranged in a polarized fashion and covered by fewer oligodendrocytes. When dorsal root ganglia (DRG) neurons were plated on top of this heterogeneous astrocyte population, the typical astrocytes promoted neuron adhesions and neurite growth consistent with prior studies (Keilhauer et al., 1985; Mason et al., 1988; Tomaselli et al., 1988). In contrast, the atypical astrocytes inhibited neuron adhesion and neurite outgrowth without impacting neuron survival. It is plausible that these two astrocyte phenotypes represent the natural heterogeneity of these glial cells in the CNS rather than an artifact of the *in vitro* methods. The atypical astrocytes may form inhibitor barriers in the developing CNS (e.g., glial wedge) and may be related to damaged or reactive astrocytes that have a well-characterized inhibitory effect on neurite growth both *in vitro* and *in vivo* (McKeon et al., 1991, 1999; Wanner et al., 2008). Liu et al. attempted to connect these *in vitro* results to an *in vivo* model by transplanting DRG neurons into either cortical gray matter or corpus callosum white matter (Liu R. et al., 2015).

They observed little neurite growth in the cortical gray matter location but robust neurite growth in the corpus callosum. They drew the conclusion that the fibrous astrocytes, which are found within the white matter, are supportive of neurite growth while protoplasmic astrocytes, the subtype found within gray matter, are not. However, since this *in vivo* experimental system does not exclude the influence of all the other differences that exist between the gray and white matter microenvironments, the effects observed on the neurite growth may be completely independent of the astrocytes within the tissue. Furthermore, the results of enhanced neurite growth in the corpus callosum are counterintuitive considering that white matter can have a high content of myelin, which is known to be repulsive to axon growth (discussed below). Clearly an important control experiment is to determine if these findings are reproducible in a rodent model with selective astrocyte ablation, which has been generated in other laboratories (Delaney et al., 1996; Sofroniew et al., 1999; Cui et al., 2001). Nonetheless, follow-up studies to examine the differences between typical and atypical astrocytes *in vitro* are warranted and should be more robustly compared to the fibrous and protoplasmic astrocytes observed *in vivo* using modern molecular techniques, such as single cell expression analysis. These data may provide candidate targets to reprogram inhibitory astrocytes to promote axon growth, which is a highly desired outcome after injury.

Astrocytes also play an active role in assembling connections of GABAergic stellate interneurons within the developing cerebellum (Ango et al., 2008; Chao et al., 2009). Bergmann glia (BG) cells, which are highly polarized astrocytes within the cerebellum, form an elaborate arborization of apical fibers that extend into the cerebellar cortex early in the postnatal brain. The stellate interneurons that reside within the upper half of the cerebellar molecular layer innervate the dendritic shafts of the Purkinje cells that reside below, and they do so by following the BG radial projections. Ango et al. observed strong expression of Close Homolog of L1 (CHL1), a member of the L1 immunoglobulin cell adhesion molecule (L1CAM) family of proteins, within both the BG apical fibers and stellate interneurons during postnatal development. Furthermore, global or BG-specific CHL1 knockout resulted in aberrant growth of stellate interneuron axons with reduced synapse formation on target Purkinje cells. The authors proposed that the disruption in stellate interneuron synapse formation on Purkinje cells may explain the poor motor performance of Chl1$^{-/-}$ mice in the Rotarod test (Pratte et al., 2003). Further probing into the electrophysiologic circuitry aberrations and functional motor differences in Chl1$^{-/-}$ mice would be an interesting follow-up study to solidify the impact of disrupting BG-mediated stellate interneuron axon migration in the cerebellum. However, another research group did not detect changes in the stellate interneuron population within the cerebellum in Chl1$^{-/-}$ mice, rather they observed a decrease in Purkinje cell number, complicating the story (Jakovcevski et al., 2009). Despite loss of Purkinje neurons, Jakovcevski et al., as well as other groups, did not detect motor deficits in Chl1$^{-/-}$ mice (Montag-Sallaz et al., 2002; Jakovcevski et al., 2007; Morellini et al., 2007), reaching the conclusion that cerebellar function is grossly preserved in these mice. These studies suggest that more refined electrophysiological measurements, as well as fine motor tasks and non-motor assessments, should be performed on Chl1$^{-/-}$ mice. While the necessity of stellate interneuron axon guidance on specific adhesion molecules is unclear at this point, it is clear that interactions between astroglia and axon growth cones are important for proper synapse formation within the cerebellum.

Oligodendrocyte-Axonal Growth Cone Interactions

Oligodendrocytes and oligodendrocyte precursor cells (OPCs) are notorious for their inhibitory effect on axon growth and guidance (Caroni and Schwab, 1988; Fawcett et al., 1989; Bandtlow et al., 1990; McKerracher et al., 1994; GrandPre et al., 2000; Chen et al., 2002a,b; Kottis et al., 2002; Wang et al., 2002; Moreau-Fauvarque et al., 2003; Vourc'h et al., 2003; Goldberg et al., 2004; Pasterkamp and Verhaagen, 2006; Shim et al., 2012; Gang et al., 2015). Early studies examining DRG axon growth in an oligodendrocyte co-culture system revealed that axons avoided growing upon oligodendrocytes unlike other glial cells such as astrocytes. Furthermore, live imaging revealed axonal growth cone stalling and collapse upon membrane contacts with oligodendrocytes (Fawcett et al., 1989; Bandtlow et al., 1990). A number of secreted and membrane proteins produced by oligodendrocytes that are in part responsible for this inhibition include Nogo (GrandPre et al., 2000), myelin-associated glycoprotein (MAG) (McKerracher et al., 1994), oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002), and the semaphorins Sema4D (Moreau-Fauvarque et al., 2003), Sema5A (Goldberg et al., 2004), and Sema6A (Shim et al., 2012). Nogo, MAG, and OMgp comprise the Nogo receptor (NgR) ligand family, which are also referred to as myelin-associated inhibitors (MAIs) that stabilize neuronal structure (Vourc'h and Andres, 2004; Schmandke et al., 2007). Interestingly, expression of many of these inhibitory molecules increases after injury, suggesting that blocking these key targets would improve axon regeneration (Pasterkamp and Verhaagen, 2006). MAIs serve seemingly redundant inhibitory activities toward axon extension, but also exhibit some key differences in function that may be important in neurodevelopment. For example, MAG does not always act as an inhibitor of axon growth as the case when applied to newborn DRG neurons (Mukhopadhyay et al., 1994). Furthermore, myelin preparations from MAG knockout mice do not inhibit neurite elongation or cause growth cone collapse compared to myelin preparations from wild type mice (Bartsch et al., 1995). Additionally, myelin preparations from adult rats actually stimulated axon growth of rodent neural progenitor cells and human induced pluripotent stem cell-derived neural stem cells, which was dependent on interactions with neuronal growth regulator 1 (Negr1) (Poplawski et al., 2018). Therefore, highlighting these differences in MAIs and downstream mediators are important when designing translational neural regeneration applications.

While it is clear that oligodendrocytes function within the adult and injured CNS, there is limited evidence for roles of oligodendrocyte-expressed inhibitors during neurodevelopment while nascent axonal growth cones are searching for their targets. *In vitro* studies on Nogo ligands suggest these inhibitory molecules expressed on oligodendrocytes and myelin may serve to prevent axon sprouting in the adult CNS (Vourc'h and Andres, 2004). Moreover, the abundance of myelin in white matter may be one explanation for its low structural plasticity in contrast to gray matter, as regions of high plasticity tend to have low myelin content (Silver et al., 2015). It is important to note that the developmental time point when myelin becomes abundant is much later than the appearance of nascent axon growth cones; thus, it is unlikely that MAIs play a major role in shaping neuronal networks (McKerracher and Rosen, 2015). Nonetheless, there are numerous studies examining the impact of several inhibitory molecules, especially the semaphorins, on various neurodevelopmental events (Iketani et al., 2016; Wang L. et al., 2017), but these are outside the scope of this review.

Of interest in this review is the neural/glial antigen 2 (NG2) integral membrane proteoglycan expressed by OPCs during neurodevelopment (Chen et al., 2002a). NG2 belongs to the family of chondroitin sulfate proteoglycans (CSPGs) that have a well-known inhibitory effect on axon growth, especially within glial scars. For example, acute treatment of DRG neurons with soluble NG2 induces growth cone collapse. Furthermore, rat ventral spinal cord explants cultured upon 3-dimensional collagen gels with membrane vesicles embedded from NG2-expressing HEK293 cells exhibited reduced neurite length and axon bundling when compared to control conditions (Chen et al., 2002b). Importantly, the authors found high expression of NG2 in the developing rat embryo in areas such as the notochord, perinotochord mesenchyme, lateral mesoderm, base of limb buds, and optic chiasm where segmental patterning was observed. β_{III}-tubulin-positive axon bundles were found in regions of low NG2 labeling, suggesting that these axons originally migrated through regions of low NG2 expression. Regions such as the perinotochord mesenchyme have been previously characterized as barriers to axon growth, and the authors suggest that NG2 expression in these regions may limit axon growth, forming repellent boundaries to prevent axon straying. Observing axon extension dynamics in live preparations with *in vivo* two-photon excitation microscopy would be very informative to detect cellular interactions during the development of this circuit. As NG2 can be expressed by a variety of immature cell types (Levine and Nishiyama, 1996) and pericytes (Laredo et al., 2019), this raises the possibility that many of the cells observed *in vivo* in this study are not OPCs nor oligodendrocytes. In fact, the role of NG2-positive glia continues to be heavily debated, and their influence on neurodevelopment and regeneration remains an open question (Silver et al., 2015).

Microglia-Axon Growth Cone Interactions

Microglia are CNS-resident macrophages that serve a number of important roles in regulating tissue homeostasis, namely phagocytic scavenging, localized immune function, modulation of synaptic transmission, synaptogenesis, and neurotrophic support (Reemst et al., 2016; Henstridge et al., 2019; Rotterman et al., 2019; Wilton et al., 2019). Microglia are implicated in both health and disease, gaining recent attention as a cell type that can be targeted for therapeutic purposes (Tay et al., 2017). Microglia are highly active in autoimmune and injury-related diseases, such as multiple sclerosis and spinal cord injury; thus, understanding their normal physiologic role in development and tissue homeostasis may provide clues for their role in disease states and offer potential targets for intervention.

Some of the hypothesized roles of microglia in axon growth and guidance were originally associated with their phagocytic properties including pathway clearance for developing axons, elimination of transient axonal projections, or clearance of axon growth and guidance factors. More recently, new evidence suggests that microglia may themselves secrete factors that mediate axon growth and guidance (Reemst et al., 2016). Within the mouse embryonic brain, microglia are positioned at decision points along specific axonal tracts rather than associated with vasculature, regions of cell death, or at progenitor zones, where they are typically located postnatally (Squarzoni et al., 2014). During development, microglia are observed near tyrosine hydroxylase (TH)-positive dopaminergic axons as they enter the subpallium. EM revealed that the microglia in the subpallium contained TH^+ axon fragments within their cytosol, suggesting a phagocytic role. Interestingly, microglial depletion resulted in enhanced growth of the dopaminergic axons within the subpallium, while maternal excess immune activation of microglia resulted in reduced axon growth. Similarly, microglia also play a role in localization of LIM/homeobox 6 (Lhx6)-positive interneurons, which originate in the subpallium, migrating tangentially into the neocortex and eventually migrating radially into the cortical plate. However, when microglia were depleted, Lhx6-interneurons entered prematurely into the cortical plate and had more diffuse localization in layer V, which persisted postnatally (Squarzoni et al., 2014). Furthermore, $Cx3cr^{-/-}$ mice exhibited expansion of TH^+ axons in the subpallium, as well as diffuse localization of $Lhx6^+$ interneurons within the neocortex as a result of impaired microglia-neuron communication. Taken together, these data provide convincing *in vivo* evidence that microglial activity plays an important role in limiting axon outgrowth of dopaminergic neurons within the subpallium, as well as interneuron distribution within the neocortex. Consistent with these findings, microglia also play a role in axon bundling and maturation, as microglial activation or knock-down resulted in defasciculation (Pont-Lezica et al., 2014). Transcriptome analysis revealed a down-regulation of genes involved in "nervous system development and function," such as *Sema3C*, *PlnxA2*, and *Vcan,* in both activated or defective microglia. These data demonstrate that microglia are important players in the wiring of the mouse forebrain, and both hyperactivation, as well as defective microglia, are likely detrimental to axon growth and guidance.

In the postnatal brain, microglia continue to be tightly associated with certain populations of maturing neurons. For example, in cats, microglia cluster within the white

matter beneath cortical areas A17/A18, which contain juvenile exuberant callosal projection neurons that project to the contralateral A17/A18 by crossing through the corpus callosum (Rochefort et al., 2002). With normal rearing (NR), many of these projections are eliminated, which is associated with amoeboid-like microglia consistent with phagocytically active cells. However, in cats raised with monocular deprivation (MD condition) from birth, most projections are stabilized and retained in the adult animal, and microglia exhibit more ramified morphology consistent with a resting, quiescent state (Rochefort et al., 2002). These data demonstrate that microglia function is regulated by postnatal visual experience, which indicates that there are likely close interactions between microglia and axons of the visual neurons. In addition, since microglia exhibited more phagocytic-like appearance in the condition where juvenile exuberant callosal projections are expected to degenerate, the microglia may be an important mediator of this postnatal axonal elimination. In contrast, another study demonstrated that microglia in mice are vital for the support and survival of callosal projection neurons through the secretion of the trophic factor IGF1 (Ueno et al., 2013), which would be in opposition to the conclusions drawn above. These seemingly contradictory results are common throughout studies of microglia in development and most likely represent their diverse function, as well as heterogeneity in methods and animal models employed.

Additional *in vitro* evidence demonstrates that microglial activation can be inhibitory to axon growth and guidance. Microglia activated by lipopolysaccharide (LPS) inhibited neuron outgrowth and induced growth cone collapse (Kitayama et al., 2011). Importantly, this effect was only observed when activated microglia and neurons were co-cultured in the same dish; culturing these two populations of cells in a transwell system, which prevents direct contact but allows for continuous bathing media, resulted in no changes in neurite outgrowth or growth cone collapse. This result suggests that the inhibitory effect of activated microglia on axon growth was not due to a secreted factor but rather direct contact via adhesion molecule or phagocytic interaction. The inhibitory effect was subsequently attributed to activated microglia expressing *repulsive guidance molecule a* (RGMa), a glycosylphosphatidylinositol (GPI)-linked glycoprotein that has been previously demonstrated to induce growth cone collapse of retinal axons (Monnier et al., 2002). Addition of RGMa-blocking antibodies or siRNA-mediated knockdown of RGMa in the activated microglia blocked their inhibitory effects on neurite outgrowth and growth cone collapse. Similar effects were observed when the activated microglia were treated with minocycline, a tetracycline antibiotic that was shown to decrease expression of RGMa. Taken together, these results indicate that activated microglia express RGMa and directly inhibit axon growth in a contact-dependent fashion, which is a potential molecular target to use in regeneration therapies.

Olfactory Ensheathing Cell-Axonal Growth Cone Interactions

Olfactory neurons are unique as they are continuously turned over so new axons must enter the CNS from the periphery throughout life. Olfactory neuron axons are supported by olfactory ensheathing cells (OECs) in both the periphery and within the olfactory bulb, which both aid in the growth and guidance of olfactory axons as well as surround groups of olfactory axons to enhance electrical conduction (Van Den Pol and Santarelli, 2003). Cell surface molecules on OECs promote axon growth, which may prove useful for regeneration after CNS injury. In the developing olfactory nerve, OECs pioneer the path for olfactory neuron axons, extending their cellular processes as much as 15 microns ahead of the axon growth cone, and olfactory processes never extend ahead of the OECs (Tennent and Chuah, 1996). *In vitro*, olfactory neurites prefer to grow upon OECs rather than surface polylysine, often leaving the surface completely to grow on top of the OECs (Van Den Pol and Santarelli, 2003). Interestingly, live imaging reveals that shortly after an axon growth cone contacts an OEC, the growth cone appears to become a passive partner remaining adherent to the migrating OEC. For example, if the OEC moves toward the neuron cell body, the neurite process will shorten as a result. Moreover, the attached axon growth cone follows their partner OEC even when the cell retracts to divide, after which the growth cone remains adherent to one daughter cell after cytokinesis. The adhesion molecules mediating this interaction include NCAM, polysialic acid (PSA)-NCAM, and N-cadherin that are expressed on the surface of OECs during all developmental stages (Miragall et al., 1989; Key and Akeson, 1990; Franceschini and Barnett, 1996; Fairless et al., 2005; Su and He, 2010). Studies performed *in situ* show that cerebellar granule cells seeded on the surface of olfactory mucosa and bulb slices preferentially grow within regions dense in OECs, such as the ventral nerve layer and lamina propria (Van Den Pol and Santarelli, 2003). These findings represent the preferable interaction between axons and OECs that supports neurite growth and introduces the unique concept that olfactory neuron axons may ride along OEC cell bodies as they migrate into the CNS. Furthermore, unique OEC populations are hypothesized due to the differences in anatomical and temporal development of the olfactory bulb (Windus et al., 2010). When primary olfactory neurons are co-cultured with central- and peripheral-derived OECs, axons grow in a dispersed pattern with central OECs but fasciculate upon peripheral OECs. Time lapse imaging showed that peripheral OECs preferably adhere to one another while central OECs display much more variable behavior of adhesion, repulsion, or crossover. The physiologic implication of these observed differences is interesting as it relates to the *in vivo* organization of the olfactory system. In the periphery, as the olfactory axons leave the olfactory epithelium, they form fascicles as they merge into the olfactory nerve mediated by OECs, which is mirrored by their behavior *in vitro*. Once in the CNS, the axons defasciculate and sort themselves dependent on their odorant receptor expression. Interestingly, the effects of central OECs *in vitro* suggest that these OECs may promote or even guide this defasciculation process. Semaphorin3A (Sema3A), a membrane-bound, cleavable chemorepellent found on the OEC cell surface, was found to mediate this defasciculating and sorting process as olfactory neuron axons avoided regions with high Sema3A expression. Furthermore, Sema3A-deficient

mice exhibited defects in olfactory neuron axon sorting within the olfactory nerve layer, which persisted into postnatal life (Schwarting et al., 2000). Overall, the relationship between OECs and olfactory neurons is an elegant demonstration of how direct glial-neuron interactions can result in changes in axonal growth behavior to have an impact on olfactory circuit development and function.

Schwann Cell-Axonal Growth Cone Interactions

Beyond astrocytes, microglia, and OECs, there is little evidence that Schwann cells or Schwann cell precursors (SCPs) play a direct role in axon growth and guidance during PNS development. Rather, the functions of SCPs during neurodevelopment are believed to include trophic support of sensory and motor axons and nerve myelination (Jessen and Mirsky, 2005). Early studies indicate that Schwann cells or SCPs do not guide axons to their target, but rather follow behind (Speidel, 1964; Carpenter and Hollyday, 1992; Bhattacharyya et al., 1994; Gilmour et al., 2002), and growing motor neurons are primarily guided by substrata composition with a preference to follow pioneer axons (Tosney and Landmesser, 1985). Specific ablation of Schwann cells or SCPs in the mouse does not affect the number of peripheral motor and sensory axons that are generated nor their ability to reach their targets to form initial synapses. However, axons without Schwann cell support do subsequently withdraw and degenerate before postnatal life (Riethmacher et al., 1997; Wolpowitz et al., 2000; Britsch et al., 2001; Jessen and Mirsky, 2005). Furthermore, specific ablation of boundary cap (BC) cells, which are neural crest-derived SCPs that reside early in neurodevelopment at the CNS:PNS junction, does not affect motor neuron axon exiting the spinal cord, but does cause displacement of their somata into the periphery (Vermeren et al., 2003). These data emphasize the importance SCPs and Schwann cells in trophic support of developing peripheral nerves and their apparent limited direct involvement in initial axon outgrowth and guidance to their peripheral targets. Typically, these studies lack detailed dynamic information about early peripheral axon growth and rely heavily on static images to draw conclusions. It remains to be determined whether the absence of SCPs or Schwann cells results in any initial aberrant axon growth cone migration that is corrected through redundant mechanisms to allow axons to reach their proper targets. Live cell *in vivo* imaging is necessary to elucidate these details in neurodevelopment.

MOLECULAR MECHANISMS OF GLIAL CELL-AXONAL GROWTH CONE INTERACTIONS

Multiple families of cell adhesion molecules (CAMs) mediate interactions between glial cells and neurons, with some being specific for certain glial subtypes (see **Table 1**). Many of these CAMs activate intracellular signaling cascades that converge on common pathways to impact cytoskeletal function and neurite outgrowth (see **Figures 2**, **3**). Many of these CAMs are extensively reviewed (Herron et al., 2009; Siebold et al., 2017; Sytnyk et al., 2017; Chooi and Chew, 2019), thus this section will highlight some of the key players and mechanisms that underlie glial cell-neuron interactions.

The immunoglobulin superfamily (IgSF) of CAMs, including L1-CAM family members NCAM and CHL1, as well as N-cadherin, mediate much of the attractive interactions between glial cells and axonal growth cones (**Figure 2**). The L1-CAM family consists of type I transmembrane proteins that have a large extracellular domain, with several immunoglobulin and fibronectin binding domains, as well as a short cytoplasmic tail of approximately 120 amino acids. These CAMs can participate in both homophilic and heterophilic binding interactions either in *cis* or *trans* that allow for a variety of functions depending on differential expression and binding interactions of the cytoplasmic tail (reviewed in Hansen et al., 2008; Herron et al., 2009; Samatov et al., 2016). There are three major isoforms of NCAM arising from alternative splicing that contain identical extracellular domains but variable cytoplasmic tails. The larger 180 kDa isoform (NCAM180) is primarily found in neurons, while the smaller 120 kDa isoform (NCAM120), which is attached to the membrane via a GPI anchor, is primarily found in glial cells (reviewed in Sytnyk et al., 2017). All L1-CAM family members contain the ankyrin-binding motif SFIGQY on their cytoplasmic tails, and the phosphorylation status of the tyrosine residue in this motif mediates binding with ankyrin. Changes in L1-CAM/ankyrin binding regulates coupling of ankyrin-associated proteins to the spectrin cytoskeleton, which can influence growth cone motility (Sytnyk et al., 2017). There is convincing evidence that both L1 and CHL1 can also bind the ezrin-radixin-moesin (ERM) family of proteins, which allow for additional linkage to the actin cytoskeleton. Interestingly, ERM binding to CHL1 was shown to be essential for Sema3A-induced growth cone collapse, providing a mechanism of crosstalk between different CAMs and axon guidance molecules (Schlatter et al., 2008). Similarly, CHL1 has recently been shown to play a role in non-canonical hedgehog signaling via interaction with PTCH1, providing a link to RhoA/ROCK signaling, which is discussed below (Katic et al., 2017). Extracellular NCAM interactions have been shown to activate membrane-linked tyrosine kinases such as Fyn, which can phosphorylate downstream targets like focal adhesion kinase (FAK) and spectrin that impact cytoskeletal dynamics (reviewed in Chooi and Chew, 2019). Finally, multiple studies have demonstrated that NCAM is able to activate fibroblast growth factor receptors (FGFR), which can influence gene expression (via MAPK) and intracellular calcium signaling (Doherty and Walsh, 1996; Niethammer et al., 2002).

Neural cadherin (N-cadherin or NCAD), which belongs to the larger family of calcium-dependent adhesion molecules, promotes cell adhesion through hemophilic binding in *trans*. The intracellular cytoplasmic tail of cadherins associate with p120 catenin complexes to link them to the actin cytoskeleton (reviewed in Hansen et al., 2008; Chooi and Chew, 2019). NCAD and NCAM share some downstream targets, such as activation of FGFR signaling cascades. Additionally, NCAD signaling through p120 both inhibits RhoA activity and activates MAPK, both of

TABLE 1 | Summary of key proteins and mechanisms underlying glial cell-axon growth cone interactions.

Glial protein	Cell type(s)	Neuron receptor(s)	Co-receptors	Signaling mechanism(s)
NCAM120	Astrocyte OEC	NCAM180 NCAM140	FGFR	Lipid raft-associated kinases FGFR signaling
CHL1	Astrocyte	L1 CHL1 Neurofascin NCAM	Sema3A	Ankyrin and ERM recruitment
N-cadherin	Astrocyte OEC	N-cadherin (homophilic)	FGFR ASTN1	Catenins FGFR signaling p120-mediated RhoA inhibition and MAPK activation
Nogo MAG OMgp	Oligodendrocyte	NgR1	p75NTR Troy Lingo-1 PirB PlexinA2 CRMP2	RhoA activation
Sema3A	OEC	Plexins	Neuropilins Integrins	R-Ras inhibition Rho-A activation
Sema4D Sema5A Sema6A	Oligodendrocyte	Plexins	Neuropilins RTKs Tim-2	R-Ras inhibition Rho-A activation
NG2	Oligodendrocyte	CSPG receptors		RhoA activation Par complex alterations
RGMa	Microglia	Neogenin	Unc5B	RhoA activation FAK-mediated Ras inactivation LMO4-mediated transcription

NCAM, neural cell adhesion molecule; OEC, olfactory ensheathing cell; FGFR, fibroblast growth factor receptor; CHL1, close homolog of L1; MAPK, mitogen-activated protein kinase; MAG, myelin-associated glycoprotein; OMgp, oligodendrocyte-myelin glycoprotein; p75NTR, neutrophin receptor; CRMP2, collapsin response mediator protein family-2; RhoA, Ras family member homolog A; RTK, receptor tyrosine kinase; NG2, neuron-glia antigen-2; CSPG, chondroitin sulfate proteoglycan; RGMa, repulsive guidance molecule A; FAK, focal adhesion kinase; LMO4, LIM domain transcription factor; ASTN1, astrotactin-1; ERM, ezrin-radixin-moesin; NgR1, Nogo receptor 1; PirB, paired immunoglobulin-like receptor B; Tim-2, T cell immunoglobulin and mucin domain containing 2.

which can positively impact growth cone motility (reviewed in Hansen et al., 2008). Recent work has emphasized the importance of co-receptors such as astrotactin (ASTN1) that are important for formation of glial cell-neuron cell adhesions mediated by NCAD (Horn et al., 2018). Finally, endocytic trafficking of NCAD has been shown to regulate neuronal migration and maturation, which depends on Rab GTPase activity (Kawauchi et al., 2010; Shikanai et al., 2011).

The inhibitory or repulsive interactions between glial cells and axon growth cones include the myelin-associated inhibitors (MAIs), semaphorins, NG2, and RGMa (**Figure 3**). The oligodendrocyte-expressed MAIs include the membrane proteins Nogo, MAG, and OMgp that signal through receptor complexes including the GPI-linked, Nogo-66 receptor NgR1 (reviewed in McKerracher and Rosen, 2015). Importantly, since NgR1 is GPI-linked, co-receptors are required to transmit signaling information within the cell, and the array of co-receptors being expressed can vary for a given neuron. These co-receptors include, but are not limited to, p75NTR, LINGO-1, TROY, PirB, PlexinA2, and CRMP2 (Sekine et al., 2019), and their common intracellular signaling mechanism is through the activation of RhoA, which is a GTPase within the Ras superfamily of proteins. RhoA is in its active form when bound to GTP, which is facilitated by guanine-nucleotide exchange factors (GEFs) that promote the exchange GDP for GTP. GEFs are regulated by a number of complex mechanisms, such as alterations in protein-lipid interactions that can change subcellular localization, release of autoinhibition by a flanking domain or region, and activation by secondary messengers or posttranslational modification (reviewed in Rossman et al., 2005; Bos et al., 2007). RhoA is also regulated via interactions with GTPase activating proteins (GAPs) that promote the conversion of GTP to GDP to inactivate RhoA, as well as guanine-nucleotide dissociation inhibitors (GDIs) that maintain GTPases in an inactive, GDP-bound state. For example, the NgR1 co-receptor p75NTR is cleaved upon ligand binding to NgR1, and the intracellular cleavage product displaces GDI from RhoA leading to its activation (Hasegawa et al., 2004; Domeniconi et al., 2005). Once activated, RhoA binds its downstream effector protein, Rho-associated protein kinase (ROCK) within the α-helical coiled-coil domain, resulting in removal of autoinhibition of ROCK and subsequent phosphorylation of substrate molecules (reviewed in Liu J. et al., 2015). ROCK phosphorylates a number of important targets that impacts axon

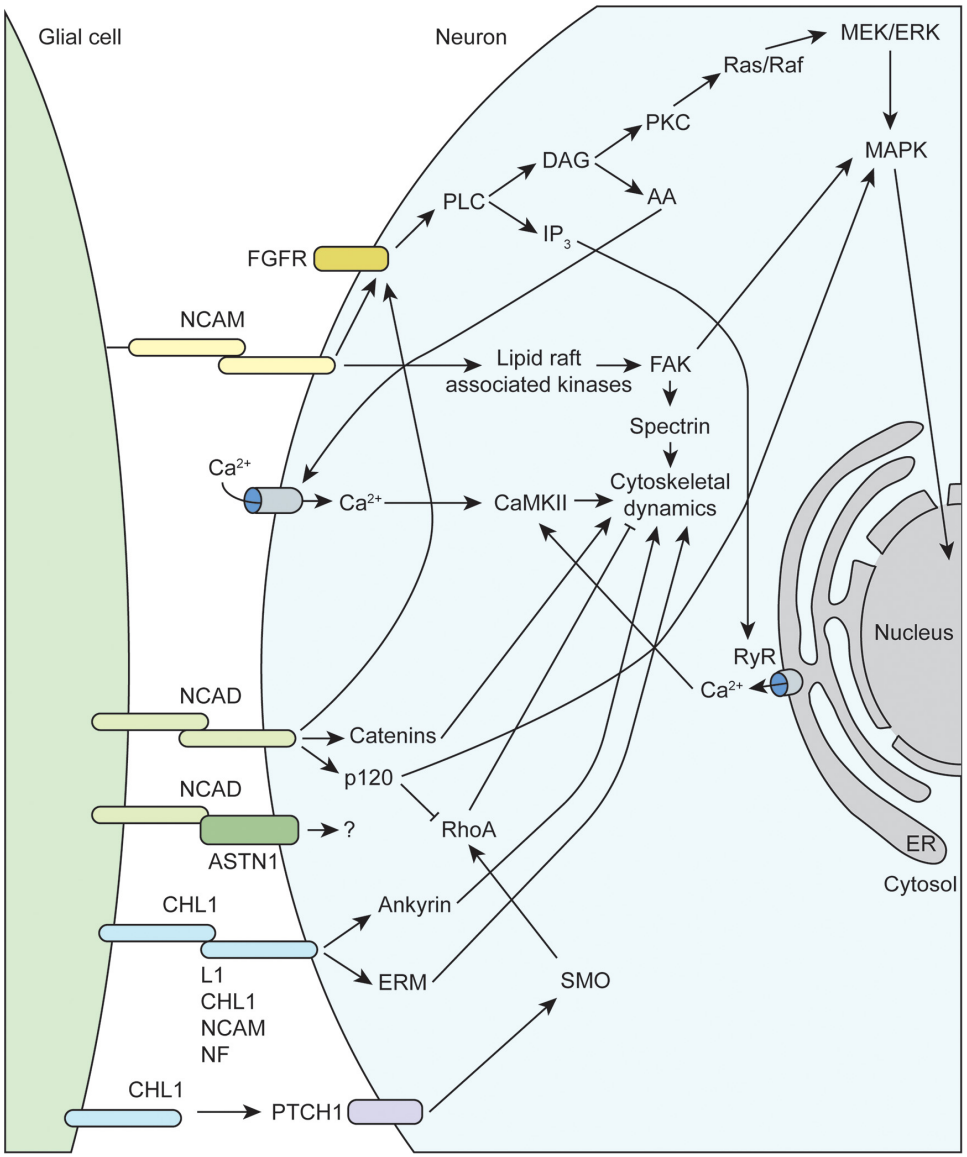

FIGURE 2 | Molecular mechanisms underlying glial-axon growth cone guidance signals. NCAM, neural cell adhesion molecule; FGFR, fibroblast growth factor receptor; CHL1, close homolog of L1; MAPK, mitogen-activated protein kinase; RhoA, Ras family member homolog A; FAK, focal adhesion kinase; NCAD, N-cadherin; NF, neurofascin; ERM, ezrin-radixin-moesin; RyR, ryanodine receptor; PLC, phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; IP_3, inositol trisphosphate; AA, arachidonic acid; ASTN1, astrotactin-1; PTCH1, protein patched homolog 1; SMO, smoothened; CaMKII, calcium/calmodulin-dependent protein kinase II; ER, endoplasmic reticulum.

guidance. For instance, ROCK promotes actin contractility by phosphorylating myosin light chain (activate) and myosin light chain phosphatase (inactivate), which can lead to growth cone collapse and neurite retraction. ROCK also phosphorylates LIM kinase (LIMK), which phosphorylates cofilin, an actin-binding protein responsible for depolymerization of actin filaments. Note that when phosphorylated, cofilin is inactivated, resulting in stabilization of actin filaments. As a result, activation of the RhoA/ROCK signaling pathway stabilizes actin filaments, which provides a substrate for ROCK-activated myosin based contractility, resulting the inhibition of axon growth.

Several other inhibitory axon guidance cues expressed by certain glia signal through the RhoA/ROCK pathway. For example, several of the previously discussed molecules above including RGMa, NG2, and many of the plexins (semaphorin receptors) signal through the RhoA/ROCK pathway to inhibit axon growth and guidance. RGMa is part of the larger family of GPI-linked repulsive guidance molecules (RGMs) that signals through interactions with the type 1 transmembrane protein neogenin (reviewed in De Vries and Cooper, 2008; Siebold et al., 2017). RGMa can act *in trans* to promote the formation of neogenin receptor dimers, which initiates

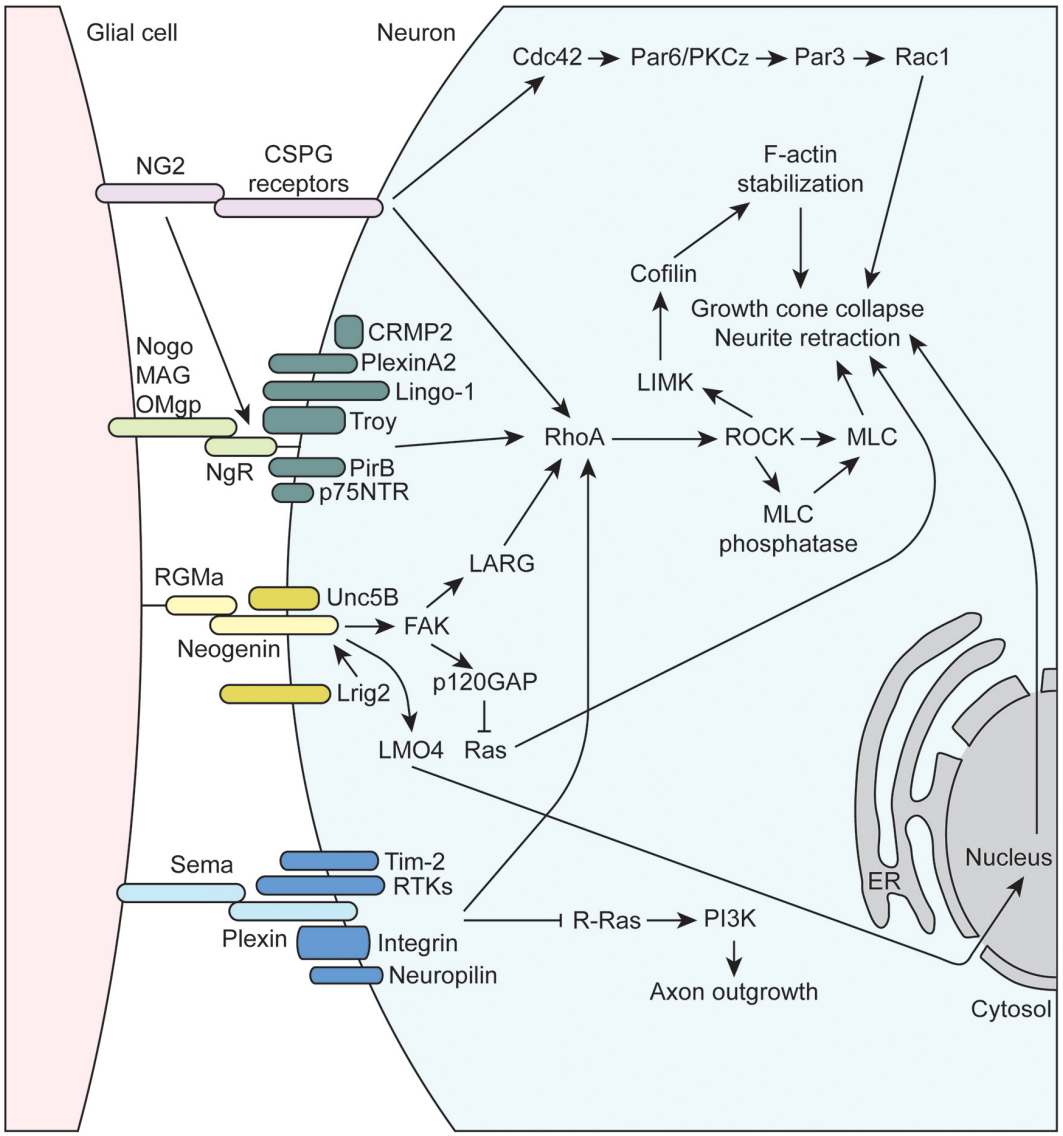

FIGURE 3 | Molecular mechanisms underlying glial-axon growth cone repulsive signals. MAG, myelin-associated glycoprotein; OMgp, oligodendrocyte-myelin glycoprotein; p75NTR, neutrophin receptor; CRMP2, collapsin response mediator protein family-2; RhoA, Ras family member homolog A; RTK, receptor tyrosine kinase; NG2, neuron-glia antigen-2; CSPG, chondroitin sulfate proteoglycan; RGMa, repulsive guidance molecule A; FAK, focal adhesion kinase; LMO4, LIM domain transcription factor; NgR1, Nogo receptor 1; PirB, paired immunoglobulin-like receptor B; Tim-2, T cell immunoglobulin and mucin domain containing 2; Lrig2, leucine rich repeats and immunoglobulin like domains 2; GAP, GTPase activating protein; PI3K, phosphoinositide 3-kinase; LARG, leukemia-associated Rho guanine nucleotide exchange factor; PKC, protein kinase C; LIMK, LIM domain kinase 1; ROCK, Rho-associated protein kinase; MLC, myosin light chain; ER, endoplasmic reticulum.

downstream signal transduction. The co-receptor Unc5B, a member of the netrin family, was found to interact with neogenin, which activates downstream signaling to the leukemia-associated Rho GEF (LARG) (Hata et al., 2009). RGMa-induced tyrosine phosphorylation of LARG by FAK has also been found to be necessary for the activation of RhoA and growth cone collapse. Interestingly, the co-expression of Lrig2 adds another layer of regulation to the RGMa-neogenin signaling cascade. Lrig2 association with neogenin prevents premature extracellular cleavage and inactivation of neogenin by A disintegrin and metalloprotease 17 (ADAM17), and RGMa disrupts the Lrig2-neogenin interaction allowing for the cleavage to occur (Van Erp et al., 2015). Thus, Lrig2 co-expression allows neurons to remain RGMa-sensitive by preventing the premature cleavage of neogenin, allowing for subsequent downstream signaling through RhoA. Several other non-RhoA-dependent mechanisms have also been shown to be important in RGMa signaling. For example, FAK has been shown to activate p120GAP, leading to the inactivation of Ras and subsequent growth cone collapse (Endo and Yamashita, 2009). Additionally, RGMa binding neogenin

also promotes the cleavage of the neogenin intracellular domain by γ-secretase. Released intracellular cytoplasmic neogenin associates with the transcriptional co-activator LIM-only protein 4 (LMO4) and affects downstream gene expression that mediates growth cone collapse (Schaffar et al., 2008; Banerjee et al., 2016). NG2 also signals to RhoA by interaction with a number of CSPG receptors such as Protein Tyrosine Phosphatase σ (PTPσ), NgR1, and NgR3 (reviewed in Ohtake and Shuxin, 2015). Additional mechanisms of NG2-mediated growth inhibition exist such as signaling through Cdc42 and atypical protein kinase C (PKCζ), which alters Par complex function (Lee et al., 2013).

Semaphorins, a large family that includes GPI-linked and membrane-bound proteins that play critical roles in axon growth and guidance, signal through both RhoA-dependent and independent pathways (reviewed in Liu and Strittmatter, 2001; Negishi et al., 2005; Derijck et al., 2010; Hota and Buck, 2012). Semaphorin signaling is mainly mediated by plexin receptors, a family cell-surface, transmembrane proteins with four subfamilies (PlexinA-D) in mammals. Additional plexin co-receptors like neuropilins, receptor tyrosine kinases (RTKs), and integrins mediate ligand binding and additional downstream signaling within target cells, allowing for diverse semaphorin-mediated signaling. All plexins contain a conserved intracellular GAP homology domain that can directly activate the GTPase activity of multiple GTPase protein families. For example, the plexin-A and B GAP homology domains inactivate R-Ras, resulting in reduced PI3K and integrin signaling (Hota and Buck, 2012). Plexins also contain a Rho binding domain (RBD) that can interact with Rho family GTPases in a number of ways to affect a complex network of downstream proteins (reviewed in Hota and Buck, 2012). Plexins can signal to RhoA by mediating the activity of GEFs, such as LARG, as well as many other possibilities as described in the above reviews.

MODULATING GLIAL CELL-AXONAL GROWTH CONE INTERACTIONS TO AID IN REGENERATION

One major motivation to improve our understanding of the roles glia play in axon growth and guidance during neurodevelopment is to direct those developmental principles to improve regeneration of the CNS or PNS after injury. For example, glia are known to mediate regeneration following spinal cord injury (SCI) (Cregg et al., 2014; Silver et al., 2015; Jin and Yamashita, 2016). This section aims to provide a few examples showing how the neurodevelopmental discoveries influenced the field of SCI for translational purposes (**Table 2**).

In an injured state, cytokines, cell fragments, and nucleic acids contribute to differentiation of CNS microglia into "classically activated" M1 or "alternatively activated" M2 subtypes (Silver et al., 2015). This decision may be influenced by a number of local factors such as interleukin-4 (IL-4) (Francos-Quijorna et al., 2016) and hemopexin (Han et al., 2018). M1 activated microglia are generally viewed as pro-inflammatory and neurotoxic, promoting axon dieback (see below). M2 activated microglia are anti-inflammatory and neuroprotective, secreting neurotrophic factor and promoting axon regeneration. For example, microglia have been shown to encourage axon elongation and presynaptic site formation following pyramidal tract section (Jiang et al., 2019) as well as promote plasticity following lesions within the visual pathways (Chagas et al., 2019). However, this binary system represents an oversimplification of microglial function. For example, a variety of cytokines are able to promote both M1 and M2 phenotypes, which adds mechanistic uncertainty into the divergent roles of M1 and M2 microglia. Nonetheless, this model provides a framework that is relevant to SCI and categorizes the multiple roles microglia may have on axon growth and regeneration.

Work described above demonstrated that deficiency of Cx3cr1 ($Cx3cr^{-/-}$), the microglial-specific fractalkine chemokine receptor, improved axonal growth of dopaminergic neurons in the subpallium (Squarzoni et al., 2014). Importantly, $Cx3cr^{-/-}$ microglia compared to WT do not exhibit an activated, M1-type morphology when stimulated with the inflammatory mediators interferon-γ (INF-γ) and LPS (Freria et al., 2017). Instead, they remained in an unstimulated, M0 "reparative" phenotype with neurotrophic potential as they expressed higher amounts of TGF-β, IGF-1, and FGF2 compared to WT microglia. After SCI, $Cx3cr^{-/-}$ mice exhibited greater regeneration of serotonergic axons, especially in the ventral horn. This may be partially attributed to the creation of a microenvironment that promoted the differentiation and survival of NG2-positive glia, which include OPCs, as greater numbers closely associate with growing axons in the $Cx3cr^{-/-}$ mice following SCI. The above data, when taken together, suggest that modulating CX3CR could be a therapeutic strategy to enhance axonal regeneration after injury through a mechanism of improved microglial support. Indeed, inhibition of CX3CR via a small molecule or monoclonal antibody has shown promise in other inflammatory conditions, such as atherosclerosis (Poupel et al., 2013), rheumatoid arthritis (Nanki et al., 2004), and multiple sclerosis (Wollberg et al., 2014). Therefore, the next logical step would be to try these interventions in a SCI clinical trial.

RGMa was identified as a major molecule involved in the inhibitory effect microglia exert on growing axons (Kitayama et al., 2011). In a mouse model of SCI, minocycline treatment, which decreases microglial RGMa expression, reduced the accumulation of microglia in the site of injury with a subsequent reduction in axonal dieback in injured corticospinal neurons. Furthermore, intrathecal administration of an antibody against RGMa in a rat SCI model promoted axonal growth and functional recovery, which may be attributed to invasion of microglia and/or macrophages in the site of injury (Hata et al., 2006). Another group developed a systemically administered, human monoclonal antibody against the N-terminus of RGMa, which both neutralized RGMa as well as prevented the RGMa receptor Neogenin from associating with lipid rafts, which is essential for its downstream functions (Mothe et al., 2017). The authors demonstrated that this treatment promoted neuron survival, corticospinal tract axonal regeneration, and improvement in motor function and gait. As such, inhibition of RGMa/Neogenin shows promise in improving clinical outcomes for SCI. In fact, AbbVie Inc. has developed the human anti-RGMa antibody

TABLE 2 | Pharmaceuticals targeting glial-neuron interactions under study for spinal cord injury.

Glial protein target	Cell type	Intervention	Clinical trial	Phase	Status	References
Nogo	Oligodendrocyte	ATI355/NG-101 (Novartis)	NCT00406016 NCT03935321 EudraCT2016-001227-31	I II II	Complete Ongoing Ongoing	Kucher et al., 2018
Nogo MAG OMgp	Oligodendrocyte	AXER-204	NCT03989440	I/II	Ongoing	
RGMa	Microglia	Elezanumab (ABT-555) Minocycline	NCT02601885 NCT03737812 NCT03737851 Not announced NCT00559494 NCT01828203	I (MS) II (MS) II (MS) I (SCI) II (SCI) III (SCI)	Completed Ongoing Ongoing N/A Completed Ongoing	Casha et al., 2012

MAG, myelin-associated glycoprotein; OMgp, oligodendrocyte-myelin glycoprotein; RGMa, repulsive guidance molecule A. The above clinical trial information was acquired from the United States (clinicaltrials.gov) and European Union (clinicaltrialsregister.eu) databases.

elezanumab (ABT-555) that is currently in Phase II clinical trials for Multiple Sclerosis (NCT03737812 and NCT03737851) with plans to start a Phase I clinical trial for spinal cord injury (see abbvie.com). Additionally, many other pre-clinical studies have demonstrated the beneficial effects of the RGMa-suppressing antibiotic minocycline in SCI (Lee et al., 2003; Wells et al., 2003; Stirling et al., 2004; Festoff et al., 2006; Wang Z. et al., 2017), and a Phase II clinical trial suggested that there may be improvement after acute SCI (Casha et al., 2012). There is an ongoing Phase III clinical trial (NCT01828203) that was expected to complete in June of 2018, but no results have been posted.

In addition to microglia, other glial cells negatively impact CNS injury with the formation of the well-known "glial scar" that is detrimental to axon regeneration. For example, astrocytes react and form a dense barrier around the lesion, stromal cells invade and form fibrous connective tissue with dense collagen and CSPG deposition, and OPCs proliferate and surround dystrophic axons (Pasterkamp and Verhaagen, 2006; Cregg et al., 2014; Dias et al., 2018). Efforts to prevent or dissociate the glial scar have been shown to improve axon regeneration (for example, see Rosenzweig et al., 2019). However, the biology of the glial scar is much more nuanced, and oversimplification of this complex healing and regeneration process can hinder advances in the field (Bradbury and Burnside, 2019). Therefore, a detailed understanding of the players involved, including the glial subtypes, can provide additional clues for intervention. As mentioned above, the expression of many inhibitory molecules such as the MAIs increase following injury, such as Nogo-A (Hunt et al., 2003), which signals through NgR/RhoA pathway to inhibit axon outgrowth. Four Nogo receptors have been identified (NgR1, NgR2, NgR3, and PirB), and $Ngr1^{-/-}$; $Ngr2^{-/-}$; $Ngr3^{-/-}$ mice exhibit improved axon regeneration following optic nerve crush injury (Dickendesher et al., 2012). Furthermore, knockout of Nogo-A in mice improved axon regeneration past the lesion following dorsal spinal cord hemisection, an effect that was not observed in MAG or OMgp knockout mice (Cafferty et al., 2010). Consistently, intrathecal administration of a Nogo-A neutralizing antibody following SCI resulted in enhanced axon growth and collateral sprouting in both rats and non-human primates (Merkler et al., 2001; Freund et al., 2006). These results led to a phase I trial (NCT00406016), which was completed in 2011 to assess the safety of intrathecal administration of ATI355 (Novartis), a recombinant human antibody directed against Nogo-A that was well-tolerated in patients with acute SCI (Kucher et al., 2018). Currently there are two ongoing phase II trials (NCT03935321 and EudraCT2016-001227-31) as a follow-up to this study, with no results posted. An additional approach to antagonize the action of MAIs is via administration of a soluble NgR fragment that can disrupt neural NgR signaling and promote axon regeneration (Fournier et al., 2002). Intrathecal administration in a rat SCI model showed promising results as demonstrated by increased axon sprouting, electrical conduction, and locomotion (Li et al., 2004). To translate this finding into humans, the drug AXER-204 (ReNetX Bio, Inc.), which is a soluble decoy for MAIs like the one previously mentioned, is being used in a current phase I/II trial for patients with chronic SCI (NCT03989440).

In addition to the identification of novel drug targets, pre-clinical studies investigating glial-axon growth cone interactions have led to the notion of implanting growth-promoting glia into the site of injury. For example, OECs can be harvested from the olfactory blub, cultured *in vitro*, and injected into SCI sites. In rodent studies, transplantation of OECs resulted in enhanced axon regeneration and even functional recovery in several animals (Ramon-Cueto et al., 2000; Keyvan-Fouladi et al., 2003; Li et al., 2003). OEC transplants provided several benefits for regenerating axons, such as functioning as a physical substrate for axon growth and secretion of soluble factors that enhanced neurite sprouting (Chung et al., 2004). Interestingly, similar behaviors were observed in spinal cord as seen in the *in vitro* models. For example, some neurons traveled along OECs as they migrated into the injury tract. This may provide a means to avoid the inhibitory signaling molecules that become enriched at injury sites, as regenerating axons may associate with OECs as they migrate through the forming glial scar. Several small clinical trials have been completed in humans (for example, NCT01327768 and NCT01231893) with mixed results, often challenged by small sample sizes and technical difficulties with cell extraction and

transplantation (Tabakow et al., 2013, 2014; Wang et al., 2016). There are currently two clinical trials actively recruiting for participants that aim to optimize the OEC harvesting procedure and implant them into patients with SCI (NCT02870426 and NCT03933072). In addition to OECs, spinal cord neural stem cell (NSC) grafts are also being developed with the potential to reconstitute components of the damaged spinal cord. Spinal cord NSCs derived from human pluripotent stem cells and transplanted into lesioned rat spinal cords develop into both neuron and astrocyte lineages, which are able to integrate into the spinal cord circuitry with improvements in functional outcomes (Kumamaru et al., 2018; Lien et al., 2019). Therefore, spinal cord NSCs are an additional cell-based therapy that show promise for translation into humans. With improvements in culturing and transplantation techniques, as well as the potential benefits from combined therapies with different mechanisms of action, there is great promise that the long history of research in glial-axonal growth cone interactions will prove worthwhile to aid in axon regeneration after SCI in human patients.

CONCLUSION

In the CNS and PNS, many glial cell types are able to affect axon growth and guidance, which has an impact on neuronal wiring in adulthood as well as the outcomes of several disease states, such as SCI (see **Figure 1**). Promising therapeutics are being developed because of the advances in knowledge of glial function in neurodevelopment, which shows the importance of further development in this area of pre-clinical research. As more specific molecular tools and labeling techniques become readily available, better correlation can be drawn from the observational data of anatomic distribution and morphology of glia with developing axonal tracts. For example, with improvements in live imaging *in vivo*, much of these developmental events will become accessible to view in real time, which could promise a plethora of important information on glial-axon growth cone interactions (Wu et al., 2013). Furthermore, with advances in cell culture techniques, such as with the multi-compartment neuron-glia co-culture platforms (Park et al., 2012), as well as widespread availability of genome editing and human stem cell differentiation protocols, more streamlined and high-throughput screening of potential drug targets will be available (Gang et al., 2015). Finally, techniques to specifically target glial populations are being further refined with application to both non-human primate models and humans (Juttner et al., 2019). In the end, there will always be a balance between the promoting and inhibiting effects of glia on growing axons, and there are likely benefits to precise molecular reprogramming of glia for both neuroprotective and regenerative applications.

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MR wrote the manuscript. TG and LP revised the manuscript.

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Differential Proliferation and Maturation of Subcortical Astrocytes During Postnatal Development

*Temitope Shoneye[1], Alessandra Tamashiro Orrego[1], Rachel Jarvis[1], Yuqin Men[1], Ming Sum R. Chiang[1] and Yongjie Yang[1,2]**

[1] Department of Neuroscience, Tufts University School of Medicine, Boston, MA, United States, [2] Graduate School of Biomedical Sciences, Tufts University, Boston, MA, United States

Astrocytes exhibit a region-dependent molecular and functional heterogeneity in the CNS. Although cortical astrocytes proliferate robustly during the first postnatal week and become proliferation quiescent, the temporal proliferation dynamics of astrocytes in subcortical regions during postnatal development remain essentially unknown. Whether subcortical astrocytes mature similarly to cortical astrocytes is also unexplored. In this current study, we examined proliferation of subcortical, especially hypothalamic, astrocytes during postnatal development using genetic labeling of astrocytes and pulse-chase EdU labeling of proliferating cells. While a lower number of proliferating astrocytes was found in the hypothalamus compared to cortex during the first postnatal week, astrocyte proliferation is much more active in hypothalamus than in cortex from P15 to P30 in both proliferating astrocyte density and percentage, indicating a persistent and distinct proliferation pattern of astrocytes in hypothalamus. This observation is further confirmed by Ki67 immunostaining with genetically or immunolabeled astrocytes in hypothalamus and cortex during P15–30. In addition, astrocytes in representative subcortical regions have a modest growth of their domain size and exhibit a significantly smaller domain size compared to cortical astrocytes at P30 when astrocytes have generally completed postnatal maturation. However, the expression of astrocyte-derived Sparc, an important synaptogenic inhibitor, is consistently higher in hypothalamic astrocytes than in cortical astrocytes throughout postnatal development. In summary, our study unveiled a distinct proliferation and maturation pattern of subcortical, especially hypothalamic, astrocytes during postnatal development.

Keywords: astrocyte, heterogeneity, development, hypothalamus, proliferation, Sparc

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***Correspondence:**
Yongjie Yang
yongjie.yang@tufts.edu

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INTRODUCTION

Astrocytes are considered important modulators of brain physiology and pathology, playing diverse and active roles in synaptogenesis, synaptic transmission, and neuronal survival (Clarke and Barres, 2013; Allen and Eroglu, 2017). Interestingly, astrocytes display region-specific differences in their mature morphology and their modulatory functions are also largely associated with local brain regions or circuits (Ben Haim and Rowitch, 2017; Chai et al., 2017; Morel et al., 2017). Several recent studies, by combining RNA-seq with ribosome pull-down (RiboTag or TRAP)

(Chai et al., 2017; Morel et al., 2017), cell surface antigen based sorting (John Lin et al., 2017), or single-cell isolation approaches (Zeisel et al., 2018), have systematically characterized transcriptomes of astrocytes from different brain regions. These studies have shown a clear molecular heterogeneity of astrocytes in the adult brain that appears to follow an anatomical dorsal to ventral and anterior to posterior axis (Farmer et al., 2016; Morel et al., 2017). Additionally, astrocytes' physiological properties, such as gap-junction coupling, inward-rectifying K^+ currents, and intracellular Ca^{2+} responses are manifested differently across CNS regions (Chai et al., 2017; Oberheim et al., 2012). By using *in vitro* mismatched neuron and astrocyte co-cultures, we further showed that astrocyte-mediated promotion of neurite growth and neuronal synaptic activity is region-conserved (Morel et al., 2017). Whether this molecular, morphological, and functional heterogeneity stems primarily from early stages of astrogliogenesis or is largely influenced by local neighboring signals during the maturation phase remains to be determined.

Subcortical brain regions have a distinct glia to neuron ratio compared with cortex with drastically different neural circuitry (Azevedo et al., 2009). These regions are also highly populated with interneurons derived from medial ganglionic eminence (MGE) progenitors in contrast to predominant glutamatergic neurons in cortex/hippocampus (Bayraktar et al., 2014). Astrocytes are derived from radial glia (RG) in the CNS when RGs transition from $GLAST^+/Nestin^+$ to $GLAST^+/Nestin^-$ progenitors during late embryonic or early postnatal stages (Bayraktar et al., 2014; Siddiqi et al., 2014). Although astrocytes proliferate robustly during the first 2 weeks postnatally to occupy the cortex, presumably through a local proliferation mechanism from newly born immature astrocytes (Ge et al., 2012), as RGs are heterogenous with a dorsoventral (DV) distribution along the ventricular zone (VZ), whether subcortical astrocytes undergo similar proliferation dynamics during early postnatal development has not been explored. In addition, newly born cortical astrocytes undergo a maturation phase to acquire their uniquely ramified morphology and express important functional proteins such as excitatory amino acid transporter (EAAT2) (Morel et al., 2014). Whether these morphological and molecular changes similarly occur for subcortical astrocytes remains to be investigated.

In the current study, we performed genetic and 5-ethynyl-2′-deoxyuridine (EdU) pulse-chase labeling to investigate astroglial proliferation dynamics in developing subcortical regions. We also examined postnatal morphological and molecular changes of subcortical astrocytes.

MATERIALS AND METHODS

Animals

The Ai14-$tdT^{f/f}$ reporter, Bac *Slc1a3*-CreERT transgenic (C57BL/6) and Bac *Aldh1l1*-eGFP mice were obtained from the Jackson Laboratory. $VGluT1^{-/-}$ mice were obtained as a kind gift of Dr. Robert Edwards (University of California, San Francisco). The EAAT2-tdTomato (tdT) transgenic mice were generated as previously described (Yang et al., 2011). Animals were deeply anesthetized with ketamine (100 mg/kg) plus xylazine (10 mg/kg) in saline by intraperitoneal injection and perfused intracardially with 4% PFA in PBS. The brains were dissected and kept in 4% PFA overnight at 4°C, then cryoprotected by immersion in 30% sucrose for 48 h. Brains were embedded and frozen in OCT-Compound Tissue-Tek (Sakura). Sagittal sections (20 μm) were prepared with a cryostat (Leica HM525) and mounted on glass SuperFrost$^+$ slides (Thermo Fisher Scientific). Mice of both sexes were used for all experiments. All procedures were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Tufts University Institutional Animal Care and Use Committee.

Tamoxifen and EdU Injection

Tamoxifen (4-OHT; Sigma-Aldrich) was suspended at 20 mg/ml in ethanol and diluted into sunflower seed oil at a final concentration of 2 mg/ml in 10% ethanol. For *Slc1a3*-$CreERT^+Ai14^{f/+}$ mice, an intraperitoneal injection of 10 μl 4-OHT (50 mg/kg) was administered from P1 to P2 for a total dose of 0.25 mg to selectively label astrocytes. The Click-iT EdU Alexa Fluor 488 Imaging Kit was suspended at 2.5 mg/ml in DMSO, a 1:10 dilution from the stock, and the final concentration was 10 mg/kg. An intraperitoneal injection of EdU was administered at different developmental time points (P3, P8, and P15) for a total dose of 0.25 mg.

Immunohistochemistry

Mice were perfused by intracardial perfusion with 4% paraformaldehyde in 1× PBS. Brains were cut into 20 μm sections with a cryostat. Slides were rinsed three times in PBS for 10 min each, then incubated with blocking buffer (1% BSA, 5% normal goat or donkey serum, and 0.2% Triton X-100 in PBS) for 1 h at room temperature (RT). Primary antibodies against Ki-67 (1:100 rabbit anti-Ki-67, Pierce #PA5-19462), Sparc (1:500 goat anti-Sparc, R&D Systems #AF942), or Sox9 (1:100 goat anti-Sox9, R&D Systems #AF3075) were incubated overnight at 4°C in the appropriate blocking buffer. After washing slides three times in PBS, secondary antibody (1:2000, donkey anti-goat Alexa Fluor 647 or goat anti-rabbit Alexa Fluor 488, Life Technology) was added for 2 h at RT. For EdU immunostaining, slides were permeabilized with 0.5% Triton X-100 in PBS for 20 min at RT. The slides were then washed with 3% BSA in PBS twice for 5 min each. The reaction cocktail was added for 30 min at RT. The sections were rinsed once in BSA for 5 min before mounting.

Acquisition of Images and Quantification of Labeled Cells

Images were obtained with a confocal laser scanning microscope (A1R, Nikon), Keyence BZ-X700 microscope, or Zeiss AXIO Imager with ApoTome. For EdU quantification, Keyence stitched images of the entire sagittal sections were taken with a 10× objective lens and all cells were manually counted using Fiji ImageJ (multi point tool). We counted all tdT^+ cells (astrocytes), EDU^+ cells (dividing cells), and tdT^+EDU^+ cells (dividing astrocytes) in cortex, thalamus, and hypothalamus. At each

time point we calculated the density of astrocytes, dividing cells, and dividing astrocytes by dividing the number of cells by the area of the respective brain region to determine the number of cells per mm^2. We also quantified the percentage of dividing astrocytes among all labeled astrocytes at P30 by dividing the number of tdT^+EdU^+ cells by the total number of tdT^+ cells. For Ki-67 quantification, Keyence stitched images of sagittal brain sections were taken with a 10× objective, and a 0.5 mm^2 grid was overlaid on the image. tdT^+ cells (astrocytes) and $tdT^+Ki\text{-}67^+$ cells (dividing astrocytes) were quantified from 20 grids in cortex and 10 grids in hypothalamus, and percentage of dividing astrocytes among labeled astrocytes was determined. Labeling efficiency of the Ai14-tdT reporter in a given brain region was estimated by dividing density of tdT^+ cells by the density of $eGFP^+$ cells in age-matched Bac *Aldh1l1*-eGFP mice.

For Sparc analysis, images were taken using the Zeiss microscope with a 20× objective. The numbers of $eGFP^+SPARC^+$ cells were individually quantified in Fiji ImageJ and the intensity of Sparc from co-localized astrocytes (indicated with eGFP fluorescence) was also measured. Sparc fluorescence-negative area in each image was selected to determine the background and subtracted. In Fiji, maximum projections were generated from Zeiss images for each channel and the merged images. The number of $eGFP^+$ labeled astrocytes in the field was manually counted using the region of interest (ROI) manager. The freehand selection tool was then used to outline labeling in Sparc images that were co-localized with $eGFP^+$ labeled astrocytes in the merged images. Measurements of average intensity within the region were calculated through Fiji's ROI manager using the polygon selection tools to draw a circle around the cells.

Astrocyte Domain Analysis

All confocal images for Imaris analysis were taken with a 40×oil-immersion objective lens. Images were taken under optimized setting to best show the astrocyte morphology. The settings were consistent across all age groups. For the morphological analysis of astrocytes, a 3D reconstruction was first generated using the original confocal *Z*-stack images in Imaris software. The surface tool was then used to build the domain. This function uses an automatic smoothing of the image with the Gaussian filter. A tdT fluorescence-negative area in each of the confocal stack images was used as the internal control to determine the background fluorescence. The sensitivity threshold (absolute intensity) was manually adjusted so that the generated astrocyte domains in the 3D images matched with the original confocal images. The cell somas were then detected based on size (≤12 μm in diameter) and used as seeding points to build the 3D domain. The quality (intensity) threshold was also manually adjusted to ensure that all cell somas were detected in a given image. The seeded watershed algorithm enables the Imaris software to recognize and split the domains of neighboring cells. Cells that were only partially included in confocal and 3D images were excluded from analysis. The volume size of individual astrocytes can be directly measured from generated 3D domains in Imaris.

Statistical Analysis

Sample size and statistical approach used for each experiment are described in figure legends. All analysis was performed using GraphPad Prism 7. All values were plotted as mean ± SEM, except for the astrocyte domain size values, which were converted to cumulative frequency. The Kolmogorov–Smirnov test was used to analyze significance for all cumulative frequency curves. For multiple groups (>2), one-way ANOVA was used to analyze the variance, followed by a Tukey *post hoc* test to compare multiple groups. For two-group comparison, an unpaired two-tailed *t*-test was used. Statistical significance was tested at a 95% ($p < 0.05$) confidence level and the exact *p*-values are presented in each figure panel and legend.

RESULTS

Proliferation of Subcortical Astrocytes During Early Postnatal Development

Previous studies found that the peak of astrocyte proliferation in the cortex occurs within the first postnatal week, after which these astrocytes become gradually proliferation quiescent (Ge et al., 2012). To examine the proliferation dynamics of subcortical astrocytes during postnatal development, we combined the genetic labeling of astrocytes using Cre-dependent $Ai14\text{-}tdT^{f/f}$ mice and EdU pulse-chase for labeling proliferating cells (**Figure 1A**). Although astrocytes can be conventionally identified by immunostaining of glial fibrillary acidic protein (GFAP) and recently aldehyde dehydrogenase L1 (ALDH1L1) (Cahoy et al., 2008; Yang et al., 2011), these immunostaining signals can be incomplete (in the case of GFAP) (Fujita et al., 2014) or weak (in the case of ALDH1L1) (Yang et al., 2011) and often more evident in astroglial processes than the cell body, making it ambiguous to clearly identify and quantify individual astrocytes. Alternatively, we bred Bac *Slc1a3* CreERT transgenic mice with Ai14-tdTomato (tdT) reporter mice in which the tdT reporter can be induced in a Cre-dependent manner in astroglial soma and processes, facilitating the confident quantification of individual astrocytes in the CNS. Glutamate transporters GLAST and GLT1 (human analog EAAT1 and EAAT2, encoded by *Slc1a3* and *Slc1a2*, respectively) are both highly and selectively expressed in astrocytes during postnatal development in the CNS (Rothstein et al., 1994). Although the *Slc1a3* genomic promoter is also active in RG during late embryogenesis (Regan et al., 2007), RG's fate is destined toward astrocytes at P1–2 (Rowitch and Kriegstein, 2010) when 4-hydroxy-tamoxifen (4-OHT) was administered. Consequently, it is unlikely that *Slc1a3*-Cre induced tdT is expressed in other CNS cell types in *Slc1a3*-$CreERT^+Ai14\text{-}tdT^{f/+}$ mice. We have also previously performed immunostaining with cell-type specific markers to confirm that tdT is indeed expressed in astrocytes, but not in other CNS cells, in cortex (Higashimori et al., 2016). In addition to astrocyte labeling, we also performed a single intraperitoneal (i.p.) injection of EdU to *Slc1a3*-$CreERT^+Ai14\text{-}tdT^{f/+}$ mice. EdU is a nucleotide analog that can be incorporated into DNA during the DNA synthesis phase of the cell cycle, thus reliably and

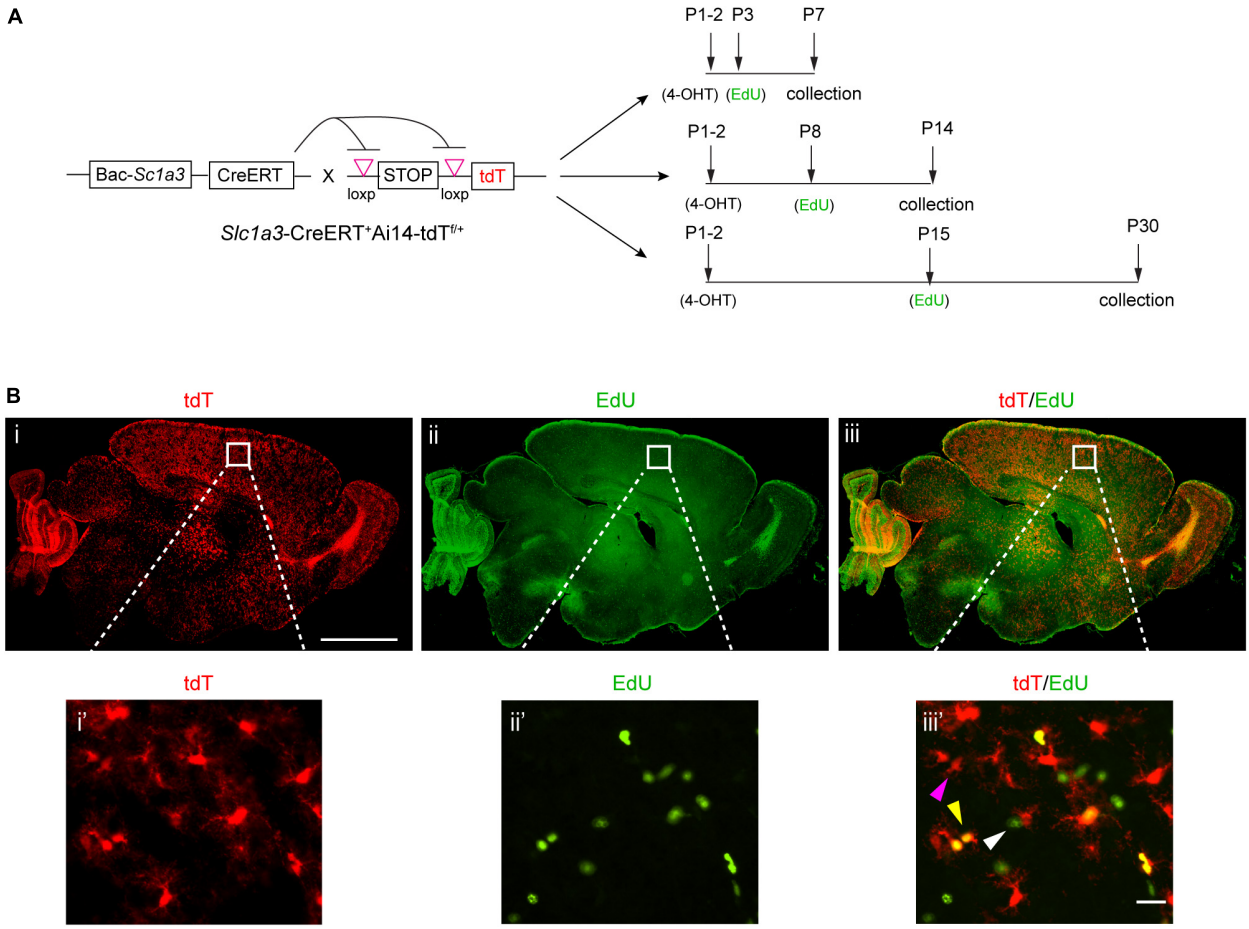


FIGURE 1 | Genetic labeling of astrocytes and EdU pulse-chase labeling of proliferating cells during postnatal development. **(A)** Experimental paradigm for genetic labeling of astrocytes and EdU pulse-chase labeling of proliferating cells at different time points postnatally; specific time points for 4-OHT and EdU injections are indicated. **(B)** Representative images of astrocyte labeling with the tdT reporter in *slc1a3*-CreERT^{+}Ai14-tdT$^{f/+}$ mice and EdU labeling of proliferating cells in P7 brain; magnified images show cortical astrocytes. Magenta arrow, tdT^{+}EdU^{-} cell; white arrow, tdT^{-}EdU^{+} cell; yellow arrow, tdT^{+}EdU^{+} cell. Scale bar: 1 mm (i–iii), 10 μm (i'–iii').

selectively labeling proliferating cells. Although EdU-mediated labeling of proliferating cells can continue for more than one cycle of division, its quantification still allows us to assess the overall proliferation activity in a given time period.

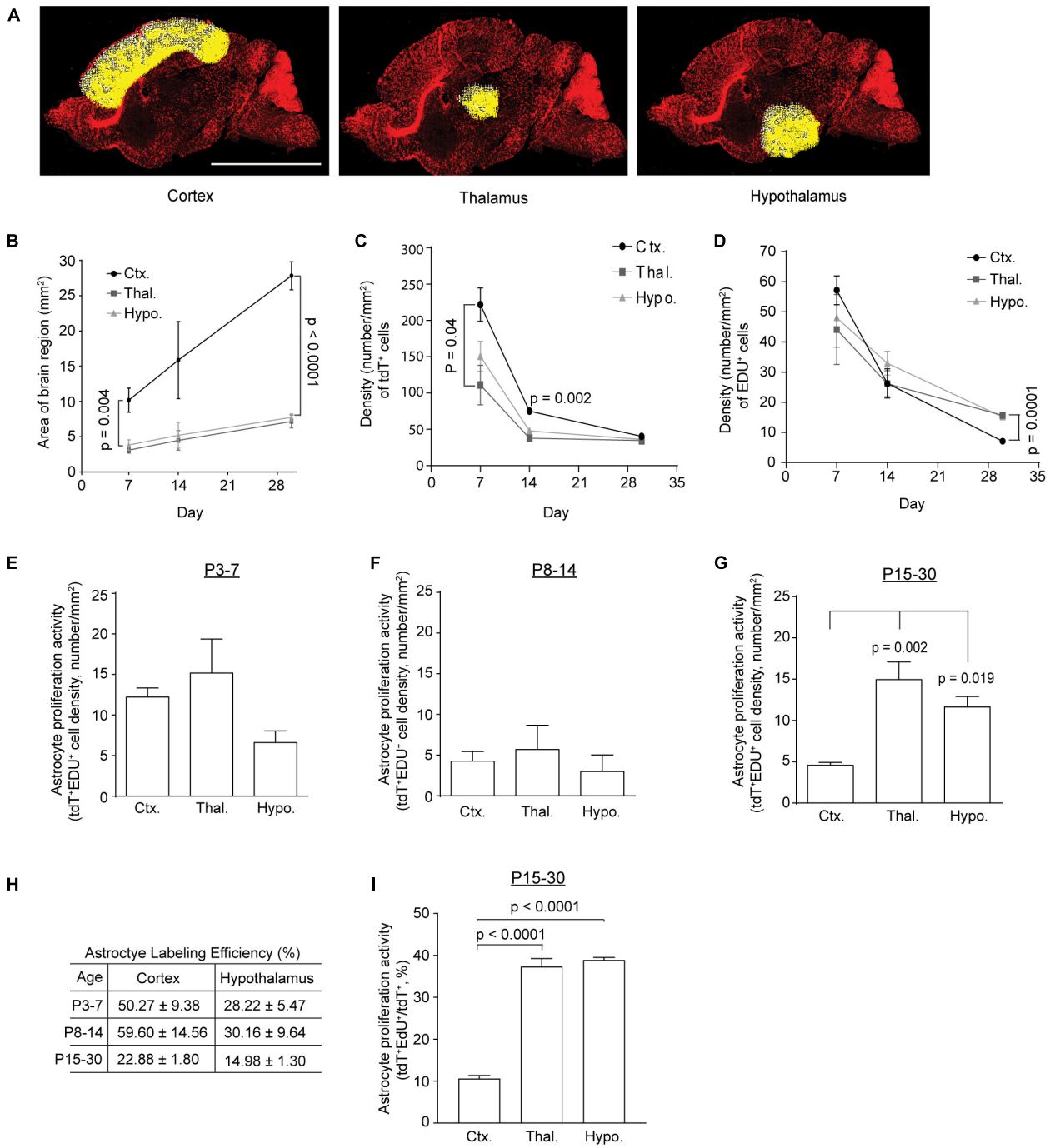
To examine astrocyte proliferation within a specified postnatal period of time, we decided to inject EdU at P3, P8, or P15 to label proliferating cells during the postnatal period from P3 to P7 (week 1), P8 to P14 (week 2), and P15 to P30 (weeks 3–4), respectively (**Figure 1A**). Based on the previously observed significant decrease of cortical astrocyte proliferation from P15 to P30, we decide to combine weeks 3 and 4 in assessing astrocyte proliferation activity during this time period. In all experimental groups, 4-OHT was injected at P1–2 to selectively induce expression of tdT in astrocytes. With the combined injections of 4-OHT and EdU, proliferating astrocytes, -likely from multiple cycles of divisions, are labeled as tdT^{+}EdU^{+} cells that can be unbiasedly quantified to reflect the proliferation activity of astrocytes during indicated periods (**Figure 1A**) of early postnatal development. We tested combinations of dose and frequency of EdU and 4-OHT injections to achieve optimal numbers of cell labeling for quantification. The combined injections of 4-OHT and EdU resulted in efficient induction of tdT expression in astrocytes (**Figure 1Bi** and the magnified view i') and sufficient labeling of proliferating cells (**Figure 1Bii** and the magnified view ii') in the CNS. Co-localized tdT and EdU labeled (tdT^{+}EdU^{+}, yellow arrow), tdT^{+} alone (tdT^{+}EdU^{-}, magenta arrow), or EdU^{+} alone (tdT^{-}EdU^{+}, white arrow) cells were all observed (**Figure 1Biii'**). Although our strategy is not designed to label all astrocytes or proliferating cells, the number of labeled astrocytes and proliferating cells is sufficient for examining astrocyte proliferation activity in both cortex and subcortical regions. On the other hand, it is known that other CNS cell types, particularly polydendrocyte NG2 cells, also actively proliferate during early postnatal development (Kang et al., 2010), thus it is not unexpected that some EdU^{+} proliferating cells do not overlap with tdT^{+} astrocytes. In addition, it is also possible that not all tdT^{+} astrocytes were sampled by the EdU injection or underwent division at the time when EdU was injected. Overall,

the combined labeling of astrocytes and proliferating cells allows temporal and spatial quantification of proliferating astrocytes, as an indication of overall astrocyte proliferation activity, during early postnatal development.

To analyze astrocyte proliferation in subcortical regions, especially thalamus and hypothalamus, we prepared sagittal sections from *Slc1a3*-CreERT^{+}Ai14-tdT$^{f/+}$ mice following 4-OHT and EdU injections at different time points. The total number of tdT^{+}, EdU^{+}, and tT^{+}EdU^{+} cells were quantified from the whole thalamus, hypothalamus, and cortex regions, highlighted with white crosses and yellow dots (indicating individual cells, **Figure 2A**) using the ROI script in ImageJ. As the brain regions analyzed undergo rapid expansion during early postnatal development, we first measured the size of the quantified brain regions and found that the cortex region expands substantially faster (slope of linear regression = 0.76 mm^2/day, **Figure 2B**) than subcortical regions (slope of linear regression = 0.17 or 0.19 mm^2/day for hypothalamus or thalamus, respectively, **Figure 2B**). The area of these brain regions was also used to calculate the density of total tdT^{+}, EdU^{+}, and tdT^{+}EdU^{+} cells in these regions. We found that the density (number of cells/mm^2) of tdT^{+} astrocytes was highest in cortex compared to thalamus and hypothalamus at P7 and P14 (**Figure 2C**). The density of astrocytes significantly decreased in all regions examined as brain volume rapidly increases in postnatal weeks 2–4 (**Figure 2C**). Although the density of EdU^{+} cells was generally comparable in all regions examined at P7 and P14 (**Figure 2D**), it was significantly higher in thalamus and hypothalamus than in cortex at P30 (**Figure 2D**), suggesting that proliferation activity in these regions remains active while cortical proliferation is much reduced from P15 to P30. It is noted that the density of EdU^{+} cells at P7 is highest in the cortex, though not significantly different from that in thalamus and hypothalamus, possibly due to the incomplete labeling of all proliferating cells. Similarly, although the density of tdT^{+}EdU^{+} cells, presumably proliferating astrocytes, is higher in cortex than that in hypothalamus at P7 (**Figure 2E**), the density of tdT^{+}EdU^{+} cells becomes significantly higher in thalamus ($p = 0.002$) and hypothalamus ($p = 0.019$) than in cortex at P30 (**Figure 2G**). The density of tdT^{+}EdU^{+} astrocytes is comparable across all examined regions at P14 (**Figure 2F**).

Although the *Slc1a3* promoter is widely active in astrocytes in the CNS, recent studies have indicated a dorsal to ventral axis heterogeneity in astrocyte gene expression (Farmer et al., 2016; Morel et al., 2017) in which the *Slc1a3* promoter could be heterogeneously activated in cortical and subcortical astrocytes. As a result, this potential *Slc1a3* promoter activity heterogeneity may become a confounding factor in quantifying genetically labeled astrocytes. We then decided to determine whether there is a comparable genetic labeling efficiency of astrocytes in cortex and hypothalamus in *Slc1a3*-CreER^{+}Ai14-tdT$^{f/+}$ mice. As it is likely that tdT^{+}-mediated labeling of astrocytes is incomplete with *Slc1a3*-CreER^{+}Ai14-tdT^{+} tamoxifen-injected mice, we employed Bac *Aldh1l1*-eGFP astrocyte reporter mice in which most if not all astrocytes are labeled with eGFP across the CNS based on previous studies (Cahoy et al., 2008), and quantified the number of eGFP^{+} astrocytes in cortex and hypothalamus respectively in a size-matched area as in *Slc1a3*-CreER^{+}Ai14-tdT^{+} mice. Interestingly, although cortex size expands significantly faster than hypothalamus during postnatal development (P7–30, **Figure 2B**), our quantification consistently found that cortical astrocyte density (# of astrocytes/mm^2) is only 74–80% of hypothalamic astrocyte density during the same time period depending on the exact time point examined. Based on eGFP^{+} and tdT^{+} astrocyte numbers from Bac *Aldh1l1*-eGFP and *Slc1a3*-CreER^{+}Ai-14-tdT$^{f/+}$ mice in corresponding regions (size-matched) and time points, we calculated the genetic labeling efficiency of astrocytes in cortex and hypothalamus from P3 to P7, P8 to P14, and P15 to P30 (**Figure 2H**) and found that the genetic labeling efficiency of astrocytes in cortex is typically 1.5 to 2-fold higher than that of hypothalamic astrocytes (**Figure 2H**). To eliminate the influence of the differential genetic labeling efficiency of astrocytes in these regions on the analysis, we calculated the percentage of proliferating astrocytes (tdT^{+}EdU^{+}/tdT^{+}) and found that both hypothalamus and thalamus have a substantially higher percentage of proliferating astrocytes ($p < 0.0001$) than in cortex from P15 to P30 (**Figure 2I**), consistent with the density-based proliferative astrocyte analysis in **Figure 2G**.

As it is likely that EdU-mediated labeling could pass several cycles of cell division, to further assess astrocyte proliferation activity in cortex and hypothalamus during early postnatal development, we performed immunostaining of Ki67, a nuclear marker of active proliferation, on cortical and hypothalamic sections of *Slc1a3*-CreER^{+}Ai14-tdT^{+} mice at different time points of postnatal development (**Figure 3A**) to provide a snapshot assessment of the proliferating astrocytes in these regions. As shown in **Figure 3B**, we observed widespread labeling of tdT^{+} astrocytes in brain and clear co-localization of tdT reporter with Ki67 immunoreactivity, an indication of proliferative astrocytes. The quantification of total tdT^{+} and tdT^{+}Ki67^{+} astrocytes showed 25% more proliferating astrocytes in cortex than in hypothalamus at P7 (**Figure 3C**), while 40% more proliferating astrocytes were observed in hypothalamus than in cortex at P14/P15 (**Figure 3D**). To assess whether astrocytes at cortex and hypothalamus still actively divide at P30 and beyond, we further performed immunostaining of Ki67 and Sox9, a recently characterized nuclear marker of adult astrocytes (Sun et al., 2017), and found essentially no Ki67^{+} cells or Ki67^{+}Sox9^{+} astrocytes at P30 in both cortex and hypothalamus (data not shown), suggesting that astrocytes in these regions become essentially proliferatively quiescent at P30 and beyond. These Ki67 snapshot analysis results further support our EdU-based analysis that more cortical astrocytes are proliferatively active than hypothalamic astrocytes at P7, but hypothalamic astrocytes are significantly more active in proliferation than cortical astrocytes from P15 to P30. Taken together, these results unveil distinct dynamics of astrocyte proliferation in subcortical regions of thalamus and particularly hypothalamus in comparison to cortical astrocytes. Consistent with previous observations (Ge et al., 2012), we found that cortical astrocytes robustly proliferate early at P7, but that the proliferation activity of these astrocytes is drastically reduced after the first week. In contrast, astrocytes in thalamus and particularly hypothalamus



Age	Cortex	Hypothalamus
P3-7	50.27 ± 9.38	28.22 ± 5.47
P8-14	59.60 ± 14.56	30.16 ± 9.64
P15-30	22.88 ± 1.80	14.98 ± 1.30

FIGURE 2 | Temporal proliferation dynamics of subcortical astrocytes during postnatal development. **(A)** Representative images highlighting the cortex, thalamus, and hypothalamus for quantifying proliferating cells; scale bar: 2 mm. Each white cross with a yellow dot represents an individual cell. **(B)** Changes in the size of cortex, thalamus, and hypothalamus during early postnatal development. One-way ANOVA with Tukey's *post hoc* test; significant differences between the means at P7 ($p = 0.001$, $F_{(2,12)} = 12.49$) and P30 ($p < 0.0001$, $F_{(2,9)} = 482.38$). Density of tdT$^+$ [significant differences between the means at P7 ($p = 0.044$, $F_{(2,7)} = 5.027$) and P14 ($p = 0.002$, $F_{(2,4)} = 49.8$)] **(C)** or EDU$^+$ [significant difference between the means at P30 ($p < 0.0001$, $F_{(2,9)} = 34.85$)] **(D)** cells in cortex, thalamus, and hypothalamus during postnatal development. *p*-values determined by one-way ANOVA and *post hoc* Tukey's test. Density of tdT$^+$EdU$^+$ cells in cortex, thalamus, or hypothalamus generated from P3–7 **(E)**, P8–14 **(F)**, and P15–30 **(G)**; one-way ANOVA followed by *post hoc* Tukey's test, significant difference between the means at P30 ($p = 0.002$, $F_{(2,9)}$=13.2). **(H)** Calculated genetic labeling efficiency of astrocytes from P3–7, P8–14, and P15–30 in cortex and hypothalamus. **(I)** Percentage of proliferating astrocytes (tdT$^+$EdU$^+$/tdT$^+$) in cortex, thalamus, or hypothalamus from P15–30; one-way ANOVA with Tukey's *post hoc* test; significant differences between the means ($p < 0.0001$, $F_{(2,8)} = 140.8$). $n = 3$ images/mouse for 4–5 individual mice. A total of 3000–5000 tdT$^+$ or EDU$^+$ cells were quantified from three images/mouse per region/time point (a total of 4–5 mice per condition). All *p*-values shown in the figure were determined by *post hoc* analysis, and if not otherwise specified represent a comparison between cortex and thalamus.

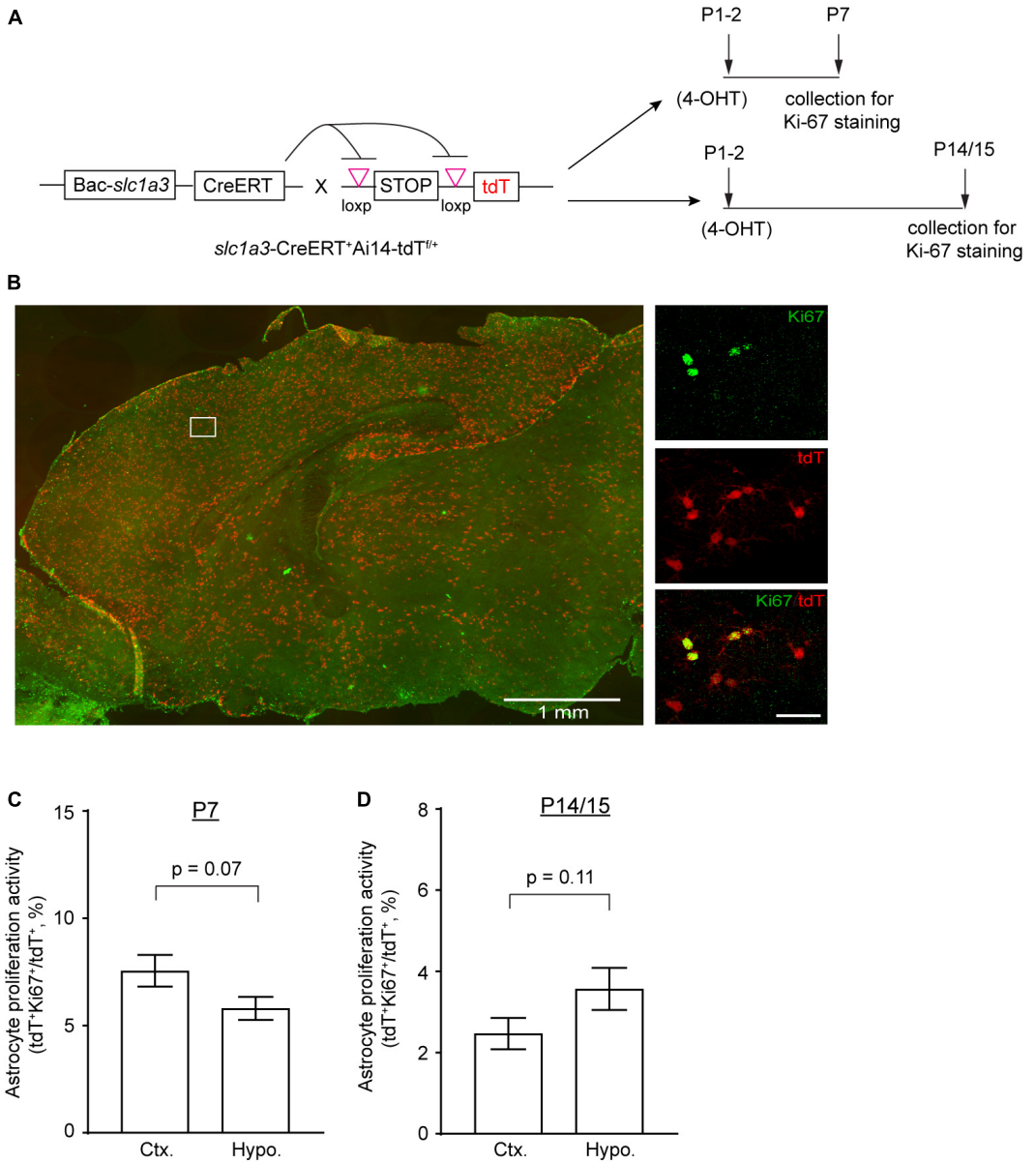


FIGURE 3 | Astrocyte proliferation activity in cortex and hypothalamus determined by genetic labeling of astrocytes and Ki67 immunostaining. **(A)** Experimental strategy for genetic labeling of astrocytes and Ki-67 staining of proliferating cells at different postnatal time points. **(B)** Representative images of astrocyte labeling with the tdT reporter in *Slc1a3*-CreERT^{+}Ai14-tdT$^{f/+}$ mice and Ki-67 labeling of actively proliferating cells in P7 brain; magnified images show cortical astrocytes. Scale bars: 1 mm (left panel), 30 μm (magnified images). Percentage of proliferating astrocytes (100 × tdT^{+}Ki-67^{+}/tdT^{+}) in cortex and hypothalamus at P7 **(C)** and P14/P15 **(D)**; n = 3 images/mouse from 3–4 individual mice per region/time point. p-values determined by unpaired two-tailed t-test.

become more active in proliferation than cortical astrocytes later in early postnatal development.

Morphological Maturation of Subcortical Astrocytes During Postnatal Development

Previous studies have found that cortical and hippocampal astrocytes undergo a maturation process during which the cell domain size significantly increases by growing abundant fine processes (Bushong et al., 2002; Morel et al., 2014). To determine whether subcortical astrocytes also undergo a similar morphological maturation process, we quantified hypothalamic astrocyte domain size from confocal images of EAAT2-tdT astrocyte reporter mice. We have previously shown that the tdT reporter is able to illustrate the full morphology of mature astrocytes, allowing direct measurement of the domain size (volume) of individual astrocytes from confocal images using Imaris image analysis software (Morel et al., 2014). Representative confocal and 3D Imaris images from

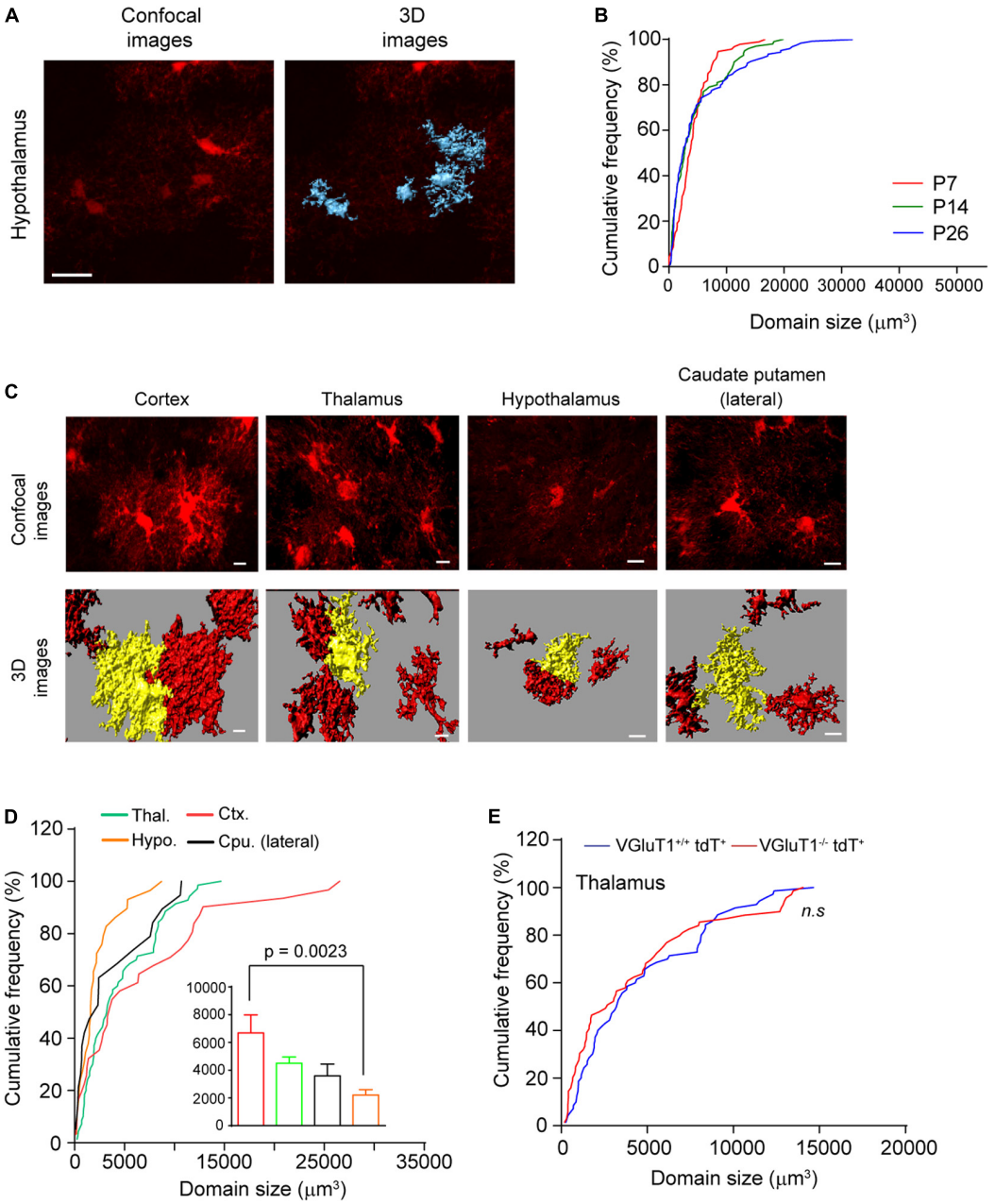


FIGURE 4 | Morphological maturation of subcortical astrocytes during postnatal development. **(A)** Representative confocal and 3D images of astrocytes from hypothalamus of *eaat2*-tdT mice. Scale bar: 20 μm; **(B)** cumulative frequency curve of astroglial domain size in hypothalamus at different postnatal developmental time points (P7, P14, and P26). *n* = 60 astrocytes/group from multiple mice; **(C)** representative confocal and 3D images of astrocytes from different brain regions of *eaat2*-tdT mice at P30. Scale bar: 20 μm; **(D)** cumulative frequency curve of astroglial domain size in these brain regions. The insert bar graph represents the average astroglial domain size (one-way ANOVA, significant differences between the means, $p = 0.002$, $F_{(3,145)} = 5.220$). *n* = 50–84 astroglia/group from multiple mice; *p*-value in the figure determined by *post hoc* Tukey's test. **(E)** Cumulative frequency curve of thalamic astroglial domain size in VGluT1$^{-/-}$tdT^{+} and VGluT1$^{+/+}$tdT^{+} mice. *n* = 70 astrocytes/group from multiple mice; n.s., not significant.

hypothalamic astrocytes of EAAT2-tdT mice are shown in **Figure 4A**. Subsequent quantification of domain size found that a majority of hypothalamic astrocytes have similar domain size at P7, P14, and P26, with only a small portion of astrocytes (∼20%) substantially growing their domain size from P7 to P26 (**Figure 4B**). These results reveal for the first time that hypothalamic astrocyte domain size only has a very modest growth during postnatal development, in sharp contrast to the dramatic growth of cortical astrocyte domain size during the same developmental period.

In addition to the small hypothalamic astrocyte domain size at P26, to gain insights about the morphological features

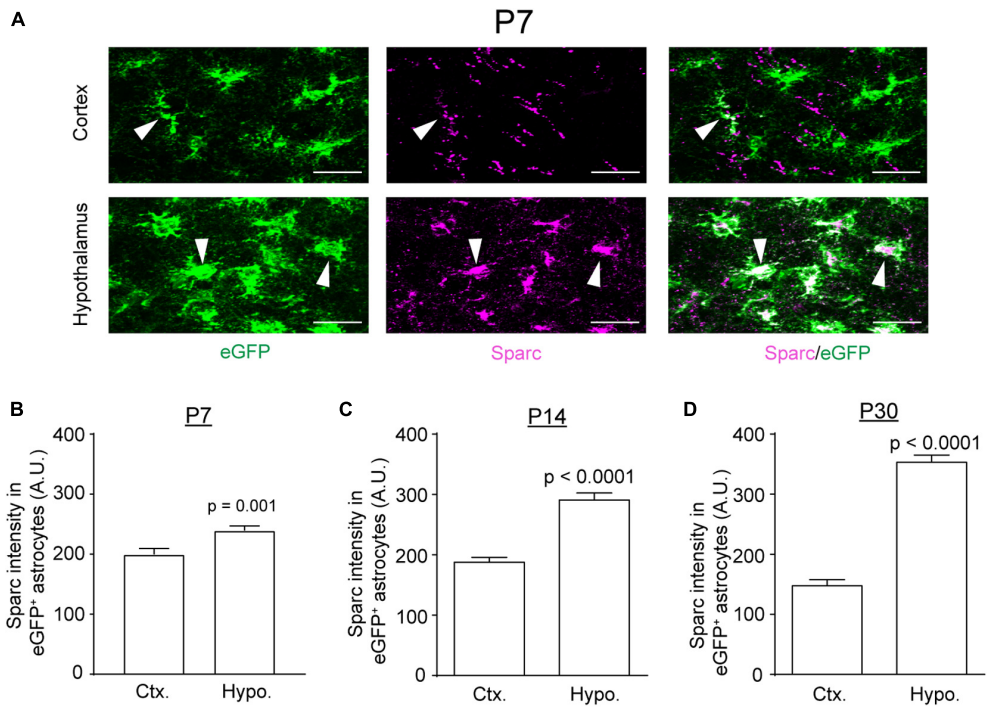


FIGURE 5 | Differential expression of Sparc in cortical and hypothalamic astrocytes during postnatal development. **(A)** Representative images of Sparc immunostaining in cortex and hypothalamus of Bac *Aldh1l1*-eGFP astrocyte reporter mice at P7; white arrows, positive Sparc immunostaining in eGFP$^+$ astrocytes; scale bar: 50 μm. Quantification of Sparc intensity in eGFP$^+$ astrocytes in cortex and hypothalamus at P7 **(B)**, P14 **(C)**, and P30 **(D)**. *n* = 118–134 astrocytes/group from multiple mice; *p*-values were determined by unpaired two-tailed *t*-test.

of astrocytes in other subcortical regions, we next measured the domain size of astrocytes from other representative subcortical regions (thalamus and lateral caudate putamen) from EAAT2-tdT mice, as shown in **Figure 4C**. The astrocytes in these subcortical regions appear morphologically less complex than those in cortex at P30 (**Figure 4C**). In particular, the enormous arborization of astroglial branches typically found in cortical astrocytes was not evident in astrocytes from other regions (**Figure 4C**). Subsequent quantification confirmed that astroglial domain size is smallest in the hypothalamus (2212 mm^3) and largest in the cortex (6687 mm^3) (**Figure 4D**). We previously showed that the arborization of cortical astroglial branches can be regulated by local VGluT1$^+$ neuronal glutamatergic signaling (Morel et al., 2014). To determine whether astrocytes in subcortical regions are regulated by the same mechanism, we quantified astroglial domain size in the thalamus from VGluT1$^{+/+}$tdT$^+$ and VGluT1$^{-/-}$tdT$^+$ mice at P30. We and others have previously shown that the loss of VGluT1 drastically reduces glutamatergic signaling in the CNS (Fremeau et al., 2004). As shown in **Figure 4E**, the distribution of thalamic astroglial domain size is similar in VGluT1$^{-/-}$tdT$^+$ and VGluT1$^{+/+}$tdT$^+$ mice, suggesting that thalamic astroglia domain size is not influenced by the loss of neuronal VGluT1$^+$ glutamatergic signaling. We previously also observed similar results in hypothalamic astrocytes of VGluT1$^{-/-}$tdT$^+$ and VGluT1$^{+/+}$tdT$^+$ mice (Morel et al., 2014). The selective effect of VGluT1$^+$ neuronal signaling on cortical but not on thalamic (and hypothalamic) astrocyte domain size indicates a region-specific regulatory mechanism for astrocyte morphological maturation during postnatal development.

We recently profiled translating mRNAs in adult astrocytes from multiple brain regions, through which we identified several genes that are differentially expressed in astrocytes across brain regions (Morel et al., 2017). In particular, we found that the expression of Sparc, one of astrocyte-secreted modulators of synaptogenesis (Allen and Eroglu, 2017), is substantially higher in adult subcortical (hypothalamic and thalamic) astrocytes than in cortical and hippocampal astrocytes. To determine whether the differential expression pattern of Sparc in these regions stems from earlier developmental stages, we performed immunostaining of Sparc in cortex and hypothalamus of Bac *Aldh1l1*-eGFP astroglial reporter mice at different developmental time points (P7, P14, and P30). As shown in **Figure 5A**, Sparc immunoreactivity is widely co-localized with eGFP$^+$ astrocytes (white arrows) in both regions at P7 (representative images for other developmental time points not shown). Quantification of Sparc immunoreactivity in eGFP$^+$ astrocytes showed that Sparc immunoreactivity in hypothalamic astrocytes is significantly higher than that in cortical astrocytes as early as P7 and persists at P14 and P30 (**Figure 5B–D**), suggesting that the differential expression pattern of Sparc in astrocytes starts in early astrogliogenesis and continues through postnatal development into the adult.

DISCUSSION

In the current study, we investigated the postnatal proliferation and maturation of astrocytes in subcortical regions by employing astrocyte genetic and EdU pulse-chase labeling. In contrast to the robust proliferation of cortical astrocytes within the first postnatal week, we found that astrocytes in subcortical regions, particularly in hypothalamus are less proliferatively active than cortical astrocytes during the same period. However, a significantly higher percentage of hypothalamic astrocytes remain proliferatively active from P15 to P30 than cortical astrocytes, indicating that hypothalamic astrocytes have a distinct temporal, particularly a more persistent proliferation dynamic in comparison to cortical astrocytes. This is also in parallel to our quantification that hypothalamic astrocyte density is 20–26% higher than cortical astrocyte density throughout postnatal development. As the area of cortex expands at a faster rate than subcortical regions (**Figure 2B**), it is unlikely that the increased percentage of proliferative astrocytes in subcortical regions from P15 to P30 is due to a greater territorial expansion of those brain regions compared to cortex.

Although our combined use of astrocyte genetic and EdU pulse-chase labeling effectively labels proliferating astrocytes, as we intended to assess the proliferation activity of astrocytes but not to label all proliferating cells at a given time period, we decide not to inject a high dose of EdU or perform repeated injections to label all proliferating cells. Similarly, our EdU injection dose also serves to sample but not label all proliferating cells. As a result, it is possible that not all dividing tdT^{+} astrocytes were sampled by the EdU injection or underwent division at the time when EdU was injected. In addition, as EdU likely labels more than one cycle of dividing cells, EdU-based quantification may include the number of proliferating astrocytes from multiple generations, which is different from the single-day snapshot quantification based on Ki67 immunostaining. Despite the difference in quantifying proliferative astrocytes, both approaches showed similar results that astrocyte proliferation activity is switched in cortex and hypothalamus during early postnatal development. Given the observation that there is a higher percentage of proliferative astrocytes at P14/P15 in hypothalamus and that there are essentially no proliferative astrocytes at P30 in both regions (based on Ki67/Sox9 staining and (Ge et al., 2012), it is likely that hypothalamic astrocytes are persistently more active than cortical astrocytes from P15 until astrocytes from both regions become quiescent near the P30 time point.

The distinct proliferation dynamics between cortical and subcortical (hypothalamic) astrocytes are likely to closely associate with the unique characteristics of synaptogenesis in each brain region. Immature (but not mature) cortical astrocytes are known to secret extracellular matrix proteins, such as thrombospondin (Thbs), hevin, and glypican, etc., (Allen and Eroglu, 2017) to actively promote the formation and function of glutamatergic synaptogenesis that is the dominant synapse type in cortex. Therefore, the massive generation of immature cortical astrocytes in a relatively short time period may potentially facilitate the supply of such extracellular proteins to promote glutamatergic synaptogenesis in the cortex. In contrast, as interneurons are more widely distributed in hypothalamus (Obrietan and van den Pol, 1995) and Thbs/hevin/glypican have no apparent effect on promoting GABAergic synaptogenesis (Allen and Eroglu, 2017), it is not unexpected that hypothalamic astrocyte proliferative activity is low at first. In addition to the distinct proliferation dynamics, we found that Sparc expression is significantly higher in hypothalamic astrocytes than in cortical astrocytes as early as P7. As Sparc antagonizes the synaptogenic effect of hevin during glutamatergic synaptogenesis (Kucukdereli et al., 2011), higher Sparc levels may also help maintain a GABAergic synaptic environment in the hypothalamus. This is also consistent with the observation that the growth of astrocyte domain size in subcortical thalamus and hypothalamus is not influenced by the loss of glutamatergic synaptic signaling in $VGluT1^{-/-}$ mice. The higher Sparc levels at P7 also support the notion that the molecular differences between adult cortical and hypothalamic astrocytes are likely to be predetermined in progenitors that are heterogeneously positioned along the VZ during late embryogenesis. Moreover, astrocytes in representative subcortical regions also show a modest growth of their domain sizes and exhibit a significantly smaller domain size compared to that of cortical astrocytes at P30 when astrocytes generally complete postnatal maturation. This likely reflects a difference in astroglial coverage on synapses in these regions, which subsequently affects how astrocytes modulate synaptic signaling.

DATA AVAILABILITY STATEMENT

No large datasets were generated from this study. All data supporting the findings of this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Tufts University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

TS bred mice and performed the tamoxifen and EdU injections, imaging, cell quantification, immunostaining, and data analysis. AO performed the tamoxifen injections, immunostaining, imaging, quantification, and data analysis. RJ bred mice and performed the immunostaining, imaging, quantification, and data analysis. YM performed the quantification, Ki67 immunostaining, and data analysis. MC performed the immunostaining and mouse breeding. YY designed the overall study, analyzed the data, and wrote the manuscript.

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Brainstem Organoids From Human Pluripotent Stem Cells

Nobuyuki Eura[1†], Takeshi K. Matsui[1,2†], Joachim Luginbühl[3†], Masaya Matsubayashi[2], Hitoki Nanaura[1,2], Tomo Shiota[1], Kaoru Kinugawa[1], Naohiko Iguchi[1], Takao Kiriyama[1], Canbin Zheng[4], Tsukasa Kouno[3], Yan Jun Lan[3], Pornparn Kongpracha[5], Pattama Wiriyasermkul[5], Yoshihiko M. Sakaguchi[2], Riko Nagata[2], Tomoya Komeda[2], Naritaka Morikawa[2], Fumika Kitayoshi[2], Miyong Jong[2], Shinko Kobashigawa[2], Mari Nakanishi[2], Masatoshi Hasegawa[6], Yasuhiko Saito[7], Takashi Shiromizu[8], Yuhei Nishimura[8], Takahiko Kasai[9], Maiko Takeda[9], Hiroshi Kobayashi[10], Yusuke Inagaki[11], Yasuhito Tanaka[11], Manabu Makinodan[12], Toshifumi Kishimoto[12], Hiroki Kuniyasu[13], Shushi Nagamori[5], Alysson R. Muotri[14,15], Jay W. Shin[3], Kazuma Sugie[1]* and Eiichiro Mori[2]**

[1] Department of Neurology, Nara Medical University, Kashihara, Japan, [2] Department of Future Basic Medicine, Nara Medical University, Kashihara, Japan, [3] Laboratory for Advanced Genomics Circuit, RIKEN Center for Integrative Medical Sciences, Yokohama, Japan, [4] Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, United States, [5] Laboratory of Biomolecular Dynamics, Department of Collaborative Research, Nara Medical University, Kashihara, Japan, [6] Department of Radiation Oncology, Nara Medical University, Kashihara, Japan, [7] Department of Neurophysiology, Nara Medical University, Kashihara, Japan, [8] Department of Integrative Pharmacology, Graduate School of Medicine, Mie University, Tsu, Japan, [9] Department of Laboratory Medicine and Pathology, National Hospital Organization Kinki-Chuo Chest Medical Center, Sakai, Japan, [10] Department of Obstetrics and Gynecology, Nara Medical University, Kashihara, Japan, [11] Department of Orthopaedic Surgery, Nara Medical University, Kashihara, Japan, [12] Department of Psychiatry, Nara Medical University, Kashihara, Japan, [13] Department of Molecular Pathology, Nara Medical University, Kashihara, Japan, [14] Department of Pediatrics, University of California, San Diego, San Diego, CA, United States, [15] Department of Cellular and Molecular Medicine, University of California, San Diego, San Diego, CA, United States

The brainstem is a posterior region of the brain, composed of three parts, midbrain, pons, and medulla oblongata. It is critical in controlling heartbeat, blood pressure, and respiration, all of which are life-sustaining functions, and therefore, damages to or disorders of the brainstem can be lethal. Brain organoids derived from human pluripotent stem cells (hPSCs) recapitulate the course of human brain development and are expected to be useful for medical research on central nervous system disorders. However, existing organoid models are limited in the extent hPSCs recapitulate human brain development and hence are not able to fully elucidate the diseases affecting various components of the brain such as brainstem. Here, we developed a method to generate human brainstem organoids (hBSOs), containing midbrain/hindbrain progenitors, noradrenergic and cholinergic neurons, dopaminergic neurons, and neural crest lineage cells. Single-cell RNA sequence (scRNA-seq) analysis, together with evidence from proteomics and electrophysiology, revealed that the cellular population in these organoids was similar to that of the human brainstem, which raises the possibility of making use of hBSOs in investigating central nervous system disorders affecting brainstem and in efficient drug screenings.

Keywords: brain organoids, brainstem, neural crest, midbrain, dopaminergic neurons, human pluripotent stem cells, melanocyte

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Harvard Medical School, United States

***Correspondence:**
Jay W. Shin
jay.shin@riken.jp
Kazuma Sugie
ksugie@naramed-u.ac.jp
Eiichiro Mori
emori@naramed-u.ac.jp

[†] *These authors have contributed equally to this work*

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INTRODUCTION

The brainstem is a posterior region of the brain between the deep structures of the cerebral hemispheres. It connects the cerebrum with the spinal cord and is divided into three parts: midbrain, pons, and medulla oblongata. They contain multiple nuclei and small fiber tracts widely projecting to the cerebrum cortex, basal ganglia, and other parts of the cerebrum. Brainstem functions such as alertness, heartbeat, blood pressure, and respiration are considered to be more vital for life than that of the cortex. Therefore, damages to or disorders of brainstem including infarction, hemorrhage, tumors, or any neurodegenerative diseases may lead to death. To investigate the pathology of these diseases and to establish novel therapies, models recapitulating brainstem tissue are needed.

Recent progress on protocols for inducing organs in-a-dish (organoids) provides potentials for the modeling of various diseases (Clevers, 2016). Organoids mimic the structure of organs composed of various cells such as the kidney (Takasato et al., 2015), brain (Dang et al., 2016), colon (Sato et al., 2009), and retina (Eiraku et al., 2011; Nakano et al., 2012). The use of brain organoids is a recognized method for the recapitulation of human fetal development during *in vitro* cultivation (Lancaster et al., 2013; Lancaster and Knoblich, 2014; Trujillo et al., 2019).

However, improvements to the protocols are still needed, particularly in aspects such as the maturity, efficiency, and the extent of recapitulation captured in the organoids. Recently, a protocol for generating human midbrain-like organoids from human pluripotent stem cells (hPSCs) was reported (Jo et al., 2016). There are also reports on the effects of reagents or growth factors on the differentiation of dopaminergic neurons (Diaz et al., 2009; Ayton et al., 2016; Lee et al., 2016). Based on these findings, we designed a new method for generating a human brainstem organoid (hBSO) model where the midbrain, surrounding brainstem parts, and neural crest region behind them are induced by the addition of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) for neuronal stem/progenitor cells expansion. This is followed by treatment with brain-derived neurotrophic factor (BDNF), glial cell line–derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), cyclic adenosine monophosphate (cAMP), and ascorbic acid for the differentiation of dopaminergic neurons. In the present study, we established a novel method for inducing hBSOs. We believe our methods will become a powerful tool in examining the pathology of neurodegenerative or neurodevelopmental diseases affecting the brainstem.

MATERIALS AND METHODS

Cell Culture

Human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) are maintained in feeder-free condition with mTeSR1 media. Human iPSC line (XY) was obtained from Takara, Kusatsu, Shiga, Japan, and human H9 ESC line (WA09) was purchased from WiCell Research Institute, Madison, WI, United States. Embryonic stem cells and iPSCs were cultivated in mTeSR1 medium (Stemcell Technologies, Vancouver, British Columbia, Canada), based on feeder-free culture protocols on six-well plates (Corning, Corning, NY, United States), coated with growth factors reduced Matrigel (BD Biosciences, San Jose, CA, United States). At the time of passage, we added ROCK inhibitor (final concentration 10 μM; Selleck Chemicals, Houston, Texas, United States). These cells were maintained with daily medium change without ROCK inhibitor until they reached approximately 70% confluence. Then, they were detached by Versene Solution (Thermo Fisher Scientific, Waltham, MA, United States) and seeded by 1:20 dilution ratio.

Human Brainstem Organoid Generation

The hBSOs were generated with some modifications on the cerebral cortical organoid protocol (Thomas et al., 2017; Trujillo et al., 2018). Human iPSCs/ESCs were gently dissociated by 10 min of treatment with 50% Accutase (Sigma A6964) in phosphate-buffered saline (PBS). Detached cells were transferred to six-well plates at the density of four million cells in 5 ml mTeSR1 medium with 5 μM ROCK inhibitor, 1 mM dorsomorphin (Wako, 040-33753) and 10 μM SB431542 (Cayman Chemical, 13031) per well in six-well plates on the orbit shaker (WakenBtech) to keep the cells in suspension. For neural induction from day 3, media was switched to one composed of neurobasal medium (Thermo Fisher Scientific, Waltham, MA, United States) and 2× Gem21NeuroPlex (Gemini Bio-Products, CA, United States), 1× non-essential amino acid solution (NEAA, Sigma-Aldrich), 1× GlutaMAX (Thermo Fisher Scientific, Waltham, MA, United States), 1 mM dorsomorphin, 10 μM SB431542, 10 μM transferrin, 5 mg/L human insulin, and 0.063 mg/L progesterone. After 9 days of exposure to dorsomorphin and SB431542, we treated the cells with 20 ng/mL bFGF (Peprotech, AF-100-18B) to induce neural progenitor cell (NPC) proliferation in the presence of neurobasal-A medium (Thermo Fisher Scientific, Waltham, MA, United States), supplemented with 2× Gem21NeuroPlex, 1× NEAA, 1× GlutaMax, 10 μM transferrin, 5 mg/L human insulin, and 0.063 mg/L progesterone until day 16. Cells were then kept in the same media containing not only 20 ng/mL bFGF, but also 20 ng/mL EGF (Wako, 059-07873) until day 22. After day 22, EGF and bFGF were replaced by ascorbic acid (nacalai, 13048-42), cAMP (nacalai, 11540-74), BDNF (Wako, 028-16451), GDNF (Wako, 075-04153), and NT-3 (Peprotech, 450-03). After day 28, cells were cultivated without any growth factors for neuronal maturation. Organoid results were combined from at least three separate batches of inductions.

Human Cerebral Organoid Generation

The human cerebral organoids (hCOs) were generated as per previously reported protocols (Lancaster et al., 2013; Lancaster and Knoblich, 2014). Human iPSCs/ESCs were detached and subjected to embryoid body (EB) induction using the protocol. After 4 days, half of the media was replaced by human EB medium without ROCK inhibitor and bFGF. After 2 days, the EBs were transferred into a neural induction media and embedded in Matrigel after 5 days. The organoids were subsequently induced by the use of an orbital shaker, following the original protocol.

Immunohistochemical Analysis

Each human cerebral or brainstem organoid was fixed in 4% paraformaldehyde in PBS overnight at 4°C, dehydrated with 30% sucrose in PBS and embedded in O.C.T. compound (Thermo Fisher Scientific, Waltham, MA, United States). Cryostat sections (14 μm) were cut and mounted onto slides (Thermo Fisher Scientific, Waltham, MA, United States). Mounted sections were incubated for 1 h at room temperature with blocking solution [3% normal goat serum + 0.3% Triton X-100 in Tris-buffered saline (TBS)] and incubated with primary antibodies (**Supplementary Table S1**) diluted in blocking solution overnight at 4°C. After three washes with TBS, corresponding fluorophore-conjugated secondary antibodies diluted in the blocking solution were added and incubated for 2 h at room temperature and followed by DAPI staining. Finally, stained slides were rinsed with TBS three times, mounted, and analyzed using a FV3000 Confocal Microscope (Olympus, Shinjuku, Tokyo, Japan).

RNA Isolation, Reverse Transcriptase–Polymerase Chain Reaction, and Quantitative Polymerase Chain Reaction

RNA from hCOs/hBSOs and ESCs was extracted according to the protocol supplied with TRIzol reagent (15596018; Thermo Fisher Scientific, Waltham, MA, United States). The concentration and purity of the RNA samples were measured using Spectrophotometer (Beckman Coulter, Brea, CA, United States). Extracted RNA samples were either shipped to bioengineering laboratory for RNA sequencing analysis or subjected to reverse transcriptase–polymerase chain reaction (RT-PCR). For RT-PCR, the extracted RNAs were reverse transcribed according to the protocol supplied with ReverTra Ace qPCR RT Master Mix (FSQ-201; TOYOBO, Osaka, Osaka, Japan). StepOne Plus Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, United States) was used to amplify and quantify levels of target gene cDNA. Real-time quantitative RT-PCR (qRT-PCR) was performed with SsoAdvanced Universal SYBR Green Supermix (172-5271; Bio-Rad Laboratories, Hercules, CA, United States) and specific primers for qRT-PCR (**Supplementary Table S2**). The cycling conditions for PCR program were 2 min at 95°C for activation followed by 40 cycles of 95°C, over a duration of 5 s for denaturation, 60°C for 30 s for annealing, 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s for melt curve stage. Reactions were run in triplicate. The expression of each gene was normalized to the geometric mean of β-actin as a housekeeping gene and analyzed using the ΔΔCT method. Mean threshold cycle values of each gene in qPCR are shown in **Supplementary Tables S3** and **S4**. Statistical significance was calculated by a two-tailed Student *t* test. A *p* value of less than 0.05 was considered statistically significant.

RNA Sequencing

Total RNA was isolated from cells using the PureLink RNA Mini Kit (12183018A) according to the manufacturer's instructions. RNA concentration was analyzed by Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States), and the purity was assessed using the Qsep100 DNA Fragment Analyzer and RNA R1 Cartridge (BiOptic, New Taipei City, Taiwan). Subsequently, total RNA was converted to cDNA and used for Illumina sequencing library preparation based on the KAPA Stranded mRNA-Seq Kit protocols (KAPA Biosystems, Wilmington, MA, United States). DNA fragments were then subjected to adapter ligation, where dsDNA adapters with 3'-dTMP overhangs were ligated to A-tailed library insert fragments by FastGene Adapter Kit (NIPPON Genetics, Bunkyo, Tokyo, Japan). The purified cDNA library products were evaluated using Qubit and dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States), followed by quality assessment using the Fragment Analyzer and dsDNA 915 Reagent Kit (Advanced Analytical Technologies, Ankeny, IA, United States) and finally by sequencing (2 × 75 bp) on NextSeq 500 (Illumina, San Diego, CA, United States).

Transcriptome Analysis

A count-based differential expression analysis "TCC" was used to identify differently expressed genes (DEGs) in the RNA-seq data with a thresholded false discovery rate of 20% (Sun et al., 2013). iRegulon (Janky et al., 2014) was used to identify transcription factors (TFs) potentially regulating the DEG with normalized enrichment scores >4 as the threshold. Genotype-Tissue Expression (GTEx) (GTEx Consortium, 2013) was used to analyze the similarity of expression pattern between organoids and various tissues in the brain.

Electrophysiology

Electrophysiological recordings of the cells in hBSOs at 3 months were performed. An organoid was transferred to a glass-bottom recording chamber on an upright microscope (Leica DM LFS; Leica, Wetzlar, Germany) and continuously perfused with an extracellular solution containing (in mM) 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 25 glucose and aeration with 95% O_2 and 5% CO_2 (pH 7.4) at a rate of 2 mL/min. The organoid was held down by a weighted net to prevent it from moving. The bath temperature was maintained at 30–32°C using an in-line heater (TC-324B; Warner Instruments, Hamden, CT, United States). Whole-cell current-clamp recordings were performed using an EPC-8 patch-clamp amplifier (HEKA, Darmstadt, Germany). Patch pipettes were prepared from borosilicate glass capillaries and filled with an internal solution containing (in mM) 120 K-methylsulfate, 10 KCl, 0.2 EGTA, 2 MgATP, 0.3 NaGTP, 10 HEPES, 10 Na_2-phosphocreatine, and 0.1 spermine, adjusted to pH 7.3 with KOH. The osmolarity of the internal solution was 280–290 mOsm/L, and the resistance of the patch electrodes was 4–8 MΩ in the bath solution. The voltage signals were low-pass filtered at 3 kHz and digitized at 10 kHz. The calculated liquid junction potential of -5 mV was corrected. The data were acquired using a pClamp 9 system (Molecular Devices, Sunnyvale, CA, United States). Voltage responses of the cells were investigated by the application of depolarizing and hyperpolarizing current pulses (400 ms in duration). Off-line analysis was performed using AxoGraph X software (AxoGraph Scientific, Berkeley, CA, United States). The input capacitance

was estimated based on the current induced by a 10-mV-voltage step from a holding potential of -70 mV. The input resistance was estimated based on the voltage change induced by an applied hyperpolarizing current pulse of -40 pA. The spike amplitude was determined by the spike height from its threshold, defined as the membrane potential at which the derivative of the voltage trace reached 10 V/s. The maximum firing frequency was obtained from cells that exhibited more than one spike and calculated as the reciprocal of the shortest interspike interval between successive pairs of spikes.

Mass Spectrometric Analysis

Human ESCs (hESCs), iPSCs, ESC-derived organoids, and iPSC-derived organoids were washed with ice-cold PBS, harvested by scraping and centrifugation, and frozen in liquid nitrogen. The frozen cells and organoids were crushed by using Multi-beads shocker (Yasui Kikai, Japan) and subsequently lysed by sonication in 9.8 M urea with protease inhibitor cocktail (cOmplete; Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (PhosSTOP; Roche, Basel, Switzerland). The clear lysate was collected by centrifugation, and protein concentration was measured by BCA protein assay. Twenty micrograms of proteins was mixed with an internal standard protein mixture (10 fmol/ml MassPREP; Waters, Milford, MA, United States) and incubated with 2 mM Tri(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) for 30 min at 37°C for reduction, followed by alkylation with 55 mM iodoacetamide for 30 min at room temperature. The mixture was then diluted fourfold with 0.1 M triethylammonium bicarbonate and subjected to trypsin digestion (1:40 trypsin: sample ratio) for 3 h at 37°C. The digestion was terminated by trifluoroacetic acid, following by desalting with SDB-XC StageTips. The samples were fractionated into eight fractions by using SDB StageTips. Each fraction was dried by vacuum and dissolved in the measurement buffer (3% acetonitrile and 0.1% formic acid). Mass spectrometry was performed as described previously (Uetsuka et al., 2015). To identify the proteins, raw data of peptides were analyzed using Proteome Discoverer 2.2 (Thermo Fisher Scientific, Waltham, MA, United States) and Mascot 2.6 (Matrix Science, London, United Kingdom). The peptide results from all eight fractions were combined and subjected to search for the matching proteins in UniProt human database (The Uniprot Consortium, 2018). Maximum numbers of missed cleavages, precursor mass tolerance, and fragment mass tolerance were set to 3, 10 ppm, and 0.01 Da, respectively. The carbamidomethylation on Cys was set as a fixed modification, whereas oxidation of Met and deamidation of Asn and Gln were set as variable modifications. A filter of false discovery rate of less than 1% was applied to the data.

The Minora Feature Detector node was used for label-free quantification, and the consensus workflow included the Feature Mapper and the Precursor Ion Quantifier nodes using intensity for the precursor quantification. The protein intensities were normalized by the total peptides intensity. In addition, annotations from the Ingenuity Knowledge Base (IKB; released in autumn, 2018; Qiagen, Redwood City, CA, United States) and the database of Ingenuity Pathway Analysis were used to determine the localization and functional categories of the identified proteins.

For downstream analysis, we used the data normalized by Proteome Discoverer 2.2. All the following analysis was calculated on R. For missing value handling, we first applied listwise deletion method and removed the rows containing missing values. The removed rows are shown in **Supplementary Table S5**. The respective correlation coefficients between iPSCs, ESCs, iPS-derived brainstem organoid, and ES-derived brainstem organoid were calculated after log transformation, and the correlation coefficient, the distributions of all genes, and scatter plots of all the genes in each sample were shown by "pairs.panels" function in psych, R package[1] (**Supplementary Figure S1A**). We performed principal components analysis (PCA) and analyzed the contribution rate of each principal component (PC) on the log-transformed data (**Supplementary Figures S1B,C**). Next, after trimmed mean of M values (TMM) normalization, we extracted DEGs between stem cells and brainstem organoids based on the likelihood ratio test by edgeR, R package[2]). The threshold of DEGs was $p < 0.05$.

scRNA-Seq and Data Analysis

To dissociate hBSOs into single cells, we incubated them for ∼30 min in Accutase (Stemcell Technologies, Vancouver, British Columbia, Canada) at 37°C. Droplet-based scRNA-seq libraries were generated using the Chromium Single Cell 3' Reagent kits V2 (10X Genomics, Pleasanton, CA, United States). Cell number and cell viability were assessed using the Countess II Automated Cell Counter (Thermo Fisher Scientific, Waltham, MA, United States). Thereafter, cells were mixed with the Single Cell Master Mix and loaded together with Single Cell 3' Gel beads and Partitioning Oil into a Single Cell 3' Chip. RNA transcripts were uniquely barcoded and reverse-transcribed in droplets. cDNAs were pooled and amplified according to the manufacturer's protocol. Libraries were quantified by high-sensitivity DNA reagents (Agilent Technologies, Santa Clara, CA, United States) and the KAPA Library Quantification kit (KAPA Biosystems, Wilmington, MA, United States). Libraries were then sequenced by Illumina Hiseq 2500 in rapid mode.

Raw sequencing data from the organoid were preprocessed using the Cell Ranger (v 2.2.0; 10X Genomics, Pleasanton, CA, United States) software (Zheng et al., 2017). Reads were aligned to the GRCh38 human reference genome using STAR. After processing by Cell Ranger, the scRNA-seq data were analyzed using the Seurat v.3.0.0 R package (Satija et al., 2015). Cells with more than 8,000 or fewer than 750 detected genes, as well as cells expressing more than 5% mitochondrial genes, were excluded. Genes expressed in fewer than three cells were excluded. We collected a total of 2,345 cells expressing a total of 19,454 genes. The data sets were log normalized and scaled to 10,000 transcripts per cell. The top 2,000 highly variable genes were determined using the variance-stabilizing transformation method. The data sets were scaled and unique molecular identifier counts, ribosomal genes, and mitochondrial

[1]https://cran.r-project.org/web/packages/psych/index.html

[2]https://bioconductor.org/packages/release/bioc/html/edgeR.html

genes were regressed out. We analyzed the data sets by using "SCTransform" function in Surat (Hafemeister and Satija, 2019). After PCA, clustering was performed based on the top 15 PCs using the shared nearest neighbor modularity optimization with a resolution of 0.8. Cluster identities were assigned based on cluster gene markers determined by the "FindAllMarkers" function in Seurat (**Supplementary Table S6**).

RESULTS

To generate hBSOs from hPSCs, we used a combination of several growth factors, including EGF/bFGF for the initial proliferation of neuronal stem/progenitor cells, and BDNF, GDNF, and NT-3 for the subsequent differentiation of dopaminergic neurons and neural crest cells. This procedure is different from the protocols in previously reported studies (**Figure 1A** and **Supplementary Figure S2**). A recent study by Muotri and colleagues reported the presence of neural networks in their cortical organoids with advanced maturity (Trujillo et al., 2018). We further modified their protocol to induce dopaminergic neurons by adding insulin, transferrin, and progesterone, all of which have been shown to be protective or induce dopaminergic neuronal differentiation in two-dimensional culture (Diaz et al., 2009; Ayton et al., 2016; Lee et al., 2016). Our novel approach to generate hBSOs yielded cells with dark granules between 22 and 28 days of cultivation (**Figure 1A**), an observation that was absent at a similar stage in previous studies (Thomas et al., 2017; Trujillo et al., 2018).

On immunohistochemistry (IHC), we detected melanin in the hBSOs by hematoxylin–eosin, Fontana–Masson, and HMB45 stainings (**Figure 1B**), showing that these dark cells were melanocytes derived from neural crest cells in the organoids. The expression of *SOX9* [12.1% (74 of 608 cells)], which plays a role in the migration of neural crest cells (Spokony et al., 2002), also supported the existence of neural crest population in the brainstem organoids (**Figure 1B**).

Based on a quantitative PCR (qPCR) analysis of 1-month-old hBSOs, we confirmed distinct expression of various markers for neuronal cells. Additionally, we detected the neural stem/progenitor cell markers *SOX2*, *ASCL1*, *SLC1A3*, and *OTX2* (**Figure 2A**), which are necessary for the development of anterior brain structures including the midbrain (Wurst and Prakash, 2014). Our analyses also demonstrated the expression of *FOXA2*, a potent inducer of midbrain dopaminergic (mDA) progenitors (Sasaki and Hogan, 1993; Norton et al., 2005; Kittappa et al., 2007; Lin et al., 2009; Ribes et al., 2010), and *NR4A2*, which is essential for both the survival and final differentiation of ventral mesencephalic late dopaminergic precursor neurons into dopaminergic neurons (Saucedo-Cardenas et al., 1998), and *SOX6*, important for the specification of substantia nigra dopamine neurons (Panman et al., 2014; **Figure 2A** and **Supplementary Figure S3**). We also detected the expression of *LMX1A*, required to trigger dopamine cell differentiation (Andersson et al., 2006), and *EN1*, required in early development of mDA neuron (Alves dos Santos and Smidt, 2011), in hBSOs, and both genes were not revealed in hESCs (**Supplementary Table S3**). Consistently, we detected the expression of mRNAs coding for the pan-neuronal marker *MAP2* and the mature dopaminergic neuronal marker *TH* (**Figure 2A**). Using IHC, we demonstrated that the organoids have midbrain components via the detection of protein expressions of SOX2 [26.8% (153 of 571 cells)], OTX2 [16.5% (55 of 333 cells)], and TH [14.7% (84 of 571 cells)], which also indicated that the organoids contain midbrain components (**Figure 2B**). Other midbrain or mDA markers were also detected in 1-month hBSOs by qPCR (**Supplementary Figure S3** and **Supplementary Table S3**).

Furthermore, we observed expressions of *ChAT* on qPCR and IHC [17.4% (83 of 478 cells)] (**Figure 3A**). The detection of ChAT, a marker for cholinergic neurons, suggests the existence of medulla population (Stornetta et al., 2013). GBX2 is a hindbrain marker that plays a role in the positioning of the midbrain/hindbrain boundary with OTX2. Its expression [in 15.2% (44 of 289) cells] suggests that the hBSOs included midbrain and hindbrain population (Waters and Lewandoski, 2006; **Figure 3B**). The expression of DBH [13.9% (63 of 453 cells)], a marker for the central noradrenergic nervous system, may indicate that pons and medulla components are contained in the hBSOs (Swanson and Hartman, 1975; **Figure 3B**). Also, we detected *VGLUT1* and *GAD67*, markers for mature and functional excitatory and inhibitory neurons, respectively (Soghomonian and Martin, 1998; Fremeau et al., 2004). The expression of *OLIG2* and *MBP* indicated that our organoids contained oligodendrocyte progenitors and mature oligodendrocytes (Wei et al., 2003), whereas the presence of S100β suggested the existence of astrocytes (**Figure 3C** and **Supplementary Figure S3**). In addition, the qPCR analysis on 3-month-old hBSOs demonstrated the expressions of a variety of neuronal components (**Supplementary Figure S4** and **Supplementary Table S4**).

To further verify the translated products in hBSOs, we performed protein mass spectrometric analysis of the hBSOs at 1 month. Finally, we identified 3,458 DEGs, of which 763 genes satisfied false recovery rate (FDR) <0.05. Of these 763 proteins, we identified genes found to be enriched in brainstem, cerebellum, or basal ganglia (**Table 1**), suggesting that hBSOs have specific components for brainstem or cerebellum (Uhlen et al., 2015, 2017; Thul et al., 2017).

To assess the electrical functionality of the neurons in the hBSOs, we performed electrophysiological characterization using the whole-cell patch clump method. Most cells displayed neither an action potential nor a membrane potential less than -40 mV immediately after patch membrane rupture. One cell showed hyperpolarizing voltage responses with an obvious voltage sag, defined as a fast hyperpolarization, followed by a slow depolarization (**Figure 4**, left-1, arrow), whereas other cells ($n = 8$) showed no sag (**Figure 4**, middle-1, right-1). In the neurons exhibiting repetitive firings, the spike overshot (52.9 ± 5.5 mV in amplitude) and its width was narrow (1.1 ± 0.3 ms of the half width). However, in the neuron exhibiting a few spikes, the spike amplitude was small (33.9 ± 7.9 mV), and the half width was wide (2.8 ± 1.6 ms), suggesting that these neurons were still in the course of development.

To further analyze the gene expression profile of hBSOs, we carried out total RNA sequencing (RNA-seq) analysis of hCOs induced using Lancaster and colleagues' protocol (Lancaster

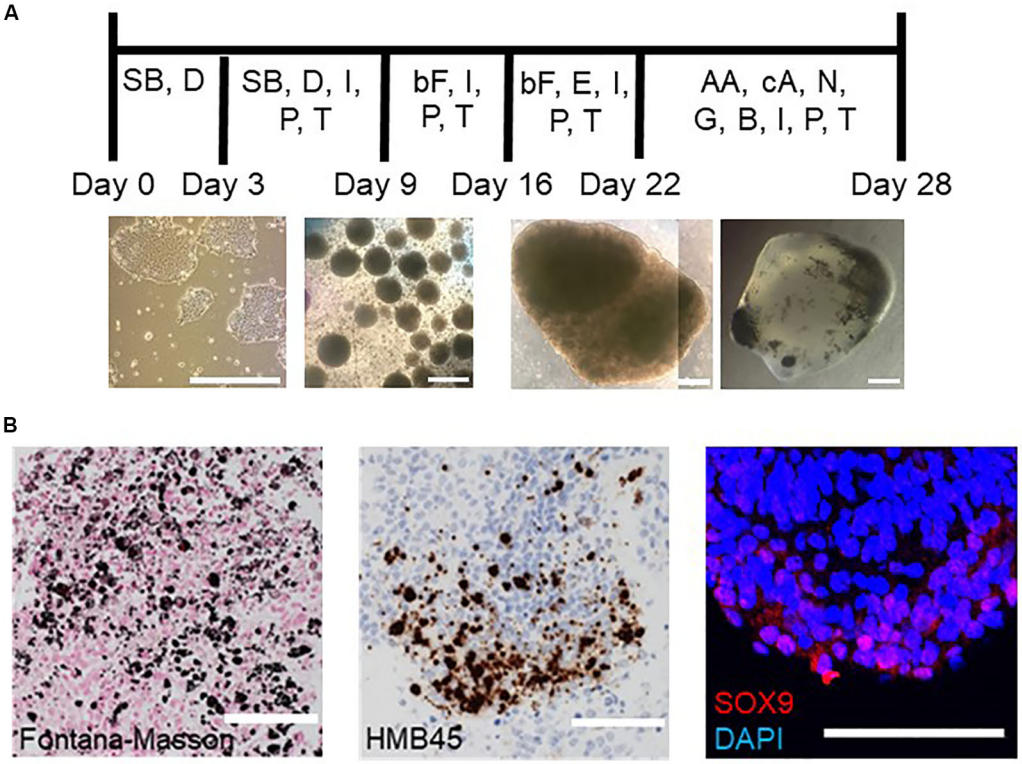

FIGURE 1 | Schematic procedure of inducing hBSOs and immunohistochemical analysis of 1-month-old hBSOs from hESCs. **(A)** Schematic procedure of generating human brainstem organoids. SB, SB431542; D, dorsomorphin; I, insulin; P, progesterone; T, transferrin; bF, basic fibroblast growth factor; E, epidermal growth factor; AA, ascorbic acid; cA, cyclic adenosine monophosphate; N, neurotrophin 3; G, glial cell line–derived neurotrophic factor; B, brain-derived neurotrophic factor. Photographs of organoids were taken on days 0, 9, 22, and 28. Bars = 500 μm. **(B)** Immunohistochemistry of hBSOs at 1-month old from hESCs for the markers of melanocyte (Fontana–Masson, HMB45) and neural crest cell (SOX9). Bars = 100 μm.

et al., 2013; Lancaster and Knoblich, 2014) and the hBSOs at day 28. RNA-seq analysis revealed that the hBSOs contained cell populations like that of a human brainstem. At the age of 1 month, the hBSOs expressed genes that were characteristic of a fetal midbrain, such as *LMX1A* and *LMX1B*, and those indicating dopaminergic neuronal property, such as *EN1*, *EN2*, *TYR*, and *TH*, whose expression was stronger than in hCOs (**Supplementary Table S7**). Additionally, *MLANA* and *MITF*, known as melanocyte-marker genes, and *MBP*, a marker for oligodendrocytes, also showed higher expressions in the hBSOs. We also observed significant expression of NGF and SOX9 specific to neural crest–stem cells. On the other hand, cortical neuron specific markers, such as Reelin and Lhx2, were lower in the hBSOs than the hCOs, indicating their distinct cellular populations.

To better understand the molecular mechanism regulating the differentiation of the hBSOs, we identified DEGs (**Figure 5A**) between the hBSOs and hESCs (**Supplementary Table S8**) and between the hCOs and hESCs (**Supplementary Table S9**). We detected 91 DEGs that were selectively regulated in the hBSOs (**Supplementary Table S10**) and 215 DEGs in the hCOs (**Supplementary Table S11**).

To analyze the correlation between the genes selectively regulated in the hBSOs, the hCOs, and various parts of the brain, we used GTEx, a comprehensive public resource to study tissue-specific gene expression and regulation (**Figures 5B,C**) (GTEx Consortium, 2013). High expressions of *EN2*, *CNPY1* reflected the link between the hBSOs, human cerebellum, and substantia nigra, whereas the expression of *EN1* and *RPE65* demonstrated the relationship between the hBSOs, substantia nigra, and hypothalamus (**Figure 5B**). Low expression of *TRIML2* in the hBSOs is characteristic of the cerebellum, substantia nigra, and hypothalamus (**Figure 5B**).

To identify TFs potentially governing the genes selectively regulated in the hBSOs and the hCOs, we applied iRegulon (Janky et al., 2014), a computational method built upon the fact that genes coregulated by the same TF contain common TF-binding sites and that uses the gene sets derived from ENCODE ChIP-seq data (Gerstein et al., 2012). *CTCF*, *RAD21*, *BRF2*, *JUND*, and *SUZ12* were identified as potential TFs for genes selectively regulated in the hBSOs (Figure 5D). This suggested that *EN1* and *CNPY1*, related to dopaminergic neurons, were controlled by *CTCF* and *RAD21*. Also, MLANA, one of the melanocyte markers, was indicated to be controlled by *CTCF*, *RAD21*, and *SUZ12*. On the other hand, in the hCOs, *FOXA1*, *FOXA2*, *HDAC2*, *EP300*, *NFIC*, *HNF4G*, *NR2F2*, *CTBP2*, and *SUZ12* were detected as potential TFs, and more complex and wide variety of factors were shown (**Figure 5E**).

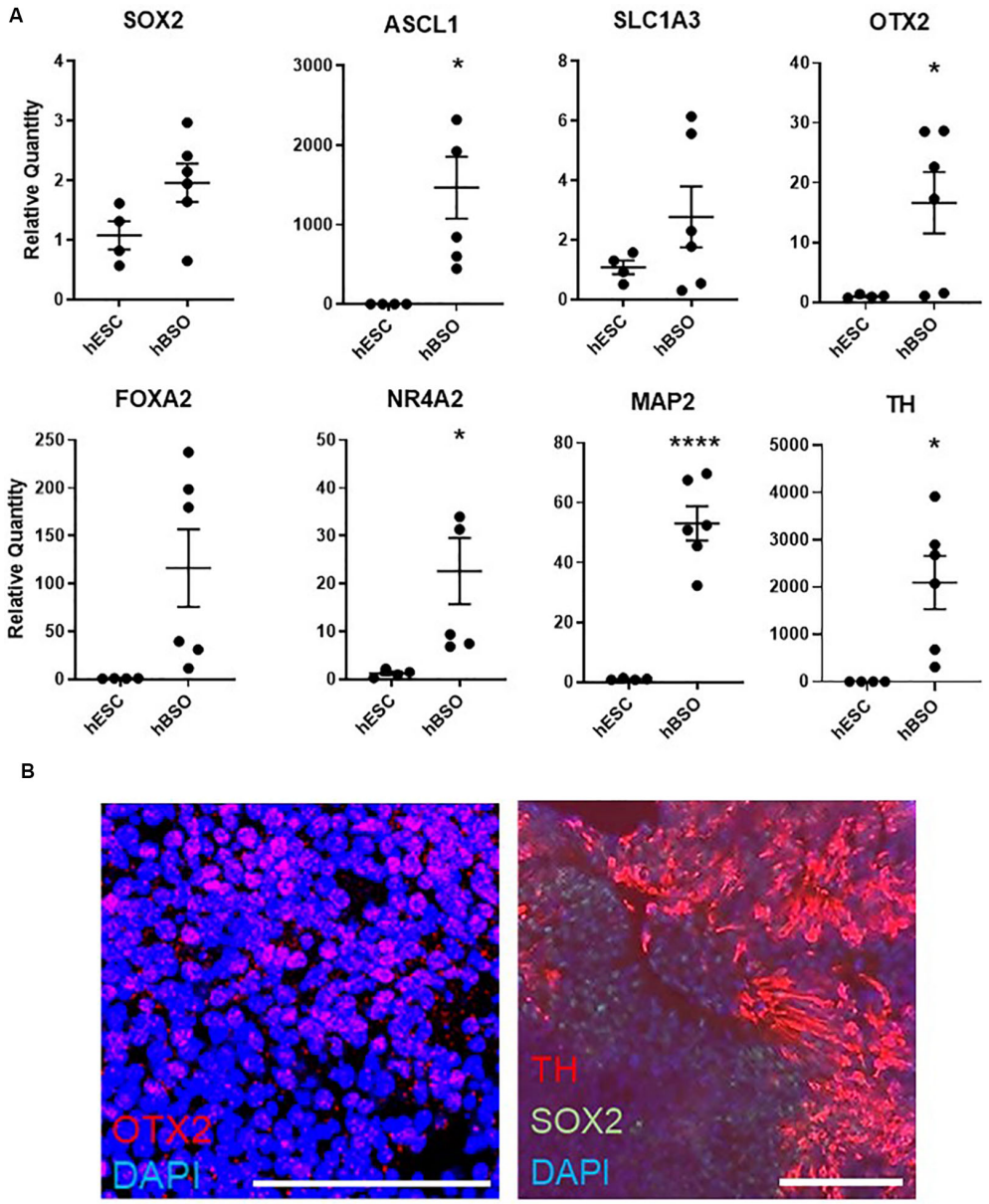

FIGURE 2 | Quantitative PCR and immunohistochemical analysis of 1-month-old hBSOs from hESCs. **(A)** Quantitative PCR analysis of 1-month-old hBSOs for the markers of neural stem/progenitor cell (SOX2, Mash1, SLC1A3), mature neuron (MAP2), midbrain (OTX2), and mDA (FOXA2, NR4A2, TH). Error bars indicate mean ± SEM; *$p = 0.0167$ (ASCL1), *$p = 0.0412$ (OTX2), *$p = 0.0387$ (NR4A2), ****$p < 0.0001$ (MAP2), *$p = 0.0178$ (TH). **(B)** Immunohistochemical staining of midbrain marker (OTX2), mDA marker (TH), and neural stem cell marker (SOX2) at 1 month. Bars = 100 μm.

Finally, to investigate heterogeneity and gene expression dynamics in hBSOs, we performed scRNA-seq analysis on 1-month-old hBSOs. After processing, quality control, and filtering, we analyzed a total of 2,345 cells expressing 19,454 genes. To identify distinct cell populations based on shared and unique patterns of gene expression, we performed dimensionality reduction and unsupervised cell clustering using uniform manifold approximation and projection (UMAP) (**Figure 6A**). The UMAP plot revealed 10 distinct cell populations composed of various cell types. Cell populations were identified based on cluster gene markers (**Supplementary Table S6**) and the expression of known marker genes. We could not annotate cluster 1 and termed this cluster as "unknown" (U). Dot plot showed a selection of genes that can be used to identify cell population types (**Figure 6B**). Each cell population expressed canonical cell type markers. Violin plots showed the expression intensity distribution of the marker genes in each cluster (**Figure 6C** and **Supplementary Figure S5**). The neuronal progenitors cluster expressed genes of cell proliferation (e.g., *MKI67*) and

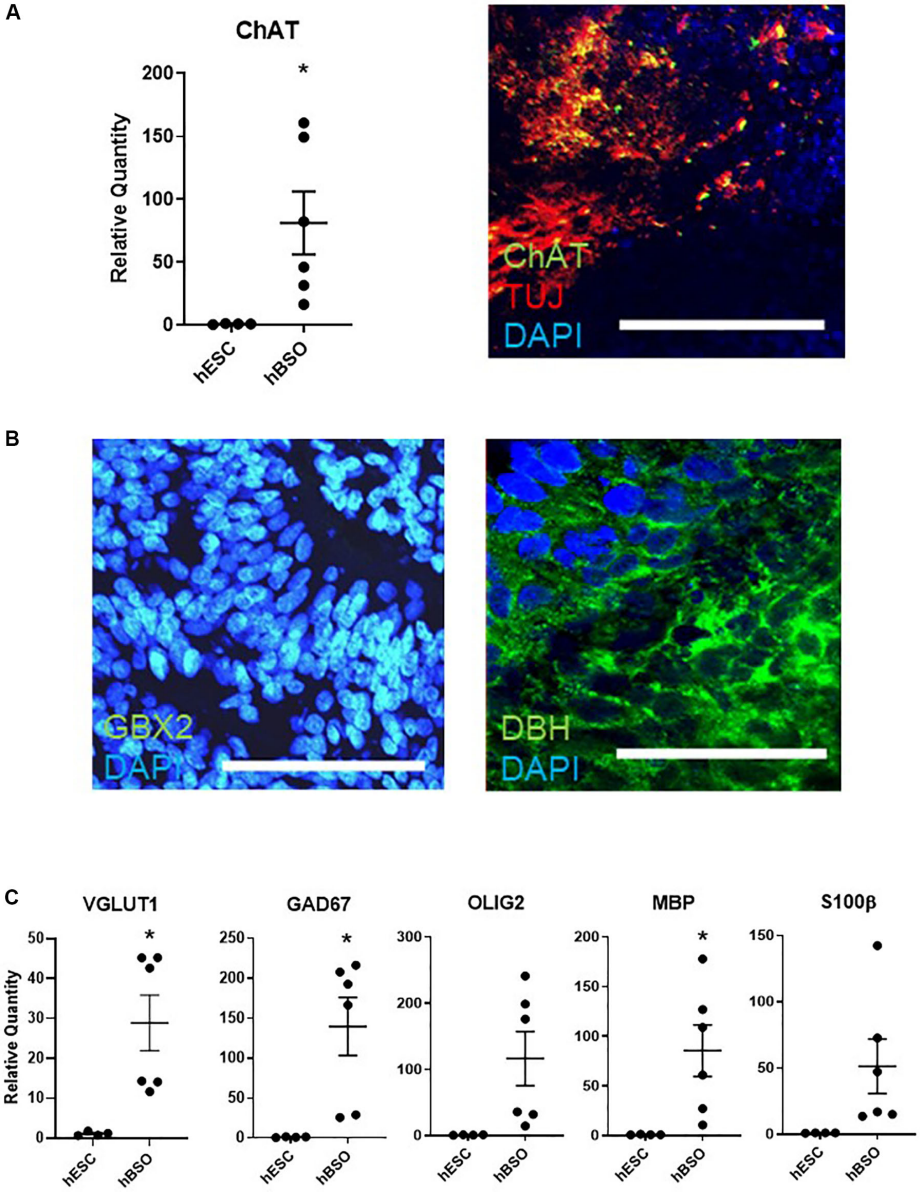

FIGURE 3 | Quantitative PCR and immunohistochemical analysis of 1-month old hBSOs from hESCs. **(A)** Quantitative PCR analysis and immunohistochemical staining of the marker of cholinergic neuron (ChAT). Error bars indicate mean ± SEM; *p = 0.0337 (ChAT). Bars = 100 μm. **(B)** Immunohistochemical staining of DBH, the marker of noradrenergic neuron, and hindbrain marker (GBX2). Bars = 100 μm. **(C)** Quantitative PCR analysis for the marker of excitatory neuron (VGLUT1), inhibitory neuron (GAD67), oligodendrocyte (OLIG2, MBP), and astrocyte (S100β). Error bars indicate mean ± SEM; *p = 0.0127 (VGLUT1), *p = 0.0155 (GAD67), *p = 0.0320 (MBP).

neural stem cell markers (e.g., *PLAGL1*). The radial glia cells cluster expressed PAX6 and the telencephalic progenitors cluster expressed genes related to telencephalon development (*FOXG1*, *LHX2*) (Godbole et al., 2018). The ependymal cells cluster expressed genes related to cilia development and formation (*FOXJ1*, *PIFO*) (Jacquet et al., 2009). The forebrain and midbrain (FB/MB) clusters expressed genes of forebrain and midbrain progenitors (*OTX2*). In addition, the FB/MB clusters expressed dopaminergic (*FGFR2*, *NR4A2*, *LMX1A*, *CALB1*), serotonergic (*HTR2C*), and melanocyte development and differentiation (MITF) markers (Quilter et al., 2012; Ratzka et al., 2012; Anderegg et al., 2015). The mature neurons (mNeu) cluster expressed pan-neuronal (*MAP2*, *SNAP25*), cholinergic (*ACHE*), and glutamatergic (*SLC17A6*) markers. The hindbrain cluster expressed genes of cerebellar or medulla formation (*ZIC1*, *ZIC4*) (Blank et al., 2011). The inflammation cluster expressed genes of microglia cells (*AIF1*) and endothelial cells (*ICAM1*). The scRNA-seq indicated that the organoids contain various cell types and neuronal subtypes.

TABLE 1 | Differentially expressed genes specific for brain in mass spectrometric analysis (FDR < 0.05).

Regional specificity	Symbol	Accession	logFC	logCPM	LR	*P* value	FDR
Pons and medulla	DSG2	Q14126	5.669599673	6.550998938	58.16620526	2.41E-14	7.57E-12
	PRPH	P41219	−6.447981469	6.171664268	44.05998978	3.18E-11	3.84E-09
	KRT8	P05787	3.759699355	10.38106493	21.58664839	0.00000338	0.0000829
	KRT18	P05783	3.725473938	10.34020675	23.87534344	0.00000103	0.000032
	PRSS8	Q16651	2.094239437	−0.279713416	8.491513239	0.003568069	0.02138368
	CFAP44	Q96MT7	−2.431897956	2.251822919	9.751540469	0.001791725	0.012801209
Cerebellum	JARID2	Q92833	8.121840088	2.982965594	69.44954272	7.84E-17	5.42E-14
	HIST3H2BB	Q8N257	−4.40083852	1.304593593	19.49310574	0.0000101	0.00020181
	HELLS	Q9NRZ9	2.112404374	6.517273007	12.01916385	0.000526563	0.004961459
	ZIC2	O95409	1.958589485	3.966265294	7.992220317	0.004697877	0.026045008
Cerebellum, midbrain, pons, medulla	MAB21L1	Q13394	−3.26983685	1.866012194	13.63006394	0.000222592	0.002412928
Basal ganglia	PCP4	P48539	−2.607799879	3.661385349	10.15879101	0.001436148	0.010890787

DISCUSSION

To the best of our knowledge, this is the first time hBSOs with dark cells such as melanocytes have been successfully induced. These cells are of neural crest origin and derived from the fetal midbrain–hindbrain boundary. Using qPCR, IHC, RNA-seq, scRNA-seq, and mass spectrometry, we observed a gene expression profile like that of a human fetal brainstem in hBSOs that were cultivated for 28 days. We built our current protocol upon existing protocols for human brain organoids. In particular, we were inspired by Lancaster and colleagues (Lancaster et al., 2013; Lancaster and Knoblich, 2014), who found that their organoids, acquired with the least use of growth factors, were composed of neuroectodermal tissues with multiple identities, such as cerebral cortex, hippocampus, and retina. In another key study, Muotri and colleagues reported a protocol where cortical organoids were induced with electrophysiologically active neurons with glutamatergic and GABAergic signaling (Trujillo et al., 2018). We designed our protocol based on these existing reports and made use of selected hormones, such as human insulin, transferrin, and progesterone, which have been found to be protective or support the survival and differentiation of dopaminergic neurons and neural crest lineage cells (Ciarlo et al., 2017; Frith and Tsakiridis, 2019). Of note, our findings suggest that the use of an orbit shaker might have contributed to quick induction of hBSOs, through the achievement of an ideal concentration gradient of growth factors. Considering that our protocol does not contain sonic hedgehog (SHH) or WNT, essential for inducing ventral midbrain (Tang et al., 2010), and that the presence of melanocytes implies the presence of dorsal tissues of brainstem, the evidence of mDA neurons in hBSOs is interesting. It is plausible that these factors were secreted from neighboring cells in the population of hBSOs.

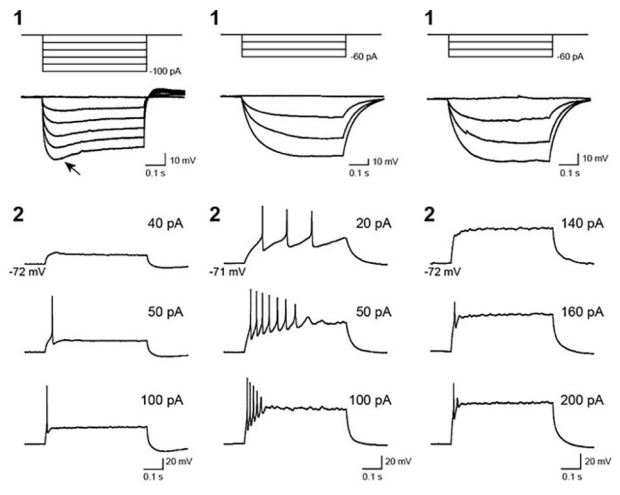

FIGURE 4 | Voltage responses of recorded cells in 1-month-old hBSOs from hESCs to current pulses. (Left, middle, right) Three types of cells exhibiting different hyperpolarizing and firing responses. (1) Voltage responses to hyperpolarizing current pulses. Arrow: voltage responses characterized by a voltage sag. (2) Firing responses to depolarizing current pulses. Firing responses with multiple spikes (left), immature spikes (middle), and a few spikes (right). The values of depolarizing current pulses are given at *right*.

The hBSOs and previously reported midbrain organoids contained characteristic dark spots mimicking the existence of neuromelanin. Given that neuromelanin is derived over a few years, through an accumulation of catecholamine-derived wastes, the dark spots observed in previously reported midbrain–organoid protocols may have melanocytes such as those found in the hBSOs. However, to our knowledge, no existing study on midbrain organoids has identified such neural crest–derived cells in their organoids.

Our findings indicate that the hBSOs mock broad fetal brainstem region and surrounding neural crest, as cranial melanoblasts are known to originate from the neural crest around the midbrain and spread to the whole cranial region (Adameyko et al., 2012; Denecker et al., 2014). Our observations of the high Wnt1 expressions in the hBSOs, which characterize the neural crest (Adameyko et al., 2012), are consistent with this idea. Our current findings also provide the basis for future research on hereditary diseases caused by neural crest migration disorders.

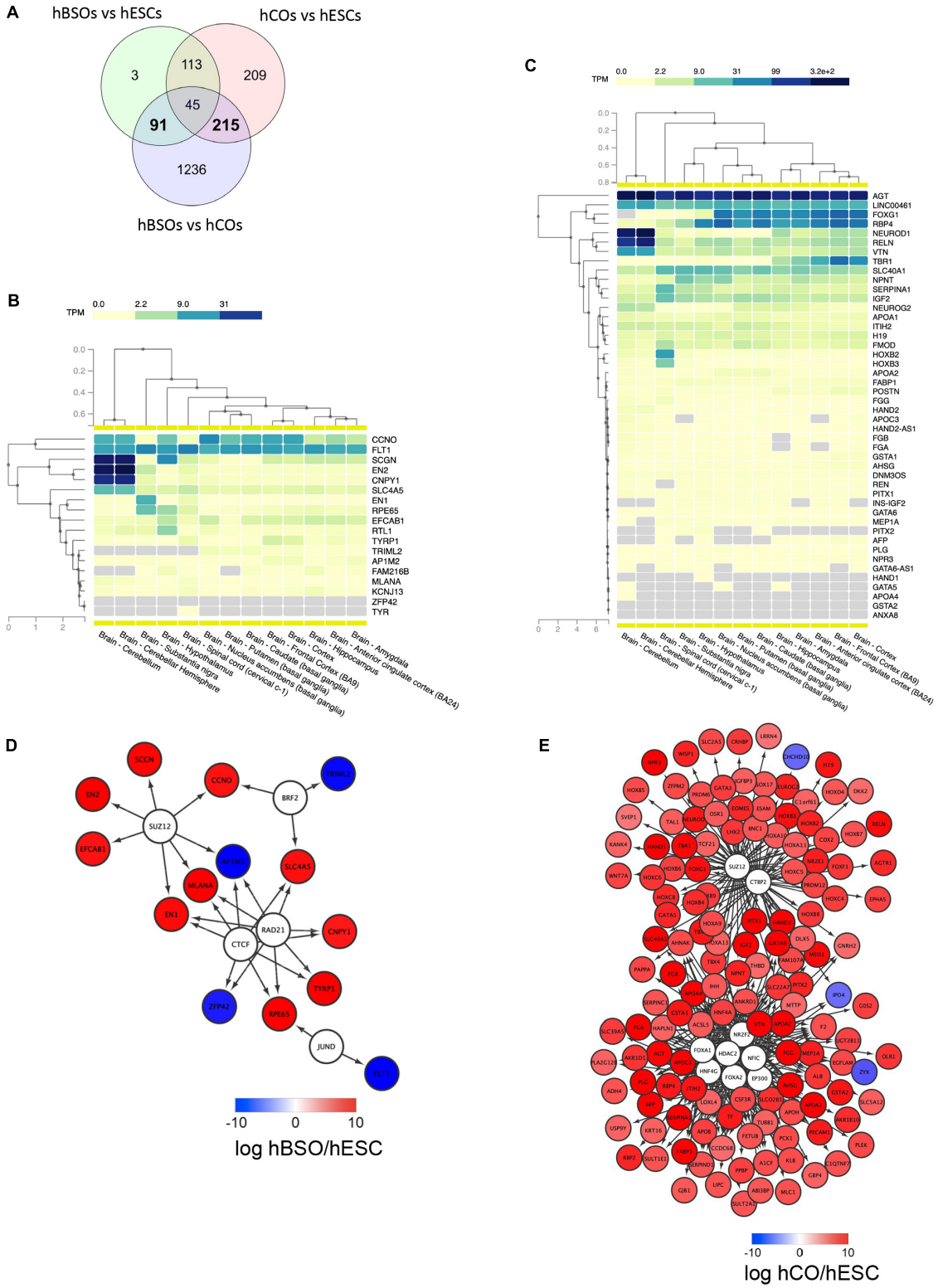

FIGURE 5 | RNA-seq transcriptomic analysis of 1-month old hBSOs from hESCs. **(A)** Venn diagrams of the number of genes differentially expressed between human brainstem organoids (hBSOs), human cerebral organoids (hCOs), and hESCs. **(B)** Clustering brain regions based on the expression of differentially expressed genes selective in hBSOs (FDR < 10%). **(C)** Clustering brain regions based on the expression of differentially expressed genes selective in hCOs (FDR < 3%). **(D)** Transcription factors that potentially regulate the differentially expressed genes in hBSOs. **(E)** Transcription factors that potentially regulate the differentially expressed genes in hCOs.

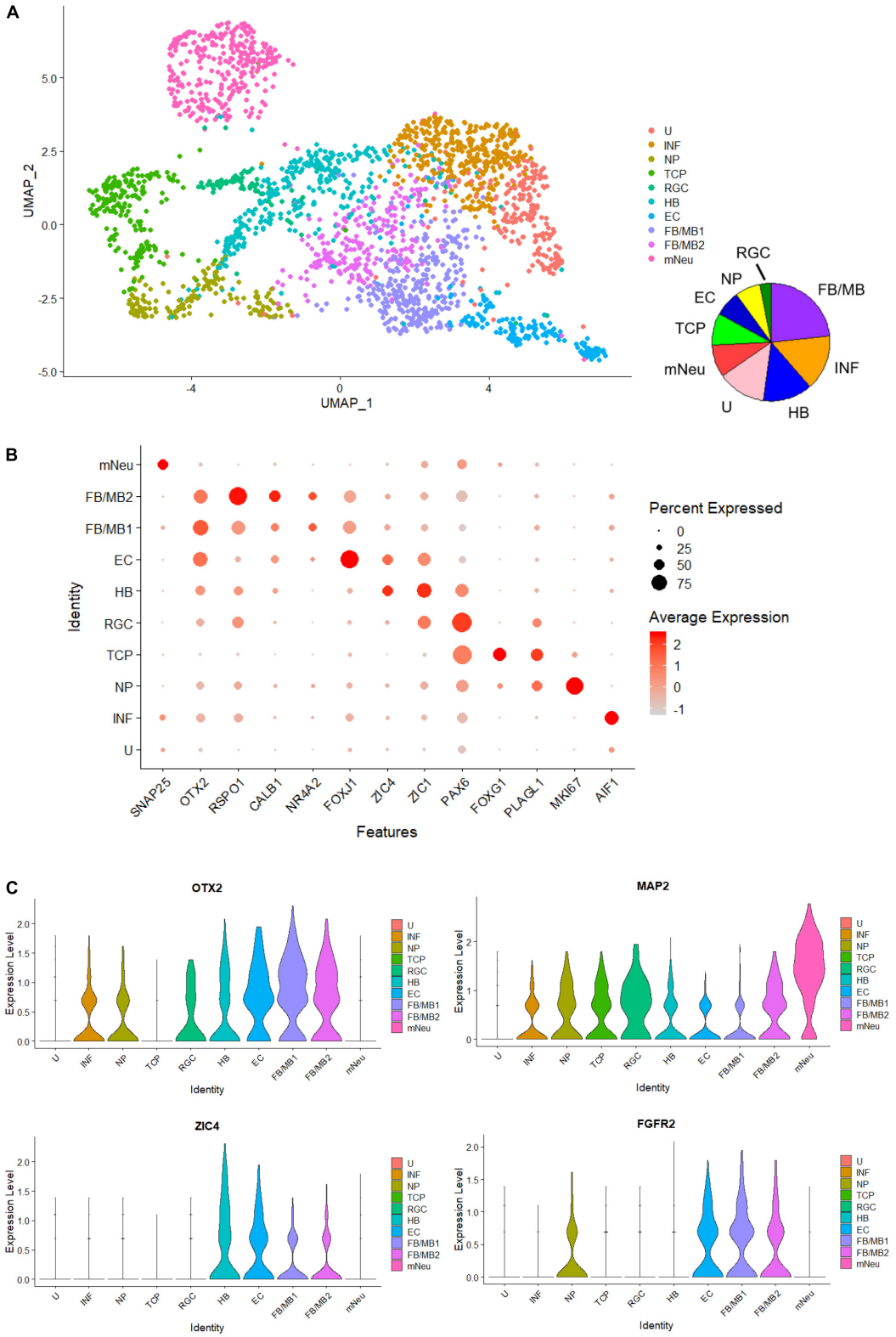

FIGURE 6 | Single-cell RNA-seq analysis of 1-month-old hBSOs from hESCs. **(A)** Unsupervised clustering of all cells from human brainstem organoids. INF; inflammation, NP; neuronal progenitors, TCP; telencephalic progenitors, RGC; radial glia cells, HB; hindbrain, EC; ependymal cells, FB/MB; forebrain and midbrain, mNeu; mature neurons, U; unknown. **(B)** Dot plots showing a selection of genes that identify cell population types. **(C)** Cell distribution plot of OTX2, FGFR2, ZIC4, and MAP2.

We would like to propose two potential applications based on our current findings: (1) to use hBSOs as a tool for drug screening and (2) apply hBSOs as an efficient tool for the modeling of neural crest disorders. First, the quick induction of hBSOs will enable more efficient drug screenings and accelerated research on the molecular mechanisms driving brainstem neurodegenerative diseases. Electrophysiological analysis of the hBSOs at the age of 1.5 months revealed neurons that exhibited action potentials and hyperpolarizing responses with voltage sags attributed to the activation of hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels (Robinson and Siegelbaum, 2003; He et al., 2014). This finding suggests that HCN channels, as well as spike generating Na^+ and K^+ channels, are expressed at an early stage of the hBSO. It also suggests the presence of a heterogeneous neuronal population that is capable of exhibiting distinct electrophysiological properties in the organoid.

A second potential application of hBSOs lies in their utility in investigations into the interaction between the brainstem and neural crest cells. For example, brainstem functions are reported to be affected in representative neural crest disorders, DiGeorge syndrome, and Waardenburg-Shah syndrome (Wang et al., 2017; Nusrat et al., 2018). Nonetheless, the disease models of such diseases have yet to be established, and their pathologies remain to be known. We see the potential in applying the hBSOs developed in our current study as they contain neural crest cells and can be powerful tools for elucidating the mechanisms driving such neural crest diseases.

DATA AVAILABILITY STATEMENT

The data sets generated and analyzed during the current study are available under following accession numbers DRA009864 (RNA-seq) in DDBJ (DNA Data Bank of Japan[3]), JPST000707 (mass spectrometry) in JPOST (Japan Proteome Standard Repository/Database[4]), and GSE145306 (scRNA-seq) in GEO (Gene Expression Omnibus[5]).

AUTHOR CONTRIBUTIONS

NE, TM, JL, JS, KS, and EM designed the study. NE, TM, JL, MMatsubayashi, HN, TShiota, NI, TKiriyama, CZ, TKouno, YL, PK, PW, YMS, RN, TKomeda, NM, FK, MJ, SK, MN, MH, YS, TShiromizu, YN, TKasai, MT, HKobayashi, YI, YT, MMakinodan, TKishimoto, HKuniyasu, SN, JS, KS, and EM conducted the research. NE, TM, JL, YS, TShiromizu, YN, TKasai, MT, SN, AM, JS, KS, and EM analyzed the data. NE, TM, JL, KK, YS, TShiromizu, YN, TKasai, MT, SN, JS, KS, and EM wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

[3] https://ddbj.nig.ac.jp/DRASearch/

[4] https://repository.jpostdb.org/

[5] https://www.ncbi.nlm.nih.gov/geo/

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00538/full#supplementary-material

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The Zebrafish Amygdaloid Complex – Functional Ground Plan, Molecular Delineation, and Everted Topology

Baylee A. Porter[1,2] *and Thomas Mueller*[1]*

[1] *Division of Biology, Kansas State University, Manhattan, KS, United States,* [2] *Department of Biochemistry and Molecular Biology, Department of Urology, SUNY Upstate Medical University, Syracuse, NY, United States*

In mammals and other tetrapods, a multinuclear forebrain structure, called the amygdala, forms the neuroregulatory core essential for emotion, cognition, and social behavior. Currently, higher circuits of affective behavior in anamniote non-tetrapod vertebrates ("fishes") are poorly understood, preventing a comprehensive understanding of amygdala evolution. Through molecular characterization and evolutionary-developmental considerations, we delineated the complex amygdala ground plan of zebrafish, whose everted telencephalon has made comparisons to the evaginated forebrains of tetrapods challenging. In this radical paradigm, thirteen telencephalic territories constitute the zebrafish amygdaloid complex and each territory is distinguished by conserved molecular properties and structure-functional relationships with other amygdaloid structures. Central to our paradigm, the study identifies the teleostean amygdaloid nucleus of the lateral olfactory tract (nLOT), an olfactory integrative structure that links dopaminergic telencephalic groups to the amygdala alongside redefining the putative zebrafish olfactory pallium ("Dp"). Molecular characteristics such as the distribution of substance P and the calcium-binding proteins parvalbumin (PV) and calretinin (CR) indicate, that the zebrafish extended centromedial (autonomic and reproductive) amygdala is predominantly located in the GABAergic and *isl1*-negative territory. Like in tetrapods, medial amygdaloid (MeA) nuclei are defined by the presence of substance P immunoreactive fibers and calretinin-positive neurons, whereas central amygdaloid (CeA) nuclei lack these characteristics. A detailed comparison of *lhx5*-driven and *vGLut2a*-driven GFP in transgenic reporter lines revealed ancestral topological relationships between the thalamic eminence (EmT), the medial amygdala (MeA), the nLOT, and the integrative olfactory pallium. Thus, the study explains how the zebrafish amygdala and the complexly everted telencephalon topologically relate to the corresponding structures in mammals indicating that an elaborate amygdala ground plan evolved early in vertebrates, in a common ancestor of teleosts and tetrapods.

Keywords: telencephalon, teleost, amygdala, hippocampus, isocortex, emotion, prefrontal cortex, prethalamic eminence

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Steffen Scholpp,
University of Exeter, United Kingdom

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Isabel Rodríguez-Moldes,
University of Santiago de Compostela, Spain
Luis Puelles,
University of Murcia, Spain
Koichi Kawakami,
National Institute of Genetics, Japan

***Correspondence:**
Thomas Mueller
muellert@ksu.edu

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SIGNIFICANCE

Based on molecular and evolutionary-developmental characteristics, the study identifies the elaborate amygdala ground plan in zebrafish and stresses the evolution of a complex emotional system in early vertebrates. A multinuclear forebrain structure, the amygdala of mammals has been viewed as a requirement for sophisticated emotions, social behavior, and emotional sentience. Comparable emotional phenomena are rarely discussed in fish in accordance with the current consensus that their amygdala is rather rudimentary and incomparable. Despite obvious morphological differences, however, we show that considerable ancestral amygdaloid building blocks are shared between fish and tetrapods including mammals. The study introduces a long-needed testable molecular reference paradigm of the mature zebrafish extended amygdaloid complex studying neural underpinnings and evolution of emotion in this important model organism.

INTRODUCTION

In mammals and other tetrapods more than a dozen telencephalic nuclei form the amygdala, the regulatory core of the emotional brain (Swanson and Petrovich, 1998; de Olmos and Heimer, 1999; Abellan et al., 2013). The early evolution of a complex amygdala, its organization and behavioral significance in basally derived vertebrates are poorly understood. Until now this heterogeneous structure, the key to emotion and social behavior, remained ill-defined in ray-finned fish (actinopterygii), because their telencephalon looks markedly different from the familiar mammalian situation. In these fish a complex developmental outgrowth called eversion (**Figure 1**) leads to a topographic rearrangement of forebrain territories relative to non-actinopterygian vertebrates (Wullimann and Mueller, 2004b; Northcutt, 2008; Nieuwenhuys, 2009b; Mueller et al., 2011). To this day, scientists have failed to consistently map pallial and subpallial (subcortical) territories even in relatively well-investigated teleost fish such as cichlids and cyprinids (carp-like fish) like goldfish or zebrafish. As a result, putative homologies across teleosts remain persistently debated (Elliott et al., 2017; Yamamoto et al., 2017) owing to inconsistencies in terminology, lack of robust molecular demarcations of brain nuclei, and erroneous annotations of molecular expression patterns.

We unraveled the zebrafish amygdala using a holistic approach that integrates molecular and chemoarchitectonic characteristics and previously published developmental data with a neuroethological framework that adopts the concepts of "the *extended amygdala*" and "*primary olfactory cortex*" originally developed by examining macrosmatic rodents that is, mammals with a pronounced sense of smell (Swanson and Petrovich, 1998; de Olmos and Heimer, 1999). The extended amygdala concept considers numerous subpallial (subcortical) regions as part of the amygdala, including subdivisions of the bed nucleus of the stria terminalis (BST) and the centromedial (CeA, MeA) nuclei. These additional territories extend the classic definition of the amygdala, which included exclusively pallial (cortical) nuclei such as the basolateral amygdala (BLA) critical for fear conditioning (Swanson and Petrovich, 1998). The concept of the primary olfactory cortex includes all pallial nuclei that receive projections from olfactory bulb neurons, several of which form part of the mammalian amygdala. For example, the posteromedial cortical nucleus (PMCo) and the composite nucleus of the lateral olfactory tract (nLOT) are amygdaloid nuclei essential for olfactory cued behavior. Usually, the entire amygdala is described as a heterogeneous collection of predominantly olfactory pallial and subpallial territories that regulate emotion and autonomic nervous system function (Swanson and Petrovich, 1998). In addition, analyzing the distributions of *lhx5*- and *vGLut2a*-driven green fluorescent protein (GFP) in the transgenic lines *tg(lhx5:GFP)* and *tg(vGlut2a:GFP)* we uncovered the ancestral relationships between the everted olfactory pallium and the thalamic eminence (EmT).

Our analysis, therefore, capitalizes on the deep evolutionary relationship between EmT, amygdala, and the sense of smell. Like rodents, zebrafish are macrosmatic animals whose reproductive behavior is hugely influenced by pheromones (Lazzari et al., 2014). Male zebrafish exposed to the female sex pheromone prostaglandin F2α (PGF2α) express stereotypic nudging behavior and conspecific male-male aggression (Sorensen et al., 1988; Yabuki et al., 2016). Molecular data in amphibians (frogs and salamanders) and sauropsids (birds and reptiles) have established a conserved amygdala blueprint across tetrapods (Martínez-García et al., 2007; Medina et al., 2011, 2017). For teleosts like zebrafish the amygdala remained vaguely defined

Abbreviations: A, amygdala; ac, anterior commissure; BG, basal ganglia; BNSM, bed nucleus of the stria medullaris; BST, bed nucleus of the stria terminalis; BSTa, anterior division of BST; BSTc, central division of BST (mouse); BSTm, medial division of BST; BSTp, posterior division of BST; Cantd, anterior commissure, pars dorsalis; Cantv, anterior commissure, pars ventralis; CeA, central amygdala; CeAa, anterior division of CeA; CeAl, lateral (migrated) division of CeA; CeAd, dorsal division of CeA; CGE, caudal ganglionic eminence; CoA, cortical amygdala (mouse); CR, calretinin; D, dorsal telencephalon (=pallium); Dc, central zone of D; Dd, dorsal zone of D; Dg, diagonal domain; Dl, lateral zone of D; dLGE, dorsal LGE; Dlv, ventral subdivision of Dl; Dlp, posterior territory of Dl; Dm, medial zone of D; DM, precomissural (vGlut2a positive) territory of Dm; DP, dorsal pallium; Dta, diencephalic tela attachment site; Dp, posterior zone of D; DTJ, diencephalic-telencephalic junction; EmT, thalamic eminence; EmT-d, EmT-derivative; EmTl, lateral EmT; EmTm, medial EmT; ENv, entopeduncular nucleus, ventral part; Fr, fasciculus retroflexus; Hy, hypothalamus; LGE, lateral ganglionic eminence; LP, lateral pallium; LTE, lateral thalamic eminence (mouse); MeA, medial amygdala; MeAa, anterior division of MeA; MeAd, dorsal division of MeA; MeAp, posterior division of MeA (zebrafish); MeApd, posterior division of MeA (mouse); MeAv, ventral division of MeA (zebrafish pendant to mouse MeApd); MP, medial pallium; MTE, medial thalamic eminence (mouse); nLOT, nucleus of the lateral olfactory tract; nLOTi (zebrafish), intermediate (vGlut2a-neg.) nLOT; nLOTp (zebrafish), posterior (*vGlut2a*-pos.) part of nucleus of the lateral olfactory tract; nLOTr (zebrafish), rostral (vGlut2a-pos.) nLOT; oc, optic commissure; PA, pallidum; pAmy, pallial amygdala; PGC, preglomerular complex; pirCtx, piriform cortex; Pit, pituitary; pLOT, posterior territory of lateral olfactory tract; PMCo, (mammalian) posteromedial cortical amygdala; PMPa, posteromedial pallial nucleus; PPa, parvocellular preoptic nucleus, anterior part; Pr, pretectum; PT, posterior tuberculum; PTh, prethalamus; sAmy, subpallial amygdala; PV, parvalbumin; SD, saccus dorsalis; SDB, base of SD; Se, septum; Str, striatum; tela attch, attachment point of tela choroidea; TE, thalamic eminence (mouse); Th, thalamus; TH, tyrosine hydroxylase; TS, torus semicircularis; V, ventral telencephalon (=subpallium); Vd, dorsal zone of V; Vdd, dorsalmost territory of V (="extended dLGE"); Vi, intermediate zone of V; Vl, lateral zone of V; vLGE, ventral LGE; VP, ventral pallium; Vv, ventral zone of V.

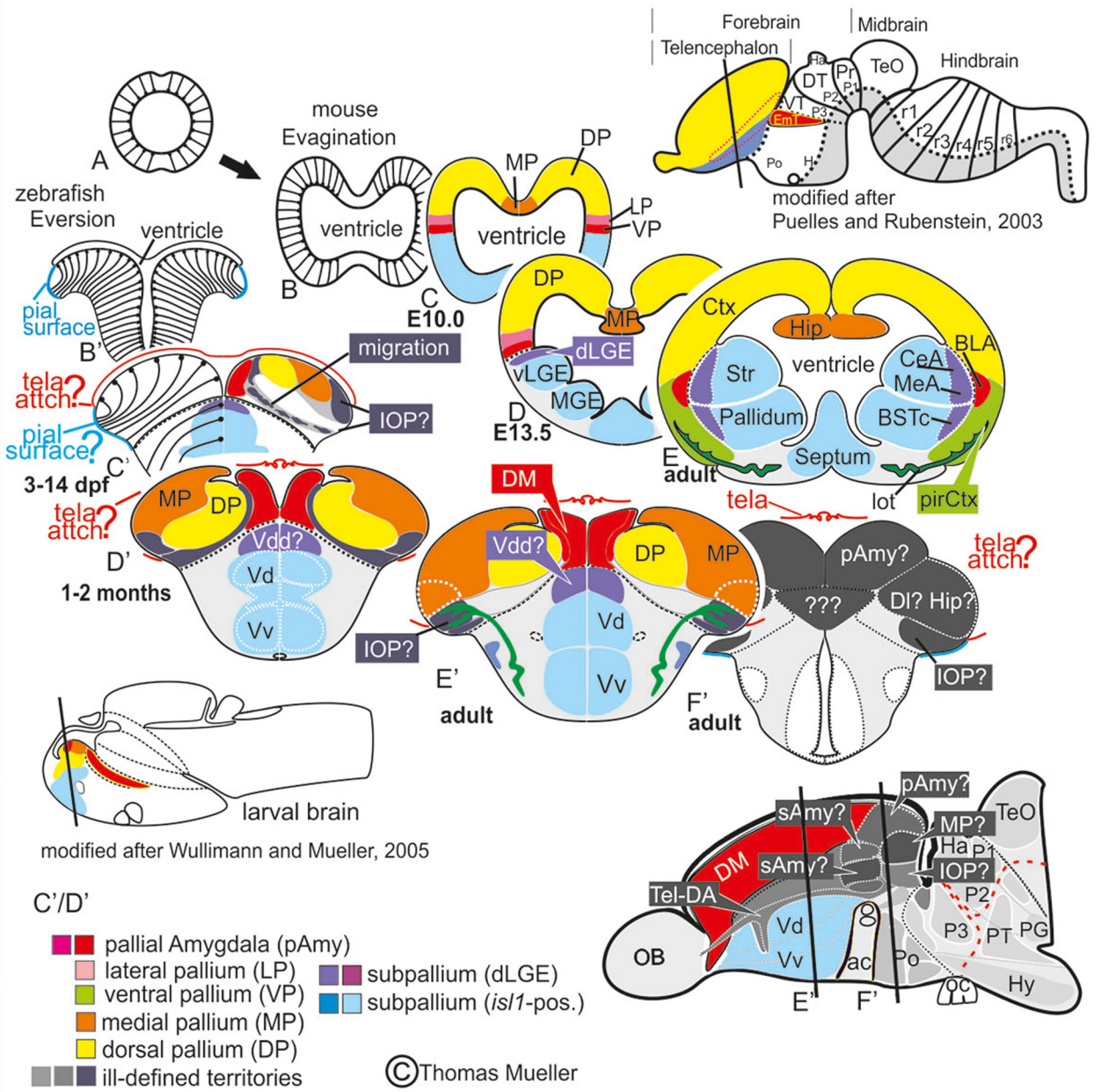

FIGURE 1 | Telencephalic eversion in zebrafish and comparison to mammals. The schematic illustrates how both the outward-growing (eversion) process of the developing telencephalon and its adult morphology of zebrafish (lower row) compares to the telencephalon development (evagination) of mammals (upper row). **(A)** The telencephalon develops from the anteriormost part of the neural tube. **(B–E)** In mammals, two bilateral hemispheres develop around a centrally located ventricle. Predominantly glutamatergic pallial zones (warm colors) develop in the dorsal telencephalon, whereas mostly GABAergic subpallial territories (cold colors) are found in the ventral telencephalon. **(B'–E')** Likewise, in teleosts like zebrafish, the dorsal and ventral telencephalon, respectively, hold pallial and subpallial territories, however, the ventricle due to the eversion comes to lie on top of the brain. **(C',D')** Proliferation patterns, BrdU-long term labeling, and gene expression studies in zebrafish indicated a complex eversion process that includes a radial migration toward the dorsoposterior pallial ("Dp") zone (Mueller et al., 2011). The zebrafish dorsal pallium (yellow) subsequently gets overgrown by the pallial amygdala and the medial pallium (MP in orange). **(F')** The teleostean eversion process is not comprehensively understood. A major obstacle in the comprehensively understanding both the eversion and comparative anatomy remains the unsolved delineation of the integrative olfactory pallium (IOP – the putative homolog to the mammalian piriform cortex) and of telencephalic entities in the posteriormost extent. Ambiguous structures and focus of this study are colored in gray.

(Wullimann and Mueller, 2004b; Northcutt, 2008; von Trotha et al., 2014; Ruhl et al., 2015). This lack of a comprehensive amygdala systems understanding hindered the study of the neural mechanisms underlying cognition, emotion, and social behavior in teleosts and hampered comparisons to mammals and other tetrapods. Our study, thus, introduces a long-needed and

testable reference paradigm of the zebrafish amygdala's functional organization, molecular definition, and evolution.

MATERIALS AND METHODS

Fish Maintenance and Stocks

We keep zebrafish (*Danio rerio*, Cyprinidae) in a LACS operated facility at Kansas State University (KSU) in Manhattan under standard conditions at 28°C (Westerfield, 2000). The University Institutional Animal Care and Use Committee (IACUC) reviewed and approved all experimental protocols of this study. Our experiments conform to the NIH Guidelines for the Care and Use of Laboratory Animals. We used four previously published transgenic lines: (1) *Tg(isl1:GFP)* (Higashijima et al., 2000); (2) *Tg(vGlut2a:GFP)* also named *Tg(slc17a6b:EGFP)* (Bae et al., 2009); (3) *Tg(lhx2a:GAP-YFP)* (Miyasaka et al., 2009), and *Tg(lhx5:GFP)* (Peng and Westerfield, 2006; Turner et al., 2016).

Immunohistology

Immunohistology on cryosectioned brain sections was performed as described (Rink and Wullimann, 2001). We used only antibodies with previously validated specificity including rabbit anti-calretinin (Swant, catalog# 7697/1:1000), mouse anti-parvalbumin (Millipore 1:5000), rabbit anti-γ-aminobutyric acid (GABA, Sigma, catalog# A2052, 1:5000), rabbit anti-substance P (SP; immunostar, catalog# 20064/lot#1003002, 1:2000), mouse anti-tyrosine hydroxylase (TH; 1:1000; catalog# 22941; lot# 1241002) Immunostar; catalog# 20066; lot# 1301001), 1:1000), chicken anti-GFP (1:1000; molecular probes/invitrogen, catalog# A10262; lot# 1729643), rabbit anti-GFAP (1:100, Immunostar), rabbit anti-DSRed antibody (Living Colors, Clontech, Cat# 632496, 1:1000). Secondary fluorescence-coupled antibodies (Invitrogen): goat anti-chicken Alexa Fluor 488, goat anti-rabbit Alexa Fluor 488/555, goat anti-mouse Alexa Fluor 488/555. We used a total of 65 adult (unsexed) zebrafish fish and stained with maximally three antibodies at the same time so that each pattern was represented by at least three samples.

Confocal and Conventional Epi-Fluorescence Microscopy and Image Analysis

To image the distribution patterns of fluorescence immunohistologically stained neuronal phenotype patterns we routinely used a ZEISS Axioplan-2 fluorescence microscope, a confocal Zeiss 700 microscope (microscope core facility KSU), and an Olympus automated epi-fluorescence microscope. Post imaging techniques included montaging, stitching, and sharpening techniques using Slidebook 6 and Adobe Photoshop. We used CorelDraw X7/8 for labeling anatomical structures and the generation of photoplates and figures.

Approach and Redefined Terminology

First, we generated a molecular atlas of the zebrafish telencephalon by imaging and analyzing numerous immunohistologically stained sections of adult zebrafish brains (both wildtype and transgenic lines. Specifically, we compared GFP distribution in reporter lines [*Tg(vGlut2a:GFP, Tg(isl1:GFP, Tg(lhx2a:GAP-YFP*), *Tg(lhx5:GFP]* in fluorescence immunostained cross sections of the telencephalon of adult fish that were additionally stained against GABA- and tyrosine hydroxylase (TH, a marker for dopaminergic neurons in the zebrafish forebrain), the neuropeptide substance P, and the calcium binding proteins parvalbumin and calretinin. We stained against up to three antigens simultaneously and always used DAPI as counterstain. This systematic multi-marker approach identifies amygdaloid nuclei based on the distribution of neurochemically defined neurons, gene expression patterns, and fiber courses that reflect topological relationships. In addition, we integrated published data on the organization and development of the zebrafish telencephalon (Castro et al., 2006; Mueller et al., 2008, 2011; Mueller and Guo, 2009; Mueller and Wullimann, 2009; Ganz et al., 2012; Ruhl et al., 2015; Lal et al., 2018). Moreover, we build on an earlier study regarding the putative isocortex-homolog (dorsal pallium) that stressed a complex pallial eversion (**Figure 1**) because our new data strongly support this previously proposed model (Mueller et al., 2011).

Similar to former studies and in accordance with established molecular data in zebrafish and functional findings in goldfish, we use the term medial pallium for the dorsolateral pallial zone of zebrafish (Dl) (Ganz et al., 2012; Ocana et al., 2017; Rodriguez-Exposito et al., 2017). To provide a meaningful framework and further improve conceptual understanding, we adapted vocabulary for the zebrafish amygdala to the terminologies used for amphibians and mammals. This is not to say that our comparison between similar molecular compositions in zebrafish and mammals suggests simple one to one equations. While we focus on comparing zebrafish to mammals, amygdaloid nuclei in this latter group are more diversified and different subdivisions can show similar molecular compositions. Mammalian amygdaloid territories, of course, also show species-specific differences and a greater cell type variety than zebrafish at large. Our modified terminology treats newly demarcated amygdaloid territories as potentially field homologs to one or more nuclei within tetrapod species and we indicate specific proposed homologies where necessary within **Table 1**. Instead of individual nuclei, we focused on the identification of common building blocks critical for understanding the underlying organizational scheme. **Supplementary Table S1** lists former topographical terminologies for goldfish and zebrafish and their labeling inconsistencies.

Of course, we build on previous knowledge that established basic functional and hodological similarities to tetrapod amygdaloid nuclei, (1) the teleostean dorsomedial pallial zone ("Dm") most often viewed as the pallial amygdala and (2) three subpallial candidate amygdaloid nuclei typically termed supra- and postcommissural nuclei (Vs, Vp, BST) (Braford, 1995; Portavella et al., 2004a; Wullimann and Mueller, 2004b; Northcutt, 2008; Ganz et al., 2014). Here, we provide additional data that either confirm, establish, redefine, or extend classical definitions of these nuclei; we discuss these in the text when needed. Last but not least, we apply the widely accepted prosomeric model as a paradigm for comparing the forebrains zebrafish and mammals (Wullimann and Puelles, 1999; Puelles and Rubenstein, 2003, 2015; Wullimann and Mueller, 2004a). Developmental studies indicated that the topological anterior

TABLE 1 | The zebrafish *extended amygdala* and *primary olfactory pallium* including putative homologies to mammals.

Probable major developmental origin	**dLGE**					**Septum (subpallial)**		**vLGE (striatal)**	**EmTr**	**MGE/pallidal**	**pAmy**	**pAmy**	**EmT-d +DP(?)**	**LP**
Anatomical/Structures Marker	**CeAa**	**CeAl**	**CeAd (+IC)**	**BSTa**	**BSTp**	**MeAa**	**MeAd**	**MeAv (*MeApd*)**	**MeAp (*MeApv*)**	**BSTm**	**DM**	**PMPa (*PMCo*)**	**nLOT**	**IOP**
Topographical location	Vdd	Vc	Vdd	T-DA	T-DA	Vdd	Vp	Vs	Vi	Vs	Dm	Dm	Div/Dp	Dl(p)
*Tg(isl-1:*GFP)	−−	−−	−−	−−	−−	−−	−−	Sparse	−−	++	−−	−−	−−	−−
Tyrosine hydroxylase (TH)	−−	−−	−−	++	++	−−	−−	+	−−	−−	−−	−−	Fibers	−−
GABA	++	++	++	++	++	++	++	++	+	++	Sparse	Sparse	1 layer	Sparse
Parvalbumin	+	−	++	+	+	−	+	+	+	−−	−−	−−	+	++
Calretinin	−−	−−	−−	+	+	+	++	++	+	+	+	++	−−	−−
substance P (SP)	−−	−−	−−	−−	−−	+	+	+	Sparse	−−	Fibers	+	−−	+
Otpa	−−	−−	−−	−−	−−	−−	−−	−	++	−−	−−	−−	−−	−−
Tg(lhx2a:GAP-YFP)	−−	−	−−	−−	−−	−−	−−	Fibers	Fibers	Fibers	−−	−−	Fibers	Fibers
Tg(gad1b:dsRed)	+	−	−−	+	+	+	−	+	Sparse	+	−/+	−/+	−/+	−/+
Tg(vGlut2a:GFP)	−−	−	−−	−−	−−	−−	−−	−−	++	−−	++	+	−−	+
Tg(lhx5:GFP)	−−	−−	−−	−−	−−	−−	−−	−−	++	−−	−−	−−	−−	Fibers
	Extended amygdala/subpallial nuclei plus EmT(r)											**Pallial nuclei**		

Molecular definitions, characteristic neuron types, and topological fiber courses differentially labeling candidate extended amygdala, nucleus of the lateral olfactory tract, and homolog to the mammalian lateral pallium (LP; entorhinal cortex, claustrum and insula) in zebrafish. Nomenclature is adapted to amphibian and mammalian terminologies to facilitate a functional systems understanding. Classical terminologies for the topographical location in the teleostean telencephalon are listed above. Note that oftentimes the part of the region "Vdd" that we consider as a GABAergic subpallial territory is mislabeled as "Dm-v" by some authors. Putative mammalian homologs in italics. For abbreviations see list of abbreviations.

tip of the prosomeric forebrain is the optic chiasm and the alar-plate derived telencephalon consists of a topologically anterior subpallium and a posterior pallium (Puelles and Rubenstein, 2003). In classical columnar paradigms of the forebrain, these entities have been mis-represented as ventral (subpallial) and dorsal (pallial) portions of the telencephalon, with the olfactory bulbs as the (topographical) anterior tip of the brain (Puelles, 2019; Puelles et al., 2019). To accommodate our terminologies to these two different perspectives, we use the terms "anterior" and "posterior" for topological annotations from the perspective of the prosomeric model. In contrast, we use "rostral" and "caudal" to refer to topographical locations in orientation to either the nose or the tail. However, as an exception to this rule, we used the terms anterior, ventral, dorsal, and posterior in the classical (topographical) sense for most amygdaloid territories simply to better relate them to similarly named structures in tetrapods.

Our iterative strategy solved the complexities of the zebrafish amygdala by starting with the identification of the nLOT and most readily identifiable nuclei, such as the medial extended amygdaloid nuclei (MeAa, MeAv, MeAd, and MeAp), and moving to more obscure regions, such as the posteromedial pallial nucleus (PMPa) most likely homologous to the mammalian posteromedial cortical nucleus (PMCo), and the anterior, lateral and dorsoposterior central amygdaloid nuclei (CeAa, CeAl, and CeAd). In addition, we propose that the dopaminergic subpallial groups belong to the newly defined anterior and posterior divisions of the bed nucleus of the stria terminalis (BSTa, BSTp). Within the new paradigm, the nLOT and newly identified rostral, medial, and lateral portions of the thalamic eminence (EmTr, EmTm, and EmTl) form pivotal landmarks for topologically relating the everted zebrafish amygdala to the evaginated mammalian telencephalon. We identified the heterogenous nLOT based on its proximity and partial convergence with thalamic (EmT and Emtl) territories within the region of the diencephalic-telencephalic junction (DTJ) as defined in this study. In fact, we consider the identification of the EmT derivatives together with the characterization of the DTJ and as pivotal for understanding the everted topology of the teleostean telencephalon. Previously, the nLOT has been misunderstood as the dorsoposterior olfactory pallium (Dp) and highest integrative olfactory center in zebrafish. We, instead, show that the highest olfactory pallial territory occupies a region that is currently considered the posteriormost part of the dorsolateral zone (Dl) most often assigned to the "hippocampal" medial pallium. As a result, we named this posterior dorsolateral zone the "integrative olfactory pallium (IOP)," which we assume is homologous to the lateral pallium (LP) and functionally comparable to the mammalian entorhinal cortex that forms part of the hippocampal formation (Watson and Puelles, 2017). The precise anatomical demarcation and molecular definitions of both the IOP and the nLOT refutes current theories on the functional organization of the olfactory pallial zones (Jacobson et al., 2018). Likewise, the GFAP and *lhx5*-driven GFP data presented do not support simple telencephalic eversion models (Butler, 2000; Nieuwenhuys, 2009a,b; Furlan et al., 2017; Yamamoto et al., 2017). Also, they are not compatible with an incomplete or partial pallial eversion as suggested earlier

(Wullimann and Mueller, 2004b). Instead they indicate that the telencephalic outward-growing process is complete and complex and probably constrained by hem-like organizing centers and the thalamic eminence and its derivatives.

RESULTS

Overview

Based on the distribution of molecularly defined neuronal phenotypes and calcium binding proteins (calretinin, parvalbumin) that reveal both territory-specific cell type abundances and intra-telencephalic connections, we identify thirteen amygdaloid territories (**Table 1**). Following established conventions in the field, **Table 1** divides predominantly GABAergic subpallial from predominantly glutamatergic pallial structures (Puelles et al., 2000; Mueller et al., 2006) as well as the predominantly glutamatergic MeAp which we consider a thalamic eminence derivative (EmT-d). Due to its dual nature as being both a part of the medial extended amygdala and derivative of the rostral otp-a positive thalamic eminence (EmTr), we refer to this structure as "MeAp/EmTr."

Table 1 highlights four key findings:

(1) Most of the subpallial amygdaloid nuclei in zebrafish (CeAa, CeAl, BSTa, BSTp, and MeAd) form part of the GABAergic, *isl1:*GFP-free region that we propose corresponds to or develop within the region of the dorsal lateral ganglionic eminence (dLGE); not to the "dorsal striatum" as previously suggested (Ganz et al., 2012). The *isl1:GFP* positive BSTm represents an extension of the striatopallidal systems, that is the teleostean counterpart of the tetrapod medial ganglionic eminence (MGE) that gives rise to the pallidum consistent with a former study that identified this nucleus (Ganz et al., 2012) as well as a study that confirmed its pallidal identity (Wullimann and Umeasalugo, 2020). In contrast, we interpret the newly identified MeAv, despite its absence of *isl1*-driven GFP, as an extension of the striatum proper based on the presence of both substance P and calretinin cells (this study). Notably, we consider the predominantly glutamatergic MeAp a derivative of the rostral EmT (MeAp/EmTr). This structure has been previously viewed as the "MeA" in zebrafish and was termed intermediate nucleus of the subpallium ("Vi") (Biechl et al., 2017). In contrast, we show that this nucleus is predominantly glutamatergic and contiguous or identical with the thalamic eminence located within what we consider the diencephalic telencephalic junction (DTJ).

(2) The presence of numerous calretinin positive neurons define medial amygdaloid nuclei (MeAd, MeAv, MeAp) whereas the lack of pronounced populations of calretinin expressing neurons is indicative of central amygdaloid nuclei (CeAa, CeAl, and CeAd) similar to the mammalian situation at early stages of development (Wojcik et al., 2013). The CeAd stands out as a laterally displaced subpallial (GABAergic) nucleus that is defined by the presence of dense parvalbumin expressing fibers and some parvalbumin-expressing neurons.

(3) The study identifies a territory in the caudalmost position of the former dorsomedial zone ("Dm") as the putative homolog of the mammalian posteromedial cortical amygdaloid nucleus (PMCo), based on substance P-fibers passing both through both the medial amygdala and posterior Dm. We termed this territory the "posteromedial pallial amygdaloid zone" (PMPa), its homology to the mammalian PMCo is also supported by its intercalated position between MeAd and integrative olfactory pallium (IOP) and many calretinin positive neurons, which potentially share developmental origins with those populating medial amygdaloid territories.

(4) We used the transgenic line *Tg(lhx2a:GAP-YFP)* to distinguish the newly identified nucleus of the lateral olfactory tract (nLOT) from the principal olfactory pallium (Dp proper), which we termed in zebrafish the "integrative olfactory pallium" (IOP) owing to its prospective higher integrative function. The heterogenous amygdaloid nucleus of the former olfactory tract (nLOT) was previously mislabeled as the dorsoposterior pallial zone ("Dp") and most likely serves olfactory and taste integration at a primary (lower) level. We propose that this composite structure in zebrafish is homologous with the mammalian nLOT, because—like its mammalian counterpart— it consists of pallial (glutamatergic), subpallial (GABAergic) and (glutamatergic) putative thalamic derivatives (EmT-d) (Huilgol and Tole, 2016). Most importantly, we show that the lateral thalamic eminence (EmTl) form the base of the nLOT and *lhx5*-driven GFP positive cells are present in the posterior lateral olfactory tract territory (pLOT) strongly resembling the mammalian situation during development (Ruiz-Reig et al., 2017). Again, as in mammals, a stream of migrating pallial neuroblasts originating from a region close to or overlapping with the pallial amygdala form part of this region in zebrafish (Remedios et al., 2007; Mueller et al., 2011). In addition, *lhx5*-driven positive elements are found within and close to Dp (Turner et al., 2016). Our results suggest that these elements represent derivatives of the thalamic eminence that, like their counterparts in mammals, may both control and contribute to the development of the nLOT and other structures at the DTJ in zebrafish. Interestingly, the heterogenous nLOT in zebrafish receives both gustatory and main olfactory input (Miyasaka et al., 2009; Yanez et al., 2017) further supporting its functional and structural heterogeneity.

PALLIAL AND SUBPALLIAL DERIVATIVES IN THE ANTERIOR TELENCEPHALON

Pallial Amygdala (pAmy)

A recent study on the developing zebrafish pallium suggests a concentric pallial growth and simple eversion (Furlan et al., 2017) as similarly postulated before (Butler, 2000; Nieuwenhuys, 2009b). This hypothesis strongly conflicts with earlier findings that showed a complex pallial eversion and identified the dorsal pallium (DP) based on molecular distinctiveness and topological relationships to two of the major pallial territories, the putative pallial amygdala [i.e., the dorsomedial (Dm) zone] and the medial pallium (MP; **Figure 1**; Mueller et al., 2011). A solid demarcation

of these pallial histogenetic units is pivotal for the complete understanding of the pallial and subpallial territories forming the entire amygdaloid complex, which is the objective of this study.

To solve conflicting views, we tested if additional molecular characteristic set the prospective dorsal pallium (DP) apart from the medial pallium (MP; roughly the dorsolateral (Dl) pallial zone in zebrafish) and the putative pallial amygdala (the dorsomedial (Dm) zone). We determined that the distribution of specific glutamatergic neurons visualized by GFP in the transgenic line *Tg(vGlut2a:GFP)* in comparisons to parvalbumin and GABA strongly supported the molecular distinctiveness and delineation of the dorsal pallium candidate. Focusing on these chemoarchitectonic characteristics, we were able to corroborate that the centralized portion of the prospective dorsal pallium is covered by the molecularly distinct DM and medial pallium (MP) at posterior sections as previously suggested (Mueller et al., 2011).

In the transgenic line *Tg(vGlut2a:GFP)*, we found that GFP is expressed only in subsets of glutamatergic neurons that differentially populated pallial derivatives. In fact, we found *vGlut2a*-driven GFP (*vGlut2a*:GFP) positive neurons heavily labeled the dorsomedial (DM) zone here considered a derivative of the pallial amygdala (pAmy), whereas the putative dorsal pallium (mammalian isocortex) entirely lacked GFP (**Figures 2A–C,H,K**). This lack of GFP in the dorsal pallium (DP) indicates that DM does not contribute neurons to the development of DP and that they indeed represent distinct histogenetic units. In addition, the hippocampal pallium (MP, medial pallium) showed *vGlut2a*:GFP exclusively in its anterior- and posteriormost extents and dense populations of parvalbumin-positive neurons throughout its entire expansion (**Figures 2E,F**). The medial pallium thus also represents a distinctive pallial unit that is easily distinguishable from both the parvalbumin-free DM and the dorsal pallium (DP) that showed parvalbumin fibers yet lacked parvalbumin-positive neurons. The further molecular characterization of these distinct pallial units thus validates previous publications on topology (homology), function, and transcription factor profiles of the three major pallial derivatives: (1) the pallial amygdala (DM, PMPa), the dorsal pallium, and (3) the medial pallium (Mueller et al., 2011; Ganz et al., 2012). This expression pattern is also visible in 2 and 3-week-old larval zebrafish (personal observation).

The validation of all of these three pallial entities as distinct developmental entities enabled us to approach the remaining pallial and subpallial entities in the everted telencephalon of zebrafish, especially those that have been controversially discussed (gray zones in **Figure 1**). Comparing *vGluta2a*:GFP and GABA patterns, we established the ventralmost cells of the *vGlut2a*-GFP positive pAmy as a landmark that defines the (topographically) dorsal side of the pallial-subpallial border (PSB) adjacent to the dense GABAergic and *isl1*:GFP-free cell populations constituting the zebrafish dLGE (**Figures 2D1–G**). These results indicate that the Dm-region widely viewed as the pallial amygdala (pAmy) in teleosts (Wullimann and Mueller, 2004b; von Trotha et al., 2014; Ruhl et al., 2015), molecularly needs to be subdivided into an anterior part (DM) of the former dorsomedial zone of the pallium ("Dm") and a posterior part. The presence of predominantly *vGLUT2a*-driven GFP expressing neurons and absence of parvalbumin-fibers and neurons define DM, which we therefore consider a derivative of the pallial amygdala. Notably, a recent study identified a group of so-called Dm120a-neurons as part of DM mediating associative fear learning (Lal et al., 2018). These Dm120a-neurons and most of DM are defined by emx3 expression whereas a posteriomost portion of Dm does not express this gene (Lal et al., 2018). This emx3-negative posteriormost part of the former dorsomedial (Dm) territory according to our results contains the posteromedial pallial nucleus (PMPa) most likely homologous to the mammalian PMCo (this study; **Figures 2K–N**). In the anterior telencephalon, *vGlut2a*:GFP positive neurons also heavily populate a portion of the nLOT consistent with the hypothesis that this portion of the nLOT is a migrated territory derived from the thalamic eminence located within the DTJ (this study).

Anterior Extended Amygdala – The Central (CeAa,CeAl) and Anterior Medial Amygdala (MeAa)

In general, dense populations of GABAergic neurons mark the zebrafish striatopallidum (Vd in **Figures 2B,C**), septal (Vv in **Figures 2B,C**), anterior (CeAa; **Figures 2B,C**) and laterally displaced (CeAl) "central amygdaloid" ("Vc") nuclei (**Figures 2B,C**). In contrast (and consistent with earlier reports), we detected sparsely distributed GABAergic neurons in pallial territories including the *vGlut2a*:GFP-positive pallial amygdala (pAmy), the olfactory pallial zone (OP), the nucleus of the lateral olfactory tract (nLOT; **Figure 2C**), as well as the territories that topologically correspond to the mammalian isocortex [=zebrafish dorsal pallium (DP)] and hippocampus [medial pallium (MP)](e.g., **Figure 2C**).

The distributions of neuronal phenotype, such as parvalbumin- and TH-expressing dopaminergic neurons was also compared to that of neurons labeled by the transgenic line *Tg(isl1:GFP)* (**Figures 2D1–G,J,N**). In this way, the *isl1*:GFP negative and GABA-positive subpallial (extended) amygdala was distinguished from positive striatopallidal territories ("Vd") (**Figures 2A–G**). Specifically, *isl1*:GFP expressing neurons are confined to a territory ventral to the *isl1*:GFP-negative TH-expressing neurons (**Figure 2F**). This indicates that the predominantly GABAergic nuclei of the subpallial (extended) amygdala, specifically the CeAa, CeAl, MeAa, and MeAd form the GABA positive and *isl1*:GFP-negative domain sandwiched between the *isl1*:GFP positive striatopallidum and the *vGlut2a*:GFP expressing DM. In other words, the absence of *isl1*:GFP labeled cells marks most of the zebrafish central amygdala (CeAa. CeAl) dorsal to the dopaminergic cell clusters (Cave and Baker, 2009). Only a small territory dorsal to the CeAa belongs to the MeAa, because substance P fibers label this territory as distinct (**Figures 4D,E**). Thus, the central amygdala (CeA) is defined based on its *isl1*:GFP negativity, its juxtaposed position to both the pAmy and MeAa, and the absence of typical MeA markers such otpa- and calretinin-positive neurons and substance P expression (**Figures 2M,L**). **Figure 3** illustrates how otpa- (**Figure 2L**) and calretinin-positive neurons (**Figure 2M**) in the supra- and postcommissural subpallial territories of

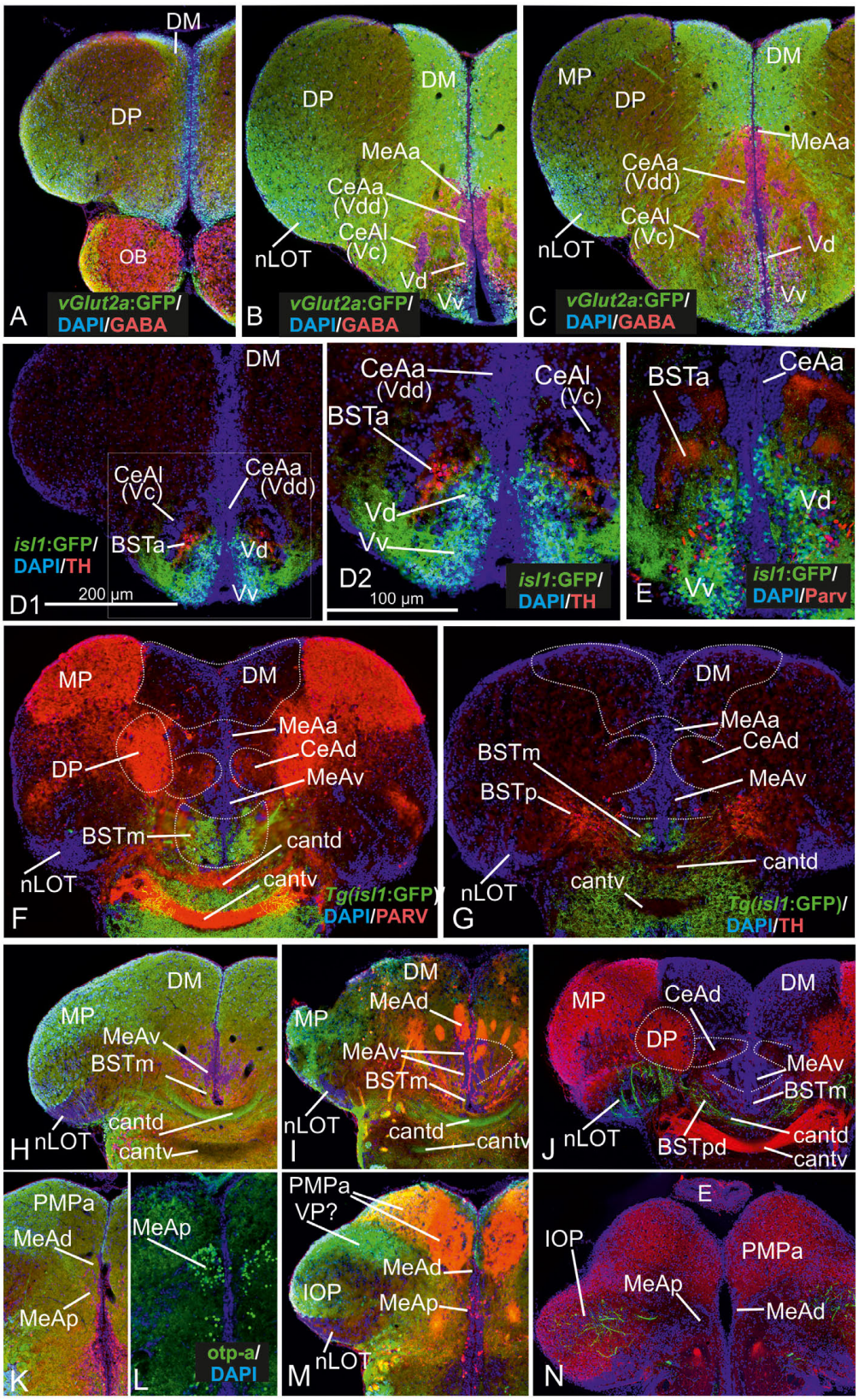

FIGURE 2 | Continued

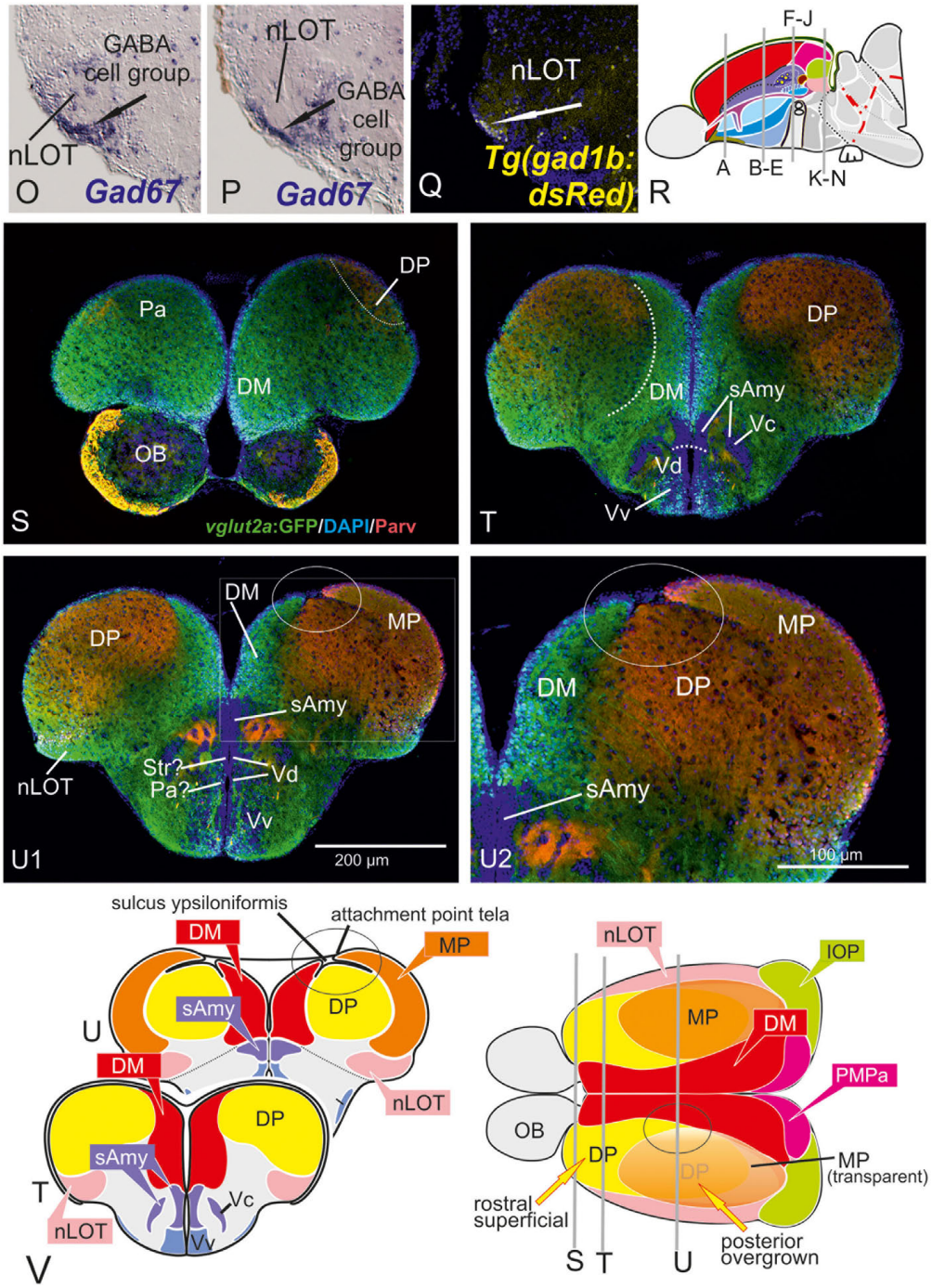

FIGURE 2 | Molecular Definitions of the Zebrafish Amygdaloid Complex. Precommissural **(A–E)**, supra- and postcommissural **(F–Q)** telencephalon, and delineation of the dorsal pallium in its periventricular {(S+T, U1/left) and central (U1, right, U2)} portions. Pallium: Analyzing the distributions of GABA **(A–C,H,K)**, parvalbumin **(E,F,J,N)**, and GFP in the transgenic line *Tg(vGlut2a:GFP)* **(A–C,H,I,K,M)** allowed to discern the dorsomedial pallial zone, the putative core region of the pallial amygdala, from the hippocampal division (medial pallium/MP) and the teleostean dorsal pallium (DP) **(A–C)**. DP lacks both *vGlut2a*-driven GFP and parvalbumin-positive neurons but shows parvalbumin positive fibers **(F,J,H,U2)**. The strongly GFP-positive DM lacks parvalbumin expression **(F,J)**. The mostly *vGlut2a*:GFP-free hippocampal division (MP) exhibits both parvalbumin-positive fibers and neurons in addition to GABA-neurons **(C,F,J)**. Only at posteriormost sections, we found *vGlut2a*:GFP positive neurons in the MP **(H)**. At anteriormost sections **(A,B)**, the DP reaches the dorsalmost periventricular zone, which holds proliferative stem cells. More posteriorly **(C,F,J)**, the DP is shown in its secondarily centralized position overgrown by both the pAmy and hippocampal division (medial pallium/MP). In addition, we found many large GABAergic interneurons in DP **(A–C)**, as well as sparsely distributed GABAergic interneurons in the pAmy and MP **(B,C)**. Subpallium: Dense population of GABAergic neurons define the subpallium **(A–C,H,K)**. Within the subpallium, *isl1*-driven GFP labels striatopallidal and septal divisions as well as the BSTm **(D1,D2,F,G)**. In contrast, GABAergic territories that lack *isl1*-driven GFP define the majority of subpallial amygdaloid territories.

(Continued)

FIGURE 2 | Continued
The anterior (precommissural) dorsalmost. GABAergic population (CeAa) together with the migrated GABAergic nuclei (CeAl = former Vc) predominantly form the zebrafish central amygdala [CeA in **(A–C)**]. The ventricular-close GABAergic territory is juxtaposed and contiguous with the former supracommissural (Vs) and postcommissural (Vp) nuclei of medial amygdala (MeA; former Vs/Vp). In addition, based on the lack of *isl1*-driven GFP in both anterior and posterior TH-positive dopaminergic and their previous reported projection to the hypothalamus, we consider as the anterior and posterior bed nucleus of the stria termalis (BSTa/BSTpd). The medial bed nucleus of the stria terminalis (BSTm) is the only *isl1*:GFP positive medial nucleus [BSTm in **(F,G)**]. We also found sparse dopaminergic neurons within the posteromedial amygdala (MeAp) that project into part of the nLOT **(B2)**. Supra- and postcommissural extended medial amygdaloid nuclei (MeAc, MeAd, MeAp, BSTm, BSTpd) are defined by the presence of numerous calretinin-positive neurons **(F,I,M)** similar to the mammalian situation. In contrast, the CeAd that comprises numerous laterally displaced GABAergic neurons shows many parvalbumin-positive fibers and few parvalbumin neurons, but lacks calretinin neurons. The MeAp was formerly assigned to the teleostean subpallium, because there are GABAergic neurons in its vicinity **(K)**. However, the otp-a **(L)** and calretinin **(I,M)** positive neurons that define the MeAp are likely glutamatergic and originate from the thalamic eminence according to our results. Discerning PMPa, IOP, and nLOT Comparing secondary olfactory projections in the transgenic line *Tg(lhx2a:GAP-YFP)* **(J,N)** with parvalbumin allowed us to identify primary olfactory pallial territories and their topological relationships. The distribution of secondary olfactory projections in comparison to parvalbumin and calretinin fibers identifies two olfactory integrative structures: (1) the parvalbumin-positive integrative olfactory pallium (IOP) **(I,J,M,N)** formerly misinterpreted as part of the medial pallium ("Dl/Dlp") in zebrafish; (2) the largely parvalbumin-free (migrated) amygdaloid nucleus of the lateral olfactory tract (nLOT). The nLOT is a predominately glutamatergic nucleus that consists of both *vGlut2a*:GFP positive (anterior; **C**) and negative (posterior) domains as well as *GAD67*-positive [arrows in **(O,P)**] and *gad1b*-driven dsRed expressing [arrow in **(Q)**] GABAergic zones resembling the mammalian nLOT that consists of alternating GABAergic and glutamatergic layers. Previously, the nLOT has been mislabeled as the posterior division of the dorsal telencephalon ("Dp") and mistakenly considered the piriform cortex homolog. The identification of the nLOT together with the piriform cortex homolog (IOP) solves their topological relationships within the complexly everted telencephalon (R indicates orientation of sections **A–N**). **(F)** Schematic figure shows orientation of section in the sagittal view. New hallmarks of the complexly everted zebrafish telencephalon – the rostral (superficial) versus posterior centralized parts of the dorsal pallium (DP). **(S–U2)** Analyzing distribution of parvalbumin versus *vGlut2a*-driven GFP shows that the dorsal pallium reaches the periventricular zone and includes its germinative layer of origin. Note, that DM and medial pallium (MP = dorsolateral (Dl) zone of the pallium) overgrow the dorsal pallium at posterior sections at the point of convergence (circled area). **(V)** Schematics of a zebrafish telencephalon from dorsal perspective illustrating levels of cross sections.

the BSTm and teleostean dLGE defines the medial extended amygdaloid nuclei as well as the pallial PMPa.

Dopaminergic Groups Form the Anterior and Posterior Division of the BST

Previous studies have classified telencephalic dopaminergic neurons in teleosts as an integral part of the striatopallidum (Rink and Wullimann, 2001; Tay et al., 2011). However, we found that these dopaminergic neurons are located within *isl1*:GFP-negative territory, in contrast to the *isl1*-driven GFP positive rest of the territory typically labeled as dorsal portion of the subpallium ("Vd") and considered here the zebrafish striatopallidum. Based on their relative position between *isl1*-negative CeAa, CeAl, and MeA amygdaloid territories and in close proximity to the newly defined nLOT, we identified them as part of the amygdaloid BST. This radical new interpretation acknowledges a recent finding that telencephalic dopaminergic neurons project into the hypothalamus in addition to the teleostean striatopallidum ("Vd") (Tay et al., 2011). Precommissural groups of dopaminergic neurons build the (topographically) anterior bed nucleus of the stria terminalis (BSTa) whereas supra- and postcommissural groups of dopaminergic neurons form the BSTp (**Figure 2G**). We also found a small number of dopaminergic neurons in the newly identified caudalmost portion of the subpallial medial amygdala (MeAv; **Figure 2G**) resembling the situation of its most probable mammalian homolog the mammalian MeApd. Likewise, dopaminergic neurons are also found in subdivision so the mammalian BST (Northcutt and Lonstein, 2011; Bupesh et al., 2014). Overall, the zebrafish dopaminergic BSTp is positioned in a laterally displaced angle and close to the *isl1*:GFP positive medial BST (BSTm; **Figures 2F–J**). Both the zebrafish CeA (=CeAa, CeAl, CeAd) and the dopaminergic BST neurons seemingly are located in the *isl1*:GFP negative and GABA-positive dorsal subpallium that corresponds to the dorsal lateral ganglionic eminence (dLGE). This interpretation is consistent with conserved regulatory gene expression patterns and developmental studies in zebrafish (Mueller et al., 2008; Mueller and Wullimann, 2009; Tay et al., 2011; Ganz et al., 2012).

NUCLEUS OF THE LATERAL OLFACTORY TRACT (NLOT) AND INTEGRATIVE OLFACTORY PALLIAL NUCLEUS [IOP; MAMMALIAN LATERAL PALLIUM (LP)]

Tg(lhx2a:GAP-YFP) Labels Main Olfactory Bulb Projections to Two Cortical Nuclei

Pivotal for assigning homologies in zebrafish is the correct interpretation of the posterior olfactory nucleus "Dp," which partially forms through a peculiar radial cell migration (Mueller and Wullimann, 2009; Mueller et al., 2011). Quite a number of authors have questioned the origin of Dp, its homology to mammals, exact delineation and position (Nieuwenhuys, 2009b). To solve this debate, we re-investigated the distribution of secondary olfactory projections using the transgenic line *Tg(lhx2a:GAP-YFP)*. According to the study that introduced this transgenic zebrafish line, olfactory bulb neurons allegedly send their projections to a large single olfactory receptive pallium, usually called the posterior pallial zone (Dp) (Miyasaka et al., 2009). However, our results showed that these neurons project not to one but to two separate pallial nuclei (**Figures 2J,N**), which in turn differ by the respective presence or absence of parvalbumin staining. The overlooked projection innervates the parvalbumin-positive integrative olfactory pallium (IOP, **Figure 2N**) previously mislabeled as a part of the dorsolateral

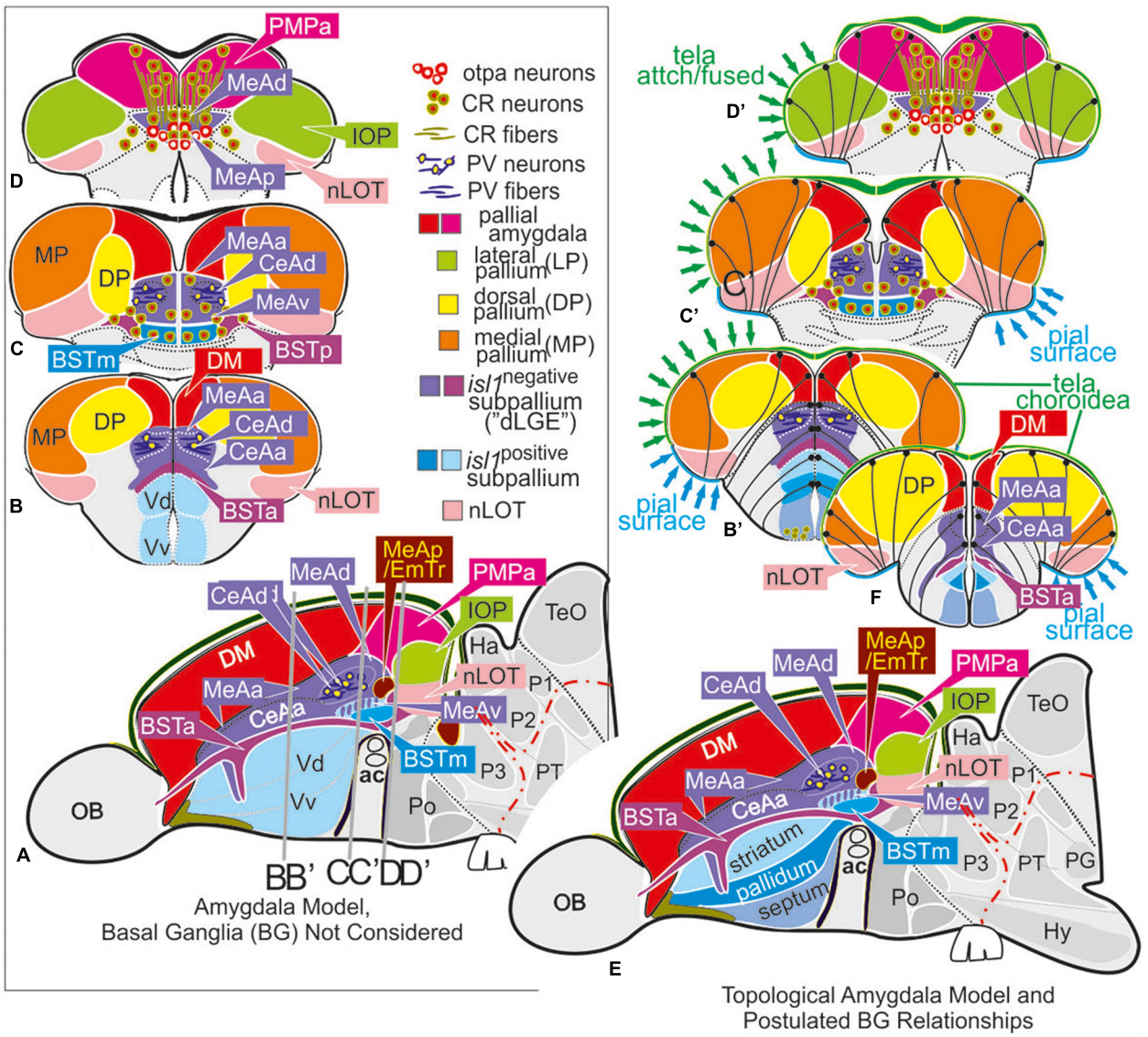

FIGURE 3 | Molecular code of the zebrafish amygdaloid complex. Definitions of amygdaloid territories in the complexly everted telencephalon: Amygdala model (basal ganglia (BG) not considered) **(A–D)** versus idealized topological amygdala model **(B',C',D',E,F)** indicating tela attachments and radial glia distribution. **(A–D)** The lateral schematic **(A)** of the amygdala model divides the zebrafish amygdaloid complex with regard to the anterior commissure (ac) into precommissural **(B)**, supracommissural **(C)**, and postcommissural **(D)** sectors. A hierarchical code defines all amygdaloid nuclei: All medial extended amygdaloid nuclei in the supra- and postcommissural sectors **(C,D)** are located within the *isl1*:GFP negative, subpallial (GABAergic) territories and comprise numerous calretinin-positive neurons. The CeAd is distinguished from such MeA-territories through the presence of parvalbumin-fibers and cells that are laterally displaced. More anterior lying CeA-territories such as the CeAa, CeAl also form part of the *isl1*:GFP negative subpallium, they are, however, distinguished from the CeAd through their lack of parvalbumin expression. In addition, all dopaminergic clusters formerly viewed to the zebrafish striatopallidum are here considered the anterior (BSTa) and posterior (BSTp) divisions of the bed nucleus of the stria terminalis (BST). These dopaminergic extended amygdaloid nuclei are also located in the zebrafish *isl1*:GFP free territory that corresponds to the mammalian dLGE (= Vdd). The medial BST(m) is the only subpallial amygdaloid territory where a large population of *isl1*:GFP forms the majority of this nucleus in addition to some calretinin-positive neurons that link this nucleus with the rest of the extended medial amygdala. The newly discovered integrative olfactory nucleus (IOP) shows secondary olfactory projections and many parvalbumin positive neurons. In contrast, the amygdaloid nLOT that also receives secondary olfactory projections lacks these parvalbumin neurons. **(E)** Topological amygdala model indicating idealized BG relationships showing tela attachment sites and radial glia distribution as revealed in this study.

pallial territory ("Dl") in the adult zebrafish brain atlas (Wullimann et al., 1996). This parvalbumin-positive region is the putative homolog to the mammalian lateral pallium (LP) and probably best viewed as a teleostean counterpart to the mammalian entorhinal cortex, which considered a part of the hippocampal formation. In contrast, the second parvalbumin-free region, as our data indicate, represents the amygdaloid nucleus of the lateral olfactory tract (nLOT; **Figure 2J**). The

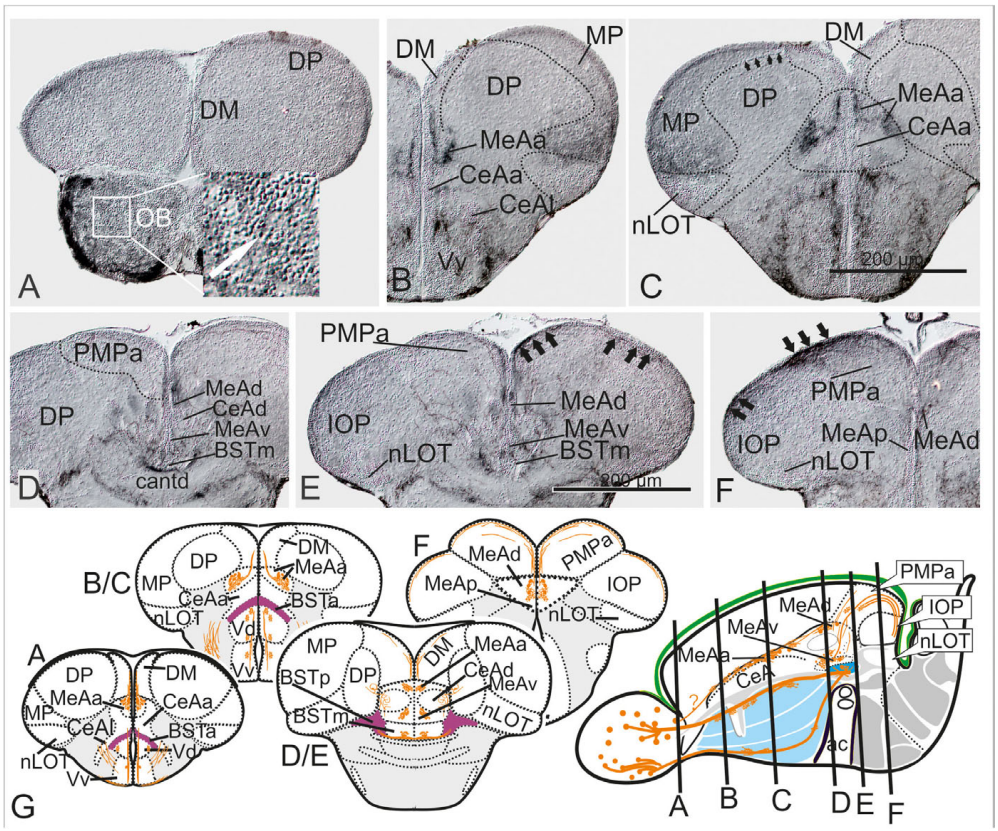

FIGURE 4 | Substance P Fiber Tracts from the Olfactory Bulb into the Telencephalon. **(A–G)** Using diaminobenzidine (DAB) as a substrate for the detection of a horseradish peroxidase coupled antibody yielded enhanced detection of substance P in the telencephalon **(A–G)**. We found substance P positive neurons in the olfactory bulb (OB in A and inlet). substance P fibers emanate from the olfactory bulb toward the lateral ventralmost part of the subpallium [Vv+Vd in **(B,C)**]. Most importantly, some SP cells and many SP fibers label the medial amygdala (MeAa) in the dorsal tier of the subpallium **(B–F)**. At the transition between supracommissural and postcommissural telencephalon **(D–F)**, substance P positive fibers passing through the dorsal medial amygdala [MeAd in **(D–F)**] enter and label the posteromedial pallial amygdala [PMPa in **(E,F)**]. Substance P fibers also pass through the PMCo into the IOP, the zebrafish putative piriform cortex homology (black arrows in IOP of H), supporting the proposed homology between the zebrafish PMPa and mammalian PMCo. Note, the anterior and dorsoposterior divisions of the CeA (CeAa + CeAd) lack substance P and therefore can easily distinguished from all medial amygdaloid nuclei (MeAa, MeAd, MeAv, MeAp). **(G)** Schematic cross sections and sagittal view of the zebrafish telencephalon to illustrate the two solid zebrafish accessory olfactory substance P fiber systems innervating the medial amygdaloid territories. Substance P fibers seemingly form also laterally displaced diffuse projections **(E)**.

zebrafish nLOT, like its mammalian counterpart, is a molecularly heterogeneous composite structure whose GABAergic and glutamatergic components originate from different primordial sources. For example, both the rostral- and caudalmost *vGlut2a*:GFP positive cell masses of the nLOT (**Figure 2C**) are most likely generated through radial migration from the *lhx5*-negative/*vGlut2a*-positive thalamic eminence (EmT; this study). In addition, other glutamatergic, *vGlut2a*-negative cells are probably derived from a region closely adjacent or overlapping with the primordial pallial amygdala (Mueller et al., 2011). In contrast, the presence of GABAergic portions of the nLOT (**Figures 2O–Q**) suggests a subpallial origin of these neurons. More posterior glutamatergic yet *vGlut2a*:GFP negative portions that are not innervated by secondary olfactory fibers may be derived from other telencephalic or thalamic sources. Previously, the nLOT was misinterpreted both as part of the teleostean ventral portion of the medial (hippocampal) pallium ("Dlv") and the so-called posterior zone of the pallium (Dp) of Wullimann (Wullimann and Mueller, 2004b; Northcutt, 2008; Ganz et al., 2012). However, the absence of substance P (this study) and previously reported absence of dorsal raphe serotonergic input (Lillesaar et al., 2009) speak against higher integrative function.

THE SUPRA- AND POSTCOMMISSURAL EXTENDED MEDIAL AMYGDALA NETWORK

The Supracommissural Extended Amygdala (CeAd, BSTm, BSTp, MeAv, pAmy)

We angled cross sections perpendicularly to the rostro-caudal axis of the zebrafish telencephalon to visualize the supracommissural BSTm/MeAv/CeAd configuration as an oval structure sandwiched between the caudalmost DM

(*vGlut2a*-driven GFP positive putative pAmy) and the anterior commissure (**Figures 2F–J**). The relative position of these territories between both the anterior commissure (ac) and pallial-subpallial border (PSB) corresponds to the topological situation in mammals, in such a way that the pallidal BSTm is located adjacent to the ac, followed by an intermediate positioned striatal MeAv, and the CeAd being closest to the PBS. We refer to this oval composite structure as the supracommissural standard configuration, which permits an easy navigation between the supracommissural and postcommissural amygdala (**Figures 2K–N**). In the latter, the MeAd merges with the posteromedial pallial nucleus (PMPa) at the caudalmost telencephalic expansion (**Figures 2K–N**). In the former, a group of *isl1*-GFP-positive neurons contribute to the medial bed nucleus of the stria terminalis (BSTm; **Figures 2F,G**), whereas *isl1*:GFP-negative TH-expressing cells form the posterior division of the BST (BSTp) (**Figures 2F,G**). A small number of *isl1*:GFP-negative and TH-and GABA-positive neurons contributes to the MeAv. The positions of the medial amygdaloid nuclei (MeAd, MeAv, MeAp) are consistent with the topological positions of the redefined BST nuclei (BSTm, BSTa, BSTp), the nLOT, the PMPa, and the IOP. In these posterior sections, numerous calretinin-positive neurons populate all MeA- and BST nuclei, which distinguish them from CeA territories that are largely free of calretinin cells. The distribution of calretinin cells in zebrafish resemble the situation described for mammals (Wojcik et al., 2013).

The Postcommissural Amygdala (MeAa, MeAd, MeAv, MeAp, PMPa, nLOT)

Next, we analyzed calretinin cell and fiber patterns in GABAergic subcortical amygdaloid territories (**Figures 2H,I,K,M**) to decipher the topological relationship between the MeA nuclei and the newly identified PMPa. We found that the PMPa shows extensive calretinin cell and fiber staining continuous with the dorsal division of the zebrafish medial amygdala (MeAd; 2I). Moreover, we define the posterior division of the MeA (=MeAp) based on the presence of a large population of otpa-positive neurons (**Figure 2L**). In addition, a contiguous band of calretinin-positive neurons defines both the MeAd and MeAv (**Figure 2M**). Connections between MeAd and PMPa are suggested by calretinin fibers that arrive via four fiber bundles in the ventralmost aspect of the MeAd (**Figure 2I**). In the dorsal GABAergic subcortical MeAd at posterior sections, these calretinin bundles fuse into one bilateral continuous band projecting into the PMPa (**Figure 2I**). The ramification patterns of these calretinin fibers suggest a functional link between MeA and PMPa. Supporting this interpretation are substance P positive fibers that emanate from the olfactory bulb and reach the medial amygdala (MeA) at posteriormost sections (**Figures 4D–F,G–J**). Specifically, these substance P positive fibers also innervate the zebrafish PMPa (black thick arrows in **Figures 4E,F** pointing within PMPa) supporting its functional relationship with the MeA. In addition, numerous calretinin positive cells in the territories of the posterior medial extended amygdala (MeAd, MeAp) support its redefined homology. We speculate that many of these calretinin positive neurons mediate odor-cued behavior and, like in mammals, emanate from hem like organizing centers including the thalamic eminence (Bielle et al., 2005; Huilgol et al., 2013; Huilgol and Tole, 2016).

Substance P (SP) in Extended Amygdala Marks Accessory Olfactory Nuclei

In mammals, the MeA mediates reproductive behavior in response to sex-pheromones and receives projections from the accessory olfactory bulb (Abellan et al., 2013). These characteristics have been used in classical studies to define the MeA in non-mammalian vertebrates with a vomeronasal organ such as amphibians and lungfish (Moreno and González, 2003; González et al., 2010). Given that zebrafish like other ray-finned fish lack a vomeronasal organ and accessory olfactory bulbs, it is generally assumed that an accessory pathway and thus the medial amygdala is absent in zebrafish. However, the olfactory epithelium of ray-finned fish contains sensory neurons with pheromone-binding vomeronasal-like as well as pheromone-binding (non-vomeronasal) olfactory receptors critical for reproductive behaviors (Ahuja and Korsching, 2014; Behrens et al., 2014). Hence, we tested the existence of several medial extended amygdaloid territories and the PMPa also through immunohistological detection of substance P (SP) in adult zebrafish. In both amphibians and mammals this neuropeptide labels accessory olfactory bulb projections to the MeA (Moreno and González, 2003; Davidson et al., 2006). Specifically, the presence of extensive SP fibers and numerous SP positive neurons separates the mammalian MeA from the central amygdala (CeA) (Emson et al., 1978). In adult zebrafish two SP-positive bulbofugal tracts innervate the medial extended amygdaloid nuclei (**Figures 4G–J**) in the rostral (**Figures 4A–C**) and caudal (**Figures 4D–F**) zebrafish telencephalon. The SP-positive MeA nuclei thus can be clearly distinguished from the negative CeA (CeAa and CeAl; **Figures 4B,C**). Even more significant is the finding that SP fibers pass through the MeA (MeAd in **Figures 4D,E**) and project to the dorsalmost regions of the PMPa and the integrative olfactory pallium (IOP; **Figures 4E,F**). This finding supports the identification of these newly defined territories. The nLOT completely lacks substance P fibers in contrast to the IOP (**Figures 4C–F**). This finding is consistent with the interpretation that the zebrafish IOP integrates accessory olfactory-like and main olfactory and gustatory information. In contrast, the absence of substance P fibers in the nLOT suggests that this structure integrates main olfactory and gustatory information only.

TELA ATTACHMENT SITES IN COMPARISON TO RADIAL GLIA CELLS AND *VGLUT2A*-DRIVEN GFP INDICATE COMPLEX TELENCEPHALON EVERSION

Our molecular data and amygdala framework as outlined above is incompatible with current eversion models, especially with the idea that the zebrafish telencephalon develops through concentric growth that causes a simple "outside-in pallial" organization as

proposed based on radial glia cell distribution and genetic fate mapping (Furlan et al., 2017). We postulated that the conclusion of these results are due to inaccurate anatomical analyses of both the periventricular radial glia and pial surfaces of the pallium and misleading claims of earlier studies suggesting that radial glia and tela attachment sites reflect a simple pallial eversion in teleosts (Nieuwenhuys, 2009a,b). To test this hypothesis and define periventricular sites versus respective pial sites, we mapped the tela attachment sites and distribution of radial glia somata and processes. For this purpose, we used antibodies against parvalbumin (PV), GFAP and GFP in the transgenic line *tg(vGlut2a:GFP)* allowing us to correctly annotate distribution of radial glia cells and their processes and tela attachment and fusion sites.

The Tela Attachment and Fusion Sites Indicate Complexly Everted Pallial Organization and Contributions of the (Pre-)Thalamic Eminence (EmT)

We found that the PV antibody visualized the telencephalic tela choroidea (**Figure 5**, specifically 5A1/A2, D1-D2B, and E1/F1). Most strikingly, and in sharp contrast with former studies, we found that the tela choroidea is fused with at the periventricular site of the dorsolateral zone ("Dl") corroborating the interpretation that this zone is the zebrafish MP (**Figures 5D1B,D2B**). This finding thus falsifies Nieuwenhuys hypothesis stating that the tela is attached solely to what he identified as (telencephalic) "nucleus taeniae": (Nieuwenhuys, 2009b). Our results, in contrast, indicate that he misinterpreted what would be traditionally viewed as a diencephalic attachment site as being telencephalic. This fact becomes particularly evident at caudalmost sections in the region that we describe as *diencephalic-telencephalic junction* (DTJ) in this study (**Figures 5E1–F3**). Within the DTJ, the zebrafish *vGlut2a*-driven GFP positive (*lhx5*-driven GFP negative) thalamic eminence (EmT) is contiguous with a portion of the regions of the dorsoposterior zone (DP) within a transitional zone between EmT, nLOT, and IOP (*vGlut2a*-driven GFP of the nLOT as visualized in **Figures 5E1–F3**). The putative GFP-positive EmT-derivative (EmT-d) extends to the pial surface of the pallium occupying a small portion of the part that is usually considered Dp or Dlp. Topographically beneath this GFP positive EmT-d, we identify the lateral thalamic eminence (EmTl; **Figures 5E1–F3**), which in mammals is an important signaling center that contributes to the development of the nLOT (Remedios et al., 2007; Huilgol and Tole, 2016; Ruiz-Reig et al., 2017). The EmTl is most pronounced and appears as a pseudo-layered or folded structure at the DTJ probably due to bending processes during development. The characteristic distribution of the *vGlut2a*-driven GFP neurons close to the pial surface of the pallium, the lateral displacement of the nLOT in connection with an indentation of the outer pallial rim, as well as the close relationship with the diencephalic tela attachment site together suggest that the *vGlut2a*-driven GFP positive portions of the nLOT represent derivatives of the thalamic eminence (EmT-d) as summarized in the schematic **Figure 8**). We postulate that *vGlut2a*-driven GFP positive cell masses of the nLOT in its most rostral (e.g., **Figures 2A–C**, 5A1–C2, D1B) and caudal aspects (are derived from the *vGlut2a*-driven portions of the EmT. Again, what is visible as a tela attachment site across the entire length of the nLOT represent a diencephalic attachment site (**Figures 5D1B,D2B**), not homologous to the one that defines the medial pallium as proposed before.

This drastic reinterpretation of the tela attachment sites as well telencephalic homologies to mammals also concerns an important structure at the DTJ, which we called the posterior medial amygdala (MeAp). Notably, we found that PV also stains the tela attachment site of what we identified as the rostral portion of the thalamic eminence (EmTr) and which we consider a part of the medial amygdala and consequently called it the *posterior medial amygdala* (MeAp/EmTr). The MeA/EmTr has been previously proposed as being the MeA and labeled as the intermediate subpallial nucleus ("Vi") that contains tangentially migrated hypothalamic otp-a positive neurons (Biechl et al., 2017). The facts that the tela choroidea is attached to this nucleus (**Figures 5E1,F1**) and that glutamatergic neurons populate this nucleus (**Figures 5E1,F1**) strongly suggest that this territory is an EmT derivative that forms a smaller part of the medial extended amygdala as visualized in schematic **Figure 8**.

Radial Glia Somata and Processes Support Complexly Everted Pallium, Contributions of the (Pre-)Thalamic Eminence (EmT), and Presence of the Dorsal Pallium

At rostral most sections (**Figures 5A1–C1**), distribution and orientation of radial glia and their processes were consistent with our delineation of both the pallial amygdala (DM) and dorsal pallium (DP). The GFAP-positive radial glia of both these territories send their processes toward the subpallium and end just at the pallial-subpallial border (PBS) that is located topographically below the GFP-positive and PV-negative nLOT. However, the somewhat laterally displaced nLOT does not show radial glia somata at its lateral superficial expansion demonstrating that this part does not contain proliferative stem cells as shown before based on proliferation patterns (Wullimann and Mueller, 2004b; Lillesaar et al., 2009; Mueller and Wullimann, 2009; Mueller et al., 2011). Thus, our results again confirm that the nLOT is located at the pial surface and not at the periventricular site as suggested in simple eversion models. This finding is consistent with the interpretation that heterogeneous migrated cell masses form the nLOT. This hypothesis is strongly supported by the somewhat lateral displacement of the nLOT which leads to an indentation between nLOT and DP or MP (**Figures 5A1–C2**). Likewise, the overall orientation and distribution of radial glia cells supports the interpretation that the centralized zone of the zebrafish pallium ("Dc") represents the dorsal pallium (DP) which extends into the topographical rostral most sector and includes its own germinative zone of origin (Mueller et al., 2011).

At mid-telencephalic sections (**Figures 5D1A,D2B**), the processes of the radial glia of both the DM and the MP cut

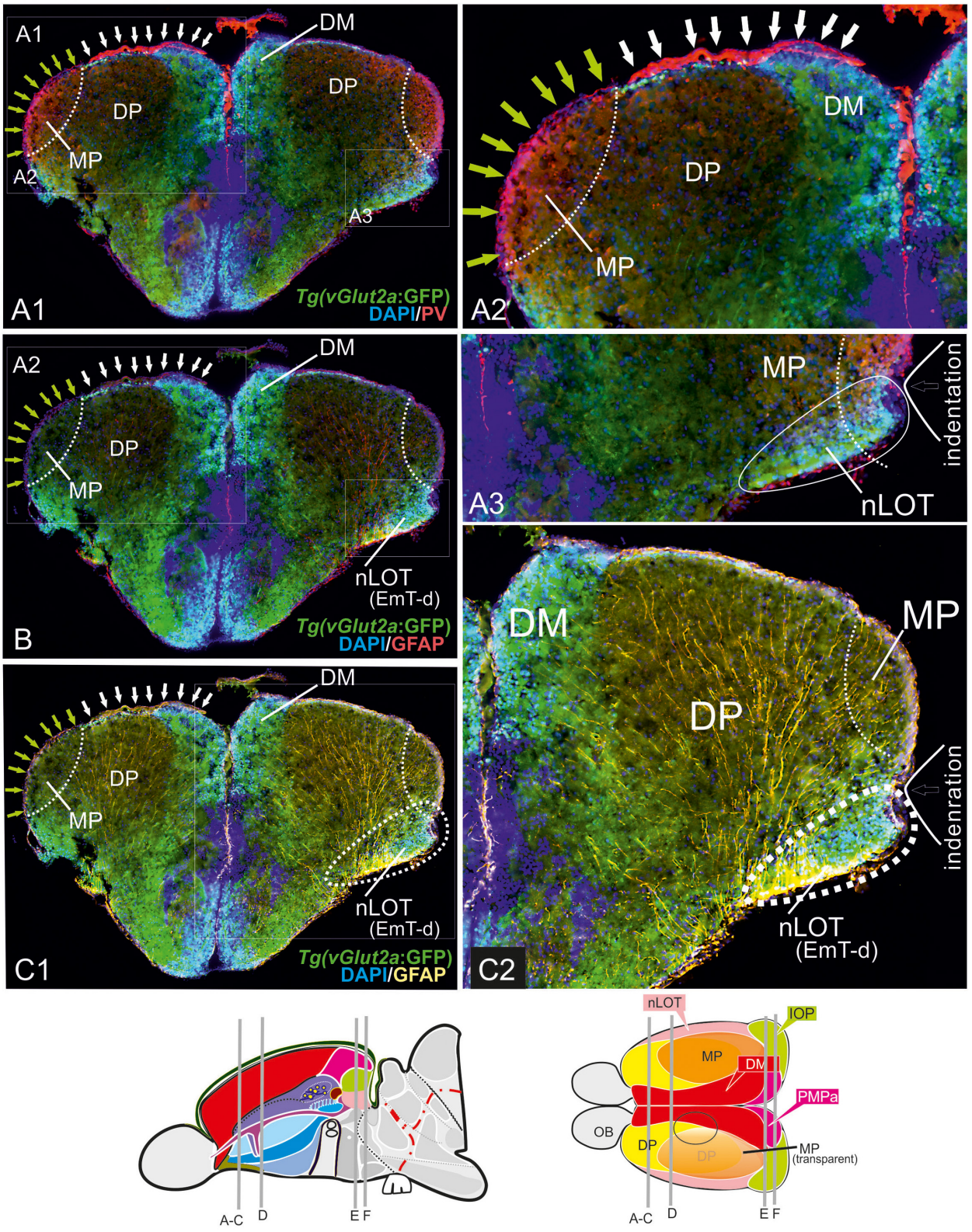

FIGURE 5 | Continued

through the DP territory and end just below the nLOT at the PSB. Interestingly, there are no radial glia cell somata nor radial glia processes visible at the lateral margins of the nLOT toward the indentation close to the tela attachement site. This finding again is consistent with our hypothesis that the *vGLut2a* positive portions of the nLOT originate from the EmTl (see schematic in **Figure 5**). Moreover, both the DM and the MP have overgrown the DP for the largest part, only few radial glia cells belong to DP (arrow pointing on radial glia cells of the dorsal pallium in **Figures 5D1B,D2B**). This finding suggests that once overgrown by DM and MP, the radial glia of DP will be replaced by those of the DM and MP.

At caudalmost sections, the processes of the radial glia located within the DM, PMPa, and IOP, again, dock onto the area topographically below the nLOT. As expected, none of the *vGlut2a*-driven GFP positive territories defined in this study as nLOT show radial glia somata at their pial site, nor do they show pronounced radial glia processes indicating that these territories have formed either through early radial migration (within a thalamic territory (EmT) or through tangential migration (late

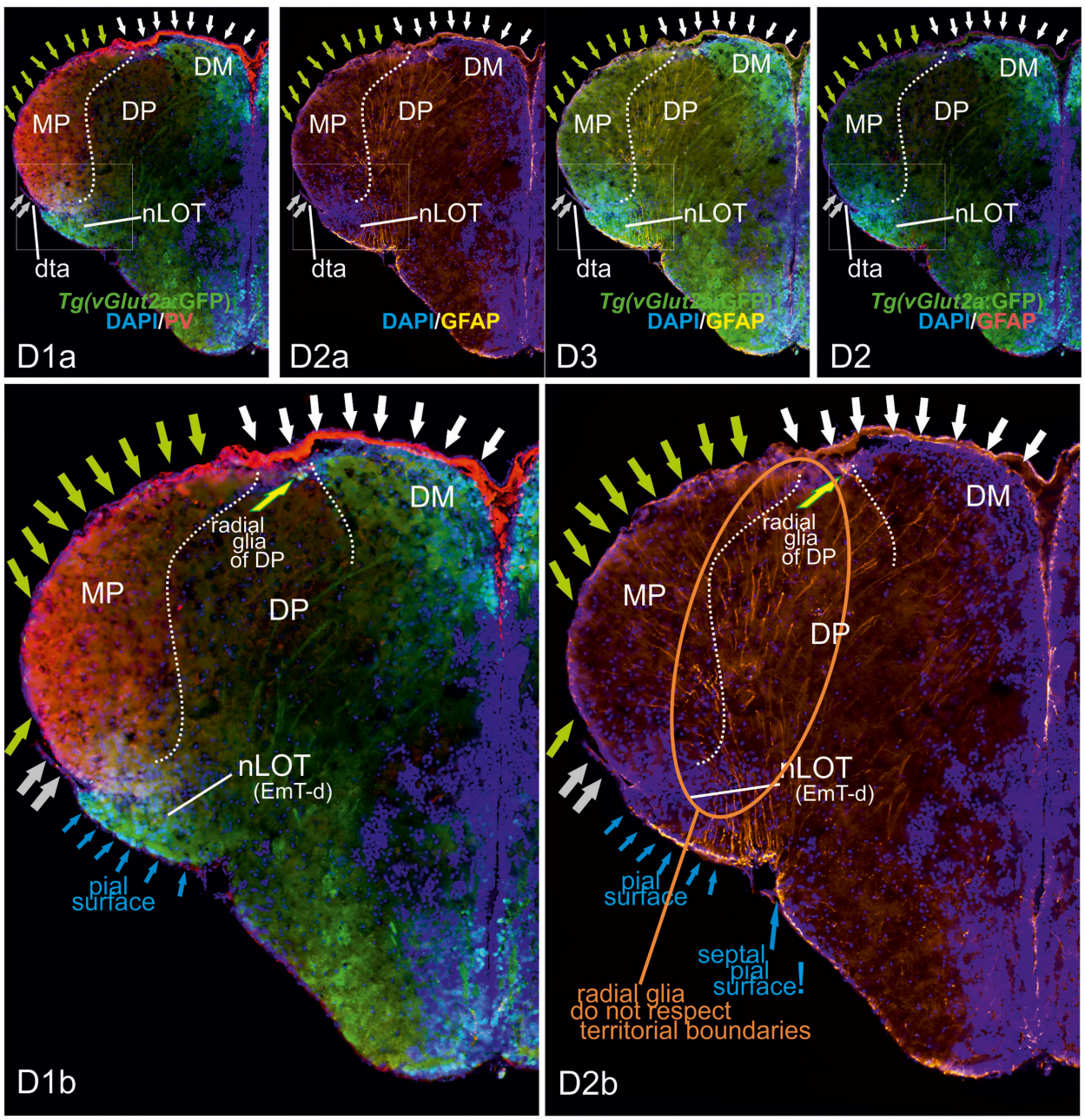

FIGURE 5 | Continued

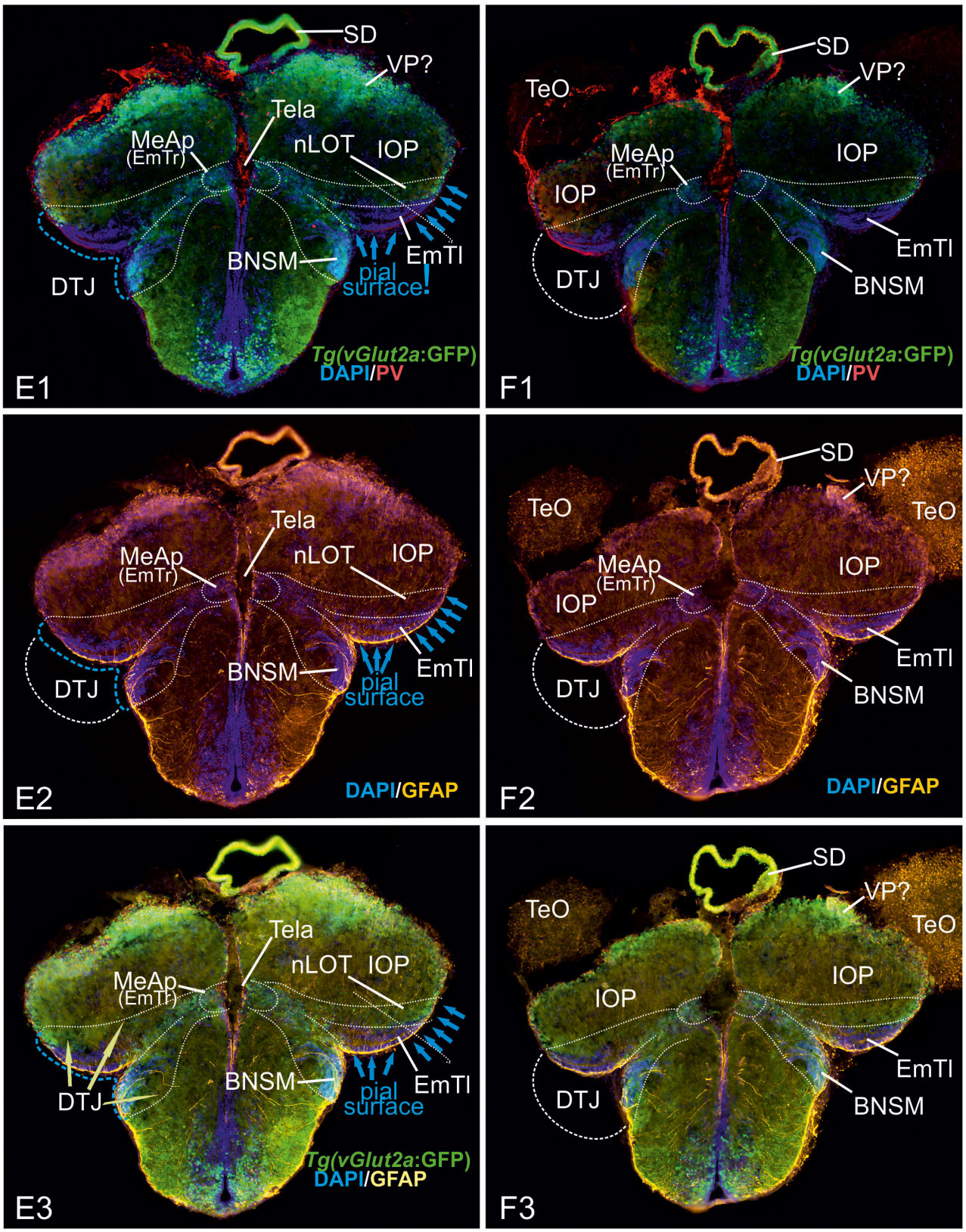

FIGURE 5 | Continued

FIGURE 5 | Radial glia cells and Tela Attachement Sites Confirm Medial, Dorsal, and Thalamic Eminence Identification.
We performed triple fluorescence immunostains against parvalbumin (PV), GFP, and GFAP in the brains of Tg(vGlut2a:GFP) counterstained against DAPI. **(A1–C2):** A1 shows different aspects of a rostral section highlighting that one large parvalbumin- (PV-) positive tela attachment site is located at the ventricular site of the dorsal pallium and pallial amygdala (white arrows in **A1,A2,B,C1**). The tela choroidea is fused (green arrows in **A1,A2,B,C1**) with the proliferative stem cell layer of the dorsolateral zone ("Dl") supporting its medial pallial ("hippocampal") identity. The nLOT tract shows a characteristic indentation **(A2,C2)** and no GFAP stained cell somata at its lateralmost aspect corroborating this area as the pial, not ventricular site. Notably, all radial glia of the candidate dorsal pallium do send their processes towards the pallial-subpallial border (PBS). These radial glia are attached to the topographically ventral side of the nLOT next to those of the medial pallium. Radial glia of the nLOT are not visible in this orientation suggesting that their radial domain is different from pallial zones. This finding supports the interpretation that the vGlut2a-driven GFP domain is a derivative of the EmT. **(D1A–D2B)** At mid-telencephalic sections, both radial glia cells and tela attachments show the same distribution. At the point where proliferative zones of the DM and medial pallium (MP) meet and grow over the dorsal pallium, a reduced number of radial glia cells of the dorsal pallium are visible. Radial glia of both MP and DM cut through the territory of DP and send their processes beneath the nLOT. The probable diencephalic attachment site of the tela choroidea is located at the nLOT (gray arrows in **D1b,D2b**). Again, there are no GFAP-positive glia cell somata at the most lateral cell groups corroborating its pial nature. The nLOT in this orientation shows no radial glia processes supporting their postulated EmT origin. At caudalmost sections, the study identifies the diencephalic -telencephalic junction (DTJ) that comprises territories formerly assigned to the telencephalon such as the posterior medial amygdala (MeAp/EmTr) and the vGlut2a-driven GFP positive nLOT territory that spans a region next to the MeAp/EmTr to the lateral (pial) surface (blue arrows in E1-E3). The BNSM was formerly identified as a derivative of the EmT in zebrafish and is often mistaken as the entopeduncular nuleus proper (ENv) Mueller and Guo 2009). Note, that the tela choroidea is attached to the MeAp/EmTr indicating its periventricular site **(E1,F1)**.

developmental stages from thalamic EmT into telencephalon). Note, that we view the *vGlut2a*-driven GFP positive portions of the nLOT as a radial domain of the EmTm or EmTl not described for earlier stages in zebrafish (Wullimann and Mueller, 2004a; Turner et al., 2016).

DIFFERENTIAL EXPRESSION OF *VGLUT2A*- AND *LHX5*-DRIVEN GFP DEFINE SUBDIVISIONS OF THE THALAMIC EMINENCE AT THE DIENCEPHALIC-TELENCEPHALIC JUNCTION

Comparing *lhx5*-driven GFP versus distributions of otp-a and *vGlut2a*-driven GFP revealed the detailed architecture of the adult zebrafish EmT and its topological relationships to both the EmTr/MeAp (former "Vi") and nLOT (**Figures 6A1–D**). Specifically, the results showed that otp-a protein distribution strongly overlaps with *lhx5*-driven GFP positive neurons within the EmTr/MeAp where the tela choroidea attaches at posteriomost sections close to the border to the habenula which is separated from the EmTr/MeAp by the base of the saccus dorsalis (SDB; **Figures 6C1,C2,D**). The *lhx5*-driven GFP expressing neurons at the posteriormost section reach to the periventricular site indicating that this proliferation zone gives rise to the *lhx5*-driven GFP positive cells, some of which are double-positive for both otp-a and *lhx5*-driven GFP (**Figures 6A3,B3**). This finding suggests that at least a fraction of these otp-a positive neurons is derived from the EmTr.

DISCUSSION

Teleost fish with more than 26,000 species comprise the largest vertebrate group and exhibit sophisticated cognitive capabilities underlying social and reproductive behaviors (Fernald, 2017). Yet to this day, the neural basis of these complex behavioral repertoires has remained elusive. Specifically, the field lacks a precise definition of the teleostean amygdala, whose counterparts in mammals form the regulatory core of the emotional brain essential to emotion, cognition, and social behavior (Northcutt, 2008). The mammalian amygdala shares many characteristics with other tetrapods and their sister group, the lungfish, which suggests that a complex tetrapod-like amygdala ground plan originated in the common ancestor of lungfish and tetrapods (Moreno and González, 2007; González et al., 2010; Abellan et al., 2013). It is currently, however, not understood how the amygdala is organized in more basally derived fish. In teleosts, eversion of the forebrain made direct comparisons difficult with tetrapod forebrains that develop through evagination (**Figure 1**; Nieuwenhuys, 2009b). We choose to focus and decipher the teleostean amygdala ground plan in zebrafish, because it represents an important model system for brain development and disease. What is more, earlier studies on zebrafish provide rich molecular information regarding pallial and subpallial entities including putative homologs of the mammalian iscortex and hippocampus (Mueller and Guo, 2009; Mueller et al., 2011; Ganz et al., 2014). Key processes, moreover, of the telencephalic eversion have been uncovered in zebrafish, providing an ideal foundation for our redefinition of the zebrafish amygdala (Mueller and Wullimann, 2009; Mueller et al., 2011; Folgueira et al., 2012).

Combining molecular characterizations with evolutionary and developmental considerations, we describe the entire zebrafish amygdala and its relationships with pallial, subpallial, and EmT territories. Our results stress that a dense almost indivisible continuum of thirteen territories forms the zebrafish amygdala. For the first time, we identify the nLOT and the posteromedial pallial nucleus (PMPa), both of which are integral elements of the zebrafish amygdala and the primary olfactory pallium. In addition, we show that the highest integrative olfactory zone ("Dp proper") occupies a sector that was previously considered a posterior portion of the dorsolateral pallium ("Dl" or "Dlp"). To avoid future misunderstandings, we call this region the integrative olfactory pallium (IOP), which we postulate represents the homolog of the lateral pallium (LP; mammalian entorhinal cortex). Despite their everted positions, each pallial amygdaloid territory is defined

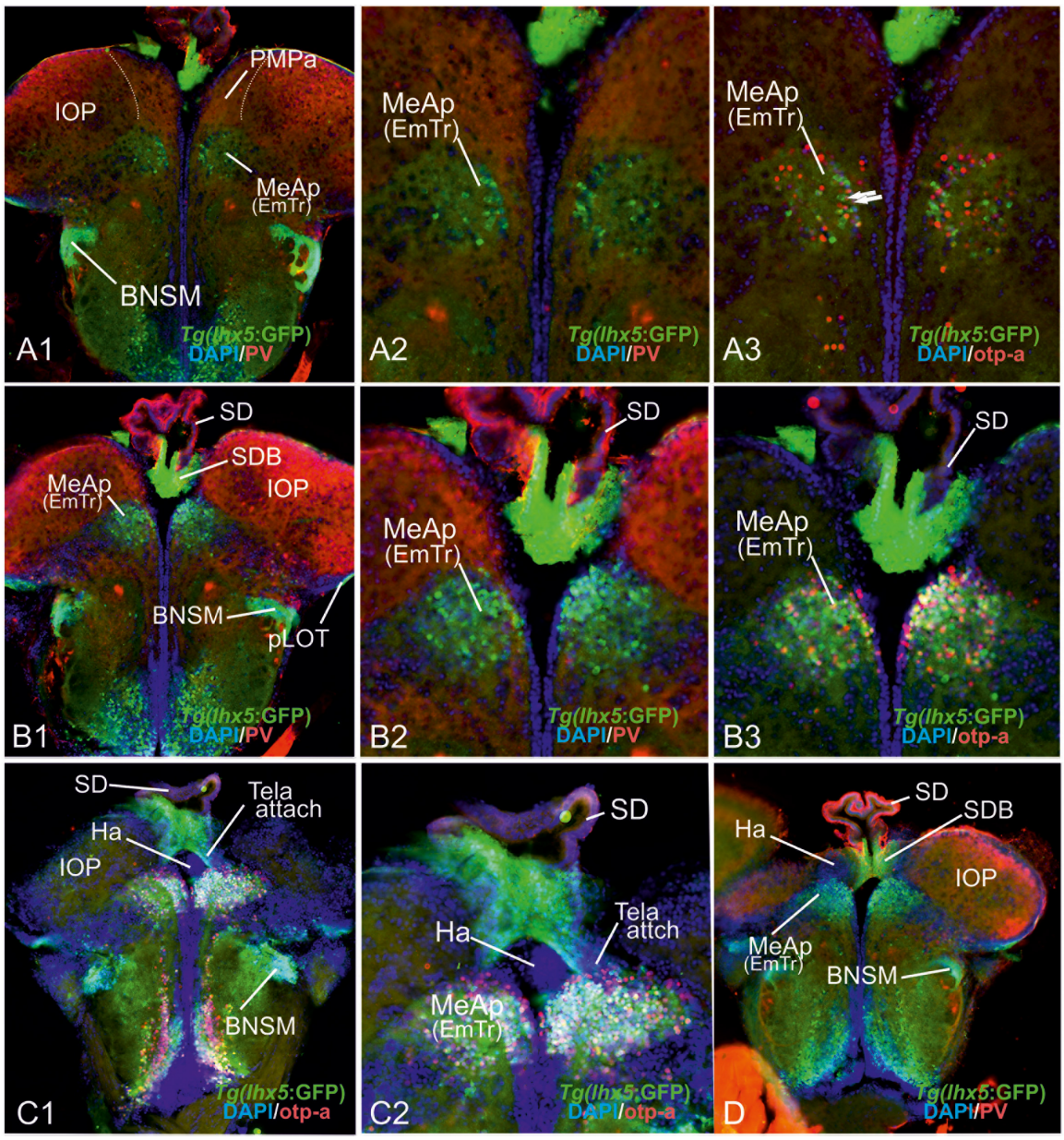

FIGURE 6 | The posterior medial amygdala (MeAp) is identical with the rostral thalamic eminence (EmTr). **(A1–D)**: The comparison between lhx5-driven GFP, parvalbumin (PV), and otp-a protein distribution shows that the lhx5-driven GFP positive EmTr reaches from its periventricular site (indicated by tela choroidea attachment in **C**). Note, the lhx5-driven GFP positive base (SDB) of the saccus dorsalis (SD) connects to both the Ha (in C1/2 and D) and the MeAp/EmTr (best visible in C1/2)hh. Like in mice, the posterior territory of the lateral olfactory tract seemingly does express lhx5 as indicated by GFP expression in Tg(lhx5:GFP) **(A1,B1,C1,D)**.

in this new framework through its conserved relationships with subpallial nuclei.

Neural Systems Organization of the Zebrafish Amygdala in Relation to Olfaction

The zebrafish amygdala consists of three functional networks similar to the situation described for mammals: (1) the main olfactory system, (2) the accessory olfactory-like or reproductive amygdala, and (3) the central and pallial amygdala (CeA-pAmy) network (mammalian BLA) regulating autonomic functions and mediating associative emotional learning. In other words, (1) the nLOT and two related nuclei of the bed nucleus of the stria terminalis (BSTa, BSTp) are part of the zebrafish main olfactory network. (2) The zebrafish accessory olfactory-like pathway consists of the PMPa, four medial amygdaloid territories (MeAa, MeAd, MeAv, MeAp/EmTr), and the medial

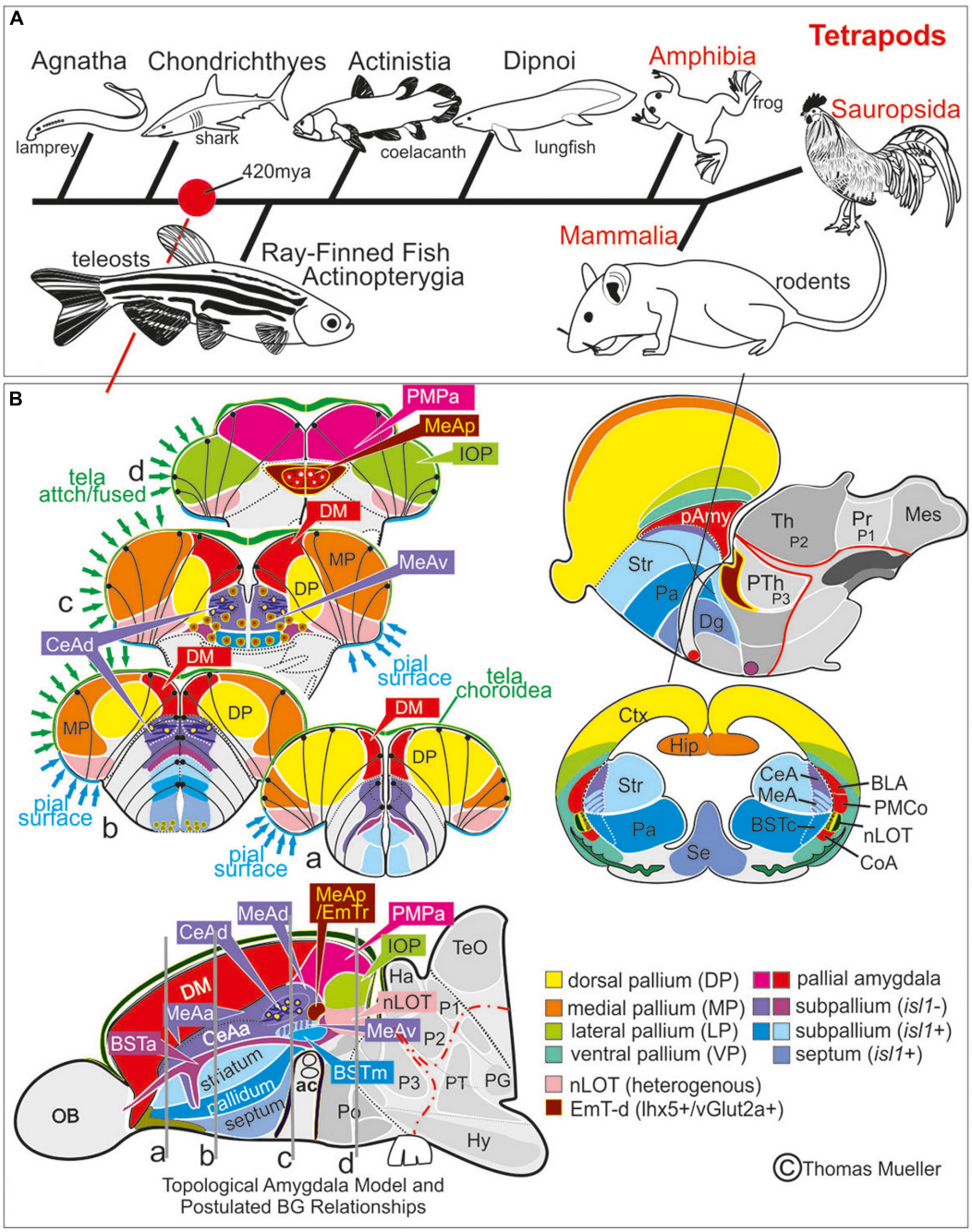

FIGURE 7 | Comparison Zebrafish – Macrosmatic Rodent Amygdala. **(A)** Cladogram indicating presence of a complex amygdala ground plan and nucleus of the lateral olfactory tract (nLOT) in the last common ancestor of teleosts and mammals. Our results indicate that the last common ancestor between ray-finned fish and mammals already showed a tetrapod-like main extended amygdala ground plan (red circle). Previously, most scientists assumed that a bipartite main versus olfactory extended amygdala evolved with a vomeronasal epithelium in the last common ancestor of lungfish and tetrapods (González et al., 2010). However, molecular evidence hint toward the presence of a bipartite olfactory system already in agnathan lamprey (Chang et al., 2013). We speculate, therefore, that a bipartite and complex amygdala may evolved with the earliest vertebrates. **(B)** Prosomeric comparisons of the zebrafish/teleostean amygdala (left) with the situation in macrosmatic rodents (right). Both the schematized zebrafish brain cross sections and the parasagittal view on the left side indicate that the zebrafish amygdala holds several previously misinterpreted territories such as the bed nucleus of the stria terminals (BST), the medial amygdala (MeA) and its anterior (MeAa), posterior (MeAv) and dorsal division (MeAd), the posteriormedial pallial amygdala (PMPa), the integrative olfactory pallium (IOP), and the nucleus of the lateral olfactory tract (nLOT). Due to the teleostean-specific outward growing process (eversion), these territories lie on top of the telencephalon and cover the zebrafish homolog to the mammalian isocortex. In mammals (right side), we find the opposite situation. Here, the amygdaloid complex is located in the anterior ventral depth of the brain covered by the enlarged isocortex (modified after Mueller et al., 2011; Mueller, 2012). For molecular definition of pallial, subpallial, and EmT-derived (EmT-d) amygdaloid territories see **Table 1**. Abbreviations see table.

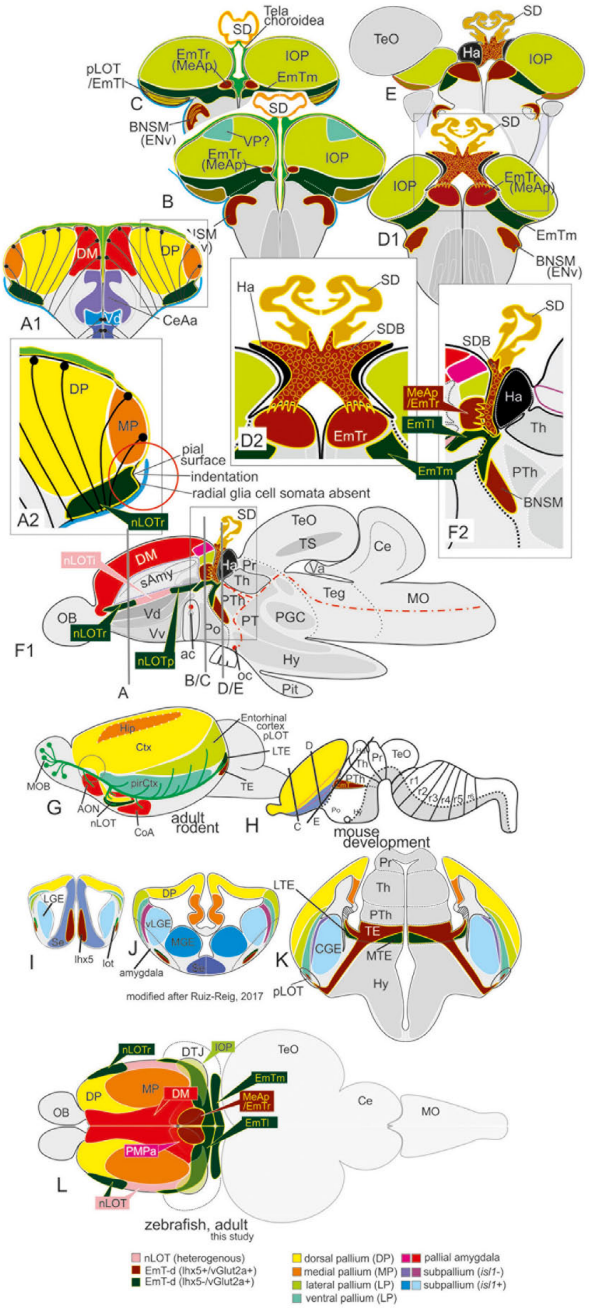

FIGURE 8 | Summary schematic explaining how the zebrafish thalamic eminence (EmT) reveals ancestral topological relationships to posterior medial amygdala and olfactory pallium. **(A–F2)** Comparing topographically rostral and posterior ends of the telencephalon in transgenic lines *Tg(vGlut2a:GFP)* and *Tg(lhx5:GFP)* with distribution of parvalbumin, otp-a, and GFAP indicated a complex EmT. In sites classically defined as "telencephalic," we identified the rostral (EmTr = *vGlut2a*+, *lhx5*+, otp-a+) and lateral (EmTl) *vlgut2a*+;*lhx5*-). At diencephalic side of the diencephalic-telencephalic junction, the EmTm can be easily identified based on *vGlut2a*-driven GFP expression and absence of *lhx5*-driven GFP, whereas the otp-a negative bed nucleus of the stria medullaris (BNSM) shows both *vlgut2a*- and *lhx5*-driven GFP. Note, that we consider the rostral and posteriormost parts of the newly defined nLOT a derivative of the EmT consistent with the absence of radial glia somata and *(Continued)*

FIGURE 8 | Continued
processes in their most lateral (pial) aspects. **(G–L)** Schematics comparing brain of adult zebrafish (dorsal view) with adult rodent (idealized side view), and developmental stage to determine various EmT derivatives. The study identifies topographically rostral and posterior EmT territories based on differential expression of *vGluta*- and *lhx5*-driven GFP in comparison to otp-a expression. The dorsal view on the zebrafish telencephalon highlights "diencephalic" medial (EmTm) versus "telencephalic" rostral (EmTr) and lateral (EmTl) at the diencephalic-telencephalic junction (DTJ). The distribution of *vGlut2a*-driven GFP and *lhx5*-driven GFP in adult zebrafish strikingly resembles the situation shown in mammals (mouse) (Ruiz-Reig et al., 2017). Our complexly everted telencephalon model considers both rostral (nLOTr) and posterior portions of the most ventro-lateral expression domains of *vGlut2a*-driven GFP as radial extensions of the EmTl. The integrative olfactory pallium (IOP) is most likely a derivative of the newly defined lateral pallium of the prosomeric model and thus homologous to the entorhinal cortex.

BST (BSTm). The MeAp/EmTr, previously termed intermediate subpallial nucleus (Vi), has already been identified as an olfactory subpallial nucleus (the "MeA") that responds to socially relevant olfactory information (Biechl et al., 2017). (3) The zebrafish central amygdala (CeA = CeAa+CeAl+CeAd) sits close to and topographically below the region that is commonly viewed as the teleostean pAmy (Portavella et al., 2004a,b; Wullimann and Mueller, 2004b; Martin et al., 2011; von Trotha et al., 2014; Ruhl et al., 2015).

This separation of the three neural systems becomes visible when looking at the molecular characteristics that define mature neurons. For example, the branching formations of both substance P and calretinin fibers reflect the topological relationship and connectivity between newly identified core territories of the extended medial amygdala (MeAd, MeAv, MeAp). As a unifying characteristic, these accessory olfactory-like territories share the presence of numerous calretinin-positive neurons. This fact clearly separates them from anterior central amygdaloid territories that lack such calretinin neurons. Likewise, among central amygdaloid territories, the CeAd stands out as a laterally displaced sector enriched with parvalbumin fibers and sparse parvalbumin-neurons. Similarly, amygdaloid structures of the main olfactory-like pathway, such as the nLOT and the dopaminergic BST territories, lack substance P fibers.

Taken together, our new amygdala framework reveals an intricate zebrafish pallial-subpallial amygdala network of heterogonous molecular structure that resembles the amygdala of mammals with prominent olfaction, such as macrosmatic rodents.

Hallmarks of our new amygdala paradigm are the identification and molecular definition of the EmT territories (EmTr; EmTl, EmTm), the nLOT, and the highest olfactory pallial zone, which we termed the integrative olfactory pallium (IOP). Their precise demarcation, chemoarchitecture and topology allowed us to clarify previously mislabeled neighboring structures (see **Supplementary Table S1** that lists labeling inconsistencies across teleosts). The zebrafish IOP, for example, was previously interpreted as part of the dorsolateral (Dl) pallial zone by Wullimann et al. (1996). As such, it was incorrectly considered as a non-olfactory extension of the teleostean medial pallium (mammalian hippocampus). Our identification of the actual Dp

region—as integrative olfactory pallium (IOP) in its redefined position in the dorsoposterior pallium—posits also a topological correspondence to the mammalian entorhinal cortex (=lateral pallium; LP). That is, we postulate that the IOP/Dp proper is not part of the "teleostean hippocampus" ("Dl") but like the mammalian entorhinal cortex an important building block of the "teleostean hippocampal formation-like system." In other words, we suspect that the IOP is critical for both olfactory integration and olfactory-related navigation similar to the mammalian entorhinal cortex. Also, the zebrafish nLOT was previously misinterpreted either as part of the "teleostean hippocampus" ("Dlv") or the posterior olfactory pallium ("Dp") (Wullimann et al., 1996; Ganz et al., 2014). Confusion about Dp has also led to functional misinterpretations and the erroneous assumption that the Dp territory represents the highest integrative olfactory pallial zone (Jacobson et al., 2018). Our identification of the zebrafish nLOT as a potentially lower integrative structure emphasizes deeply conserved evolutionary links between the teleostean and mammalian amygdala and primary olfactory pallium. The identification of various EmT territories and their structural relationship to the zebrafish amygdala clarifies the unusual (compared to non-actinopterygian vertebrates) everted topology of the teleostean forebrain that has been subject of debate among comparative neurologists for over a century (Nieuwenhuys, 1963, 2009b; Wullimann and Mueller, 2004b; Yamamoto et al., 2007; Northcutt, 2008; Mueller and Wullimann, 2009).

Our data also indicate deep and unexpected relationships between the DM, the PMPa, the integrative olfactory pallium (IOP), and the nLOT. For example, we show that the PMPa needs to be viewed as a pallial passage functionally linked to the extended medial amygdala and cannot simply be described as an extension of DM (anterior Dm) (Northcutt, 2008; von Trotha et al., 2014; Ruhl et al., 2015). Critically positioned between the DM (important for motivational states), the IOP and the extended medial amygdala (processing pheromones and social cues), the PMPa, we postulate, integrates accessory olfactory information and motivational states. In contrast, the newly identified dopaminergic BST nuclei (BSTa and BSTpd) form a functional relationship with the nLOT. As a part of the main olfactory system, the BSTa and BSTpd most likely modulate the activity of the nLOT. We postulate that the nLOT itself integrates main olfactory and gustatory information, given that this structure receives projections from both systems (Vaz et al., 2017; Yanez et al., 2017).

Molecular Amygdala Characteristics and Functional Organization

Our drastically revised zebrafish amygdala-olfactory systems paradigm is also supported by previous findings about the expression patterns of conserved gene regulatory genes and the development of the zebrafish telencephalon. The interpretation of the *isl1*:GFP negative BSTa; BSTp, CeA, MeAd as mainly dLGE-derived territories is compatible with conserved regulatory gene expression patterns and developmental studies of the zebrafish telencephalon (Costagli et al., 2002; Mueller et al., 2008; Mueller and Guo, 2009; Ganz et al., 2012; Folgueira et al., 2012). A study has shown as well that the adult *isl1*-driven GFP expression strongly resembles the larval expression (Baeuml et al., 2019). This fact supports our interpretation that the isl1-free GABAergic zone in fact represents an evolutionarily expanded dLGE territory. However, we cannot exclude the possible presence of pallidal and striatal neurons that may have migrated from the *isl1*-positive territory and subsequently suppressed *isl1*-expression. We suspect also that amygdaloid territories might be even more heterogeneous than this study proposes. For example, the zebrafish BST may include a territory with isl1-negative and nkx2.1 positive neurons as has already been suggested (Ganz et al., 2012).

Equally important for understanding the ground plan is that the distribution of mature neuronal phenotypes conforms with the function of these amygdaloid territories. For example, even though the overlapping distribution of *vGlut2a*-driven GFP (this study) and *emx3* mRNA (Ganz et al.) render the zebrafish DM a relatively homogenous histogenetic unit, the region most likely subdivides into functionally distinct sectors. A recent study showed that emx3-positive glutamatergic subpopulations, called "Dm120A," innervate zebrafish hypothalamic territories (Lal et al., 2018)(Ganz et al., 2014). In contrast, the mammalian central amygdala projects to the hypothalamus via GABAergic neurons. Here the zebrafish pallial amygdala candidate (DM) seems to deviate, at least partially, from the mammalian one. Mammals except marsupials also do not possess an emx3 ortholog (Kuraku, 2010) and instead express Emx1 in specific nuclei of the pallial amygdala (Medina et al., 2017). In fact, the mammalian basolateral amygdala (BLA)—critical for associative emotional learning and in all likelihood the most comparable region to the zebrafish DM region – contains Emx1 positive neurons (Cocas et al., 2009) (Medina et al., 2017). Other studies also support this comparability between mammalian BLA and part of the zebrafish DM. In particular the significant expression of cannabinoid receptor 1 in the zebrafish DM (Lam et al., 2006), is also a key characteristic of the mammalian BLA (Katona et al., 2001). These findings point to deeply conserved neurophysiological and functional parallelisms between ray-finned fish and tetrapods. Previous studies showed also that the zebrafish DM is involved in associative learning and endocannabinoid signaling critical for its function (von Trotha et al., 2014; Lau et al., 2011; Ruhl et al., 2015, 2017). As a result, the zebrafish amygdala needs to be understood as a mosaic of conserved and acquired characteristics much like other parts of the zebrafish brain (Costagli et al., 2002; Wullimann and Mueller, 2004b; Adolf et al., 2006; Northcutt, 2008; Mueller, 2012).

Zebrafish Eversion and Amygdala – Complex and Constrained

What is more, our data drastically extend a complex telencephalon eversion model characterized by a centralized isocortex-homolog (dorsal pallium) and a migratory stream across the pallium only in an earlier study (Mueller et al., 2011). Our identification here of several previously overlooked structures, including the principal olfactory pallium (IOP), the nLOT, and a set of EmT territories alongside documentation of

radial glia distribution and attachment sites of the tela choroidea, paint a new and complex picture of the zebrafish eversion. Our results indicate, that a complete and complex outward-growing (eversion) process rearranges the zebrafish forebrain constrained by the thalamic eminence (EmT), the organizing center sitting at the diencephalic-telencephalic junction (DTJ, **Figure 8**). The presence of the thalamic eminence in the newly demarcated diencephalic-telencephalic junction (DTJ) of adult zebrafish supports a highly regulated outward-growing process that starts during early development. This hypothesis is consistent with embryonic eversion processes as well (Folgueira et al., 2012). During larval and juvenile stages, the eversion gains in complexity through radial migratory processes and the overgrowing of the dorsal pallium by both the DM (putative pallial amygdala) and the MP (Mueller et al., 2008, 2011; Mueller, 2012).

The Evolution of Cognition and Emotion

For the first time, our study identifies the DTJ with the EmT derivatives and their relationship to the amygdala thereby solving the zebrafish eversion for teleost ray-finned fish (actinopterygians) at large. What this study makes particularly clear is that the zebrafish telencephalon resembles the mammalian forebrain even though the teleostean everted morphology causes a markedly difference in appearance. Our findings show as well that the here newly identified EmT territories represent the missing link between the everted zebrafish forebrain and the evaginated telencephalon of mammals. Like in mammals, zebrafish EmT territories form highly conserved and intertwined forebrain elements, a fact previously overlooked causing misinterpretations of the forebrain evolution in ray-finned fish. This study also validates the prior discovery of the zebrafish dorsal pallium (mammalian iscortex), which already suggests a conserved forebrain ground plan between teleosts and tetrapods (Mueller et al., 2011). The presence of both a considerable dorsal pallium and a complex zebrafish amygdala, of course, is pivotal for the development of phenomenal consciousness, emotional sentience, and the perception of pain (Key, 2015; Sneddon, 2019). This study, therefore, stresses the need to discuss the presence and implications of emotion in fish.

Important for a better understanding of the early evolution of vertebrate amygdala and isocortex-homolog will be the identification of EmT territories in more basally derived fish. The probably highly conserved territories in actinopterygian and non-actinopterygian fish, such as agnathans (lamprey) and chondrichthyes (sharks and manta rays), might allow to solve disagreements about their forebrain evolution (Puelles, 2001; Pombal et al., 2009; Mueller, 2011; Northcutt, 2011; Schluessel, 2015; Docampo-Seara et al., 2018). Similarly, the discovery of EmT and nLOT enabled in this study to effectively compare the everted zebrafish with the evaginated forebrain of mammals. Notably, the mammalian amygdala is evolutionarily not only related to EmT and nLOT but shows also strong reciprocal connections with the prefrontal cortex, critical for cognition and goal-directed behaviors. Currently, most comparative neurobiologists assume that the prefrontal cortex, the highest integrative cortical center, represents a distinguishing characteristic of the mammalian isocortex, most pronounced in humans. However, based on the prefrontal cortex's connections to the amygdala in mammals and conservatisms in the zebrafish amygdala, we postulate that basic amygdala-prefrontal cortex-like circuits exist in teleost fish, too. In this context, our study provides role model and rationale for the neural substrates of cognition and emotion in fishes or basally derived vertebrates. The predictable presence of amygdala-prefrontal cortex-like circuits in teleosts may eventually allow identifying the neural basis of emotional sentience in the important genetic model zebrafish.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the IACUC Committee Kansas State University, University Research Compliance Office, Manhattan, KS, United States.

AUTHOR CONTRIBUTIONS

TM planned the study and designed the experiments, wrote the manuscript, and carried out a portion of the immunohistological procedures and imaging. BP conducted immunhistological stainings, imaging, and statistical analyses. Both authors discussed the results and scientific interpretations.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00608/full#supplementary-material

FIGURE S1 | substance P fiber tracts from the olfactory bulb into the telencephalon. **(A–D)** The antibody against substance P labeled fiber bundles originating in the olfactory bulb and projecting to all extended medial amygdala (MeA) territories, posteromedial pallial amygdala (PMPa), and integrative olfactory pallium (IOP). The bulbo-telencephalo projections form *two solid tracts*; one smaller dorsally located one and a larger ventral one, plus a diffuse tract less visible in sagittal sections. The ventral substance P positive tract most likely corresponds to the lateral medial olfactory tract of cyprinids conveying pheromone information. The dorsal ascending substance P tract extends along the pallial-subpallial border (PSB) and most likely represents a derivative of the septum. Note, the nLOT, as defined in this study, lacks substance P fibers.

FIGURE S2 | Sagittal brain section stained against GFAP (red), *vGlut2a*-driven GFP (green), and parvalbumin (yellow). **(A1,A2)** The distribution of *vGlut2a*-driven GFP shows the extent of the pallial amygdala DM in relation to the dorsal pallium (DP) and integrative olfactory pallium (IOP) both of which lack *vGlut2a*-driven GFP at large. The tela choroidea is closely attached (white arrows) to the DM an extents up to the olfactory bulb. Also, note that dense population of GFAP-positive fibers surround the anterior commissure similar to the situation in mammals.

TABLE S1 | Nomenclature for teleostean telencephalic territories and examples for former labeling inconsistencies. *None of the former studies did establish molecularly defined histogenetic units as this study does making it impossible to perfectly compare conflicting interpretations. **After Biechl and colleagues (Biechl et al., 2017).

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