





RESEARCH ARTICLE

In search of common developmental and evolutionary origin of the claustrum and subplate

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Abstract

The human claustrum, a major hub of widespread neocortical connections, is a thin, bilateral sheet of gray matter located between the insular cortex and the striatum. The subplate is a largely transient cortical structure that contains some of the earliest generated neurons of the cerebral cortex and has important developmental functions to establish intra- and extracortical connections. In human and macaque some subplate cells undergo regulated cell death, but some remain as interstitial white matter cells. In mouse and rat brains a compact layer is formed, Layer 6b, and it remains underneath the cortex, adjacent to the white matter. Whether Layer 6b in rodents is homologous to primate subplate or interstitial white matter cells is still debated. Gene expression patterns, such as those of *Nurr1/Nr4a2*, have suggested that the rodent subplate and the persistent subplate cells in Layer 6b and the claustrum might have similar origins. Moreover, the birthdates of the claustrum and Layer 6b are similarly precocious in mice. These observations prompted our speculations on the common developmental and evolutionary origin of the claustrum and the subplate. Here we systematically compare the currently available data on cytoarchitecture, evolutionary origin, gene expression, cell types, birthdates, neurogenesis, lineage and migration, circuit connectivity, and cell death of the neurons that contribute to the claustrum and subplate. Based on their similarities and differences we propose a partially common early evolutionary origin of the cells that become claustrum and subplate, a likely scenario that is shared in these cell populations across all amniotes.

KEYWORDS

claustrum, development, dorsal endopiriform nucleus, evolution, insular cortex, lateral amygdala, layer6b, subplate

Abbreviations: ABADP, Allen Brain Atlas Data Portal; ac, anterior commissure; acp, posterior limb of anterior commissure; Am, amygdala; C, caudate nucleus; Cl, claustrum; CP, caudate/putamen; DPall, dorsal pallidum; Ec, external capsule; EPd, dorsal endopiriform nucleus; EPv, ventral endopiriform nucleus; extc, extreme capsule; ic, internal capsule; Ins, Insular cortex; LPall, lateral pallidum; MPall, medial pallidum; P, putamen; Pir, piriform cortex; rf, rhinal fissure; SPall, subpallidum; St, striatum; VPall, ventral pallidum.

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1 | INTRODUCTION

The claustrum received its name from the Latin for “enclosure,” due to its unique anatomical position (Molnár, 2004; Willis, 1664). The cells of the claustrum form a sheet of gray matter which is embedded in the white matter, separated from the putamen by the external capsule and the ventral pallidum (VPall) radial domain (medially), and from the insula by the extreme capsule (superficially). The function of the claustrum has received much interest due to its presumed role in higher brain functions, such as salience-attention, pain perception, speech production, emotion processing, coordination of slow wave activity, interoception, and consciousness (Atlan et al., 2018; Bickel & Parvizi, 2019; Crick & Koch, 2005; Koubessi, Bartolomei, Beltagy, & Picard, 2014; Krimmel et al., 2019; Mathur, Caprioli, & Deutch, 2009; Nariyo et al., 2018; Smythies, Edelstein, & Ramachandran, 2012). The claustrum has extremely widespread reciprocal connections with the cortex, which prompted ideas about its potential function in human perception and delusional phenomena (Patru & Reser, 2015).

The subplate is a transient compartment of the developing cerebral cortex, with migrating neurons that populate the overlying cortical plate after passing through cortical afferent and efferent fibers (Judaš, Sedmak, & Kostović, 2013; Kostović & Judas, 2010). The subplate contains some of the earliest born neurons of the cerebral cortex (Angevine & Sidman, 1961; Bayer & Altman, 1990; Duque, Krsnik, Kostović, & Rakic, 2016; Kostović et al., 2014; Price, Aslam, Tasker, & Gillies, 1997; Rakic, 1974; Rickmann, Chronwall, & Wolff, 1977). Subplate cells have important developmental functions that establish intra- and extracortical connections through transient connectivity that will eventually be dismantled at later stages (Allendoerfer & Shatz, 1994; Kanold & Luhmann, 2010). In most species assessed to date, some subplate cells undergo regulated cell death and their transient connectivity is demolished, although it remains a subject of debate as to whether this occurs in the majority or just of few of the cells. In humans and macaque monkeys, some cells remain as interstitial white matter cells. In mice and rats a compact layer, Layer 6b, is formed from the earliest born subplate neurons and remains at the base of the cortex adjacent to the white matter into adulthood (Duque et al., 2016; Hoerder-Suabedissen & Molnár, 2012, 2013, 2015; Price et al., 1997). That Layer 6b in rodents is homologous to the primate subplate and interstitial white matter cells is a likely possibility; although it warrants further investigation, for the purposes of this review we shall assume it as a given. Undisputed is the fact that the transient subplate compartment is considerably enlarged in primates as compared to other mammals (Kostović & Judas, 2010; Wang et al., 2011). It has been argued that the subplate and its postnatal remnant serve as the main area for the evolution of developmental shifts, such as increased brain size, neuron number, connectivity, and shaping of circuits in response to their social environment. Judaš et al. (2013) also proposed that a more complex and protracted development, including intricate transient circuits, might have contributed to more sophisticated cortical organization and consequently more advanced cognitive functions, such as language, self-awareness, and cognition.

In the adult mouse neocortex, a thin sheet of neurons, Layer 6b, appears beneath Layer 6a in the cortical plate, located immediately superficial to the white matter, and has a continuous transition with the deepest part of the claustrum, with which it shares various gene expression patterns (Montiel et al., 2011; Puelles, 2014; Wang et al., 2011; Watakabe, Ohsawa, Ichinohe, Rockland, & Yamamori, 2014; see Section 1.5). In addition to overlapping gene expression patterns, the claustrum and Layer 6b share other developmental features. Notably, neurons in these structures are born earlier than all other cortical pyramidal neurons (Hoerder-Suabedissen & Molnár, 2013). The development of the mutual connectivity of the claustrum, dorsal endopiriform nucleus (EPd), lateral amygdala, and insular/piriform cortices is highly dependent on the precise choreography of early axonal guidance mechanisms, which include the formation of reciprocal thalamocortical projections at the pallial-subpallial boundary (González-Arnav, González-Gómez, & Meyer, 2017; Molnár, 1998; Molnár & Butler, 2002; Molnár, Garel, López-Bendito, Maness, & Price, 2012). The relatively early histogenesis of all these structures may be highly relevant for our understanding of the development and evolution of forebrain circuits. Here, we appraise the comparative data on cytoarchitecture, evolutionary origin, gene expression, cell types, birthdates, neurogenesis, lineage, migration, connectivity, and cell death of the neurons that contribute to the claustrum and subplate. While we have made every effort to address each topic individually, often multiple strands of evidence are required to understand historic viewpoints or current conclusions. In this review we note the marked similarities between claustrum and subplate neurons and raise the possibility of a partially common developmental and evolutionary origin.

1.1 | Cytoarchitecture of the claustrum

The claustrum-insular complex forms the lateral pallidum (LPall) radial domain (Puelles, 2014), with its immediate dorsal neighbor being the dorsal pallidum (DPall), where layers 1-6a/b of the neocortex can be found (6b being equivalent to subplate in rodents). In most mammals, including marsupials and most eutherian species, the claustrum is located near the insular cortex, which in humans and other primates lies beneath the Sylvian fissure between the temporal and parietal/ frontal cortices (Figure 1). While in humans and other primates the extreme capsule is easily detected between the claustrum and insular cortex, in other mammals, such as rodents, microchiropterans, and small marsupials, these fibers are less prominent or even absent and the claustrum lies directly under the deep layers of the insular cortex. The claustrum (CI) and EPd together are sometimes referred to as the claustral complex (Puelles, 2014). The EPd lies beneath the piriform cortex within the VPall. A distinct bundle of VPall radial glia crosses the EPd to reach the piriform cortex. At the same level there is a separate ventral endopiriform nucleus next to the putamen, which originates from the VPall and is unrelated to the claustrum. Both the claustrum and the EPd derive from the lateral pallidum (Puelles, 2014; Watson & Puelles, 2017), and serve as major hubs of neocortical and

limbic circuits, respectively. In general, while the shape and size of the claustrum is variable across mammals, its size increases in proportion with the neocortical volume (Baizer, Sherwood, Noonan, & Hof, 2014; Druga, 2014; Kowiański, Dziewiatkowski, Kowiańska, & Moryś, 1999; Figure 1).

1.2 | Cytoarchitecture of the subplate/Layer 6b

The subplate is a largely transient cortical structure that contains some of the earliest generated neurons of the cerebral cortex (Allendoerfer & Shatz, 1994; Kostović & Rakic, 1990). The subplate can be identified in primate brains by its expression of proteoglycans, with cortical afferents passing through this region and transient and migratory cell populations (Judaš et al., 2013; Kostović & Judas, 2010). The lower subplate boundary is not as easy to identify in the primate cortex as it is in the rodent cortex, and in primates the subplate also lacks a clear boundary with the intermediate zone. In the rat and the mouse the subplate appears as a thin, compact cell layer which is increasingly separated from the cortical plate at embryonic (E) stages E14–16 according to an antero-posterior and ventro-dorsal gradient (Figure 3b,e; Oeschger et al., 2011). In the putative primary visual cortex of the macaque monkey and human, the subplate divides into upper and lower compartments with a thin, dense, transient cell layer forming at their boundary (Hoerder-Suabedissen & Molnár, 2015; Kostović & Rakic, 1990). Such transient compartmentalization has not been observed in other species to date. The subplate is also a compartment in which thalamocortical afferents wait and form early synapses, before growing into the overlying cortex (Kostović & Rakic, 1990).

The constituent cells of the transient subplate compartment may survive at least in part as interstitial white matter neurons in monkey and human brains (Duque et al., 2016). In postnatal and adult rodent brains the location of the former subplate is occupied by a densely packed band of cells—Layer 6b—that is distinct from the underlying white matter and separated from the overlying cortex by a thin, cell sparse region.

1.3 | Evolutionary comparison of the claustrum

The claustrum is an evolutionarily ancient structure, with a putative claustrum having been described in mammals, birds, and reptiles. Despite its presence in most mammals studied to date, the existence of a claustrum in monotremes (i.e., egg-laying prototherian mammals) has been long debated. Abbie (1940) examined the brains of platypus and echidna but failed to find a cytoarchitecturally distinct claustrum, and concluded that therefore no associated insular cortex was present. Butler and colleagues argued that the apparent lack of the claustrum in monotremes posed challenges to the understanding of pallial evolution and argued that this might just be a cytoarchitectonic absence, rather than the true absence of the particular cell group (Butler & Molnár, 2002; Butler, Molnár, & Manger, 2002; Molnár &

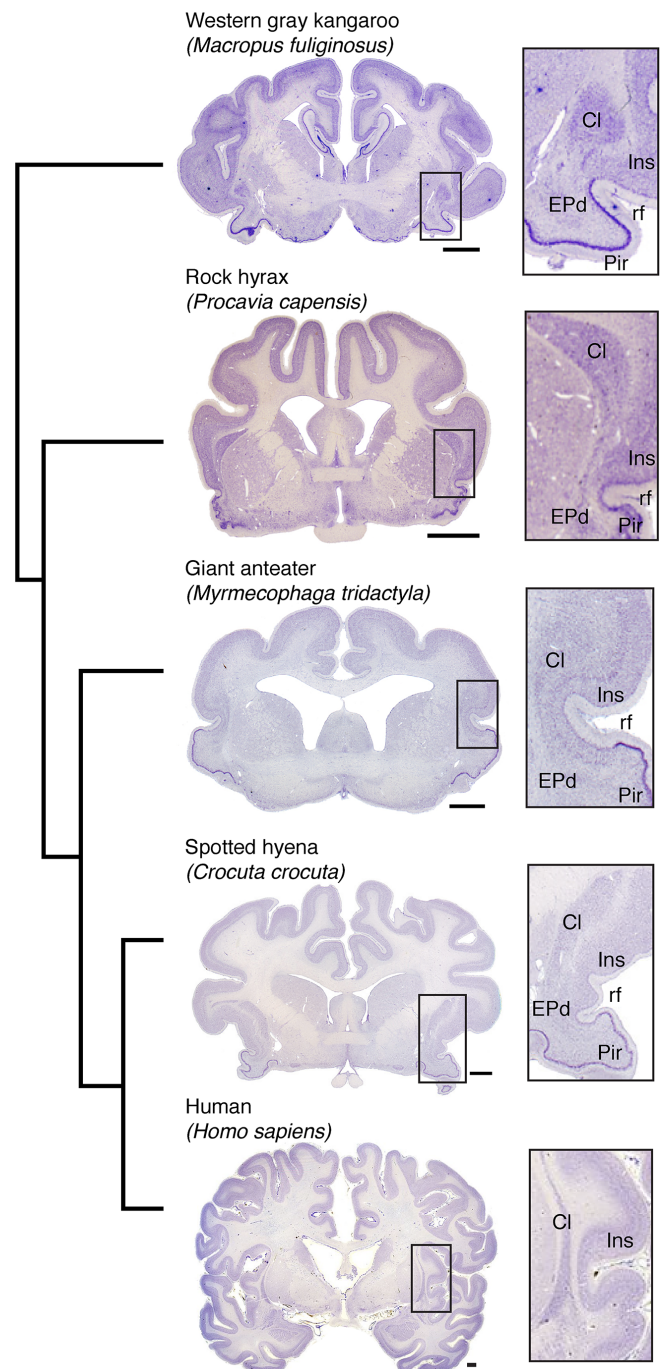


FIGURE 1 Nissl-stained coronal brain sections and phylogenetic relationships of representative mammalian species (western gray kangaroo, rock hyrax, giant anteater, spotted hyena, and human). Insets on the right were taken from the right hemisphere from the region of the rhinal fissure (rf) near the insular cortex (Ins). The claustrum (Cl) is present in most therians immediately adjacent to the insular cortex and forming a cytoarchitectonic complex with the dorsal endopiriform nucleus (EPd), which lies deep to the piriform cortex (Pir). Scale bar: 3000 μ m. Images taken from brainmuseum.org [Color figure can be viewed at wileyonlinelibrary.com]

Butler, 2002). Subsequently Ashwell, Hardman, and Paxinos (2004) reported the presence of both the claustrum and EPd in platypus and echidnas based on the examination of cytoarchitectural,

histochemical, and fiber stained material. Puelles (2014) agreed with the evidence that there is a cryptic claustrum in monotremes, and commented that this structure is difficult to distinguish from the insular cortex using cytoarchitectonic or myeloarchitectonic methods, as has been reported in microchiropteran bats (Humphrey, 1936; see references in Puelles, 2014), tree shrews (Tigges & Totada, 1969) and other therian mammals. A full unequivocal elucidation of the monotreme claustral complex is yet to be achieved, and would likely involve

complementing comparative analysis of cytoarchitecture (see Figures 1 and 2) with enriched or specific gene expressions (see Section 1.5). In Figure 2 we present high-resolution Nissl images of the platypus brain in coronal and sagittal views indicating tentatively the putative claustrum (Figure 2). Figure 2 also clearly indicates the EPd and piriform cortex (Pir). In the sagittal plane, the claustrum seems to comprise cells scattered within the white matter above the external capsule and possibly intermingled with an extreme capsule. According to Ashwell

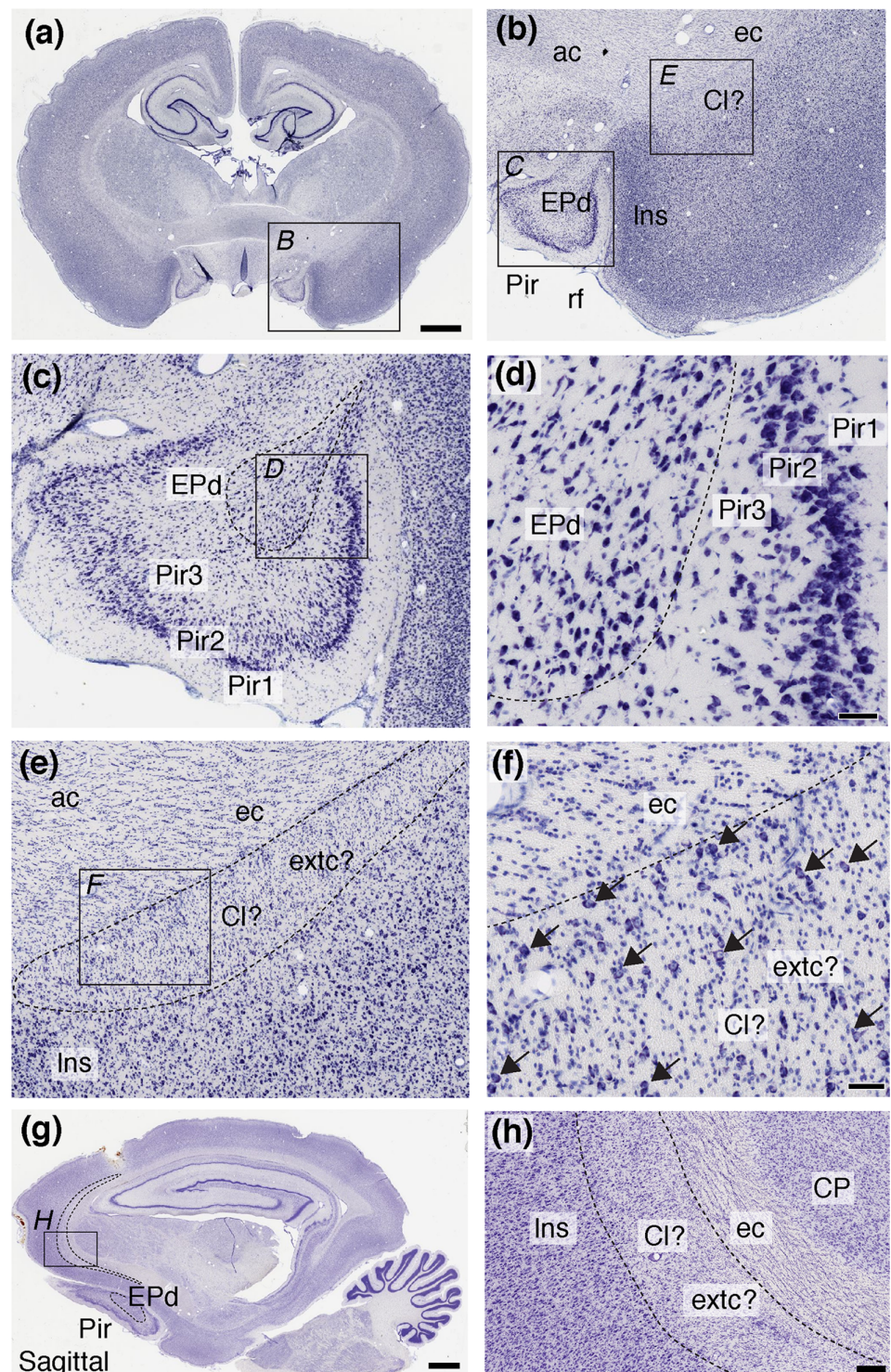


FIGURE 2 Nissl-stained coronal and sagittal sections of the adult platypus (*Ornithorhynchus anatinus*, Monotremata) brain. (a–f) coronal sections showing the location of the piriform cortex (Pir), rhinal fissure (rf), insular cortex (Ins), anterior commissure (ac), and external capsule (ec). (c, d, f) are higher magnification images of the square area highlighted in (a), (c), and (e), respectively. The dorsal endopiriform nucleus (EPd) lies deep to piriform cortex Layers 1–3 (Pir1–3 in c and d), and the putative claustrum (Cl) includes neuron-sized cells (arrows in f), possibly intermingled with white matter of an extreme capsule (extc). (g, h) sagittal sections reveal the EPd deep to Pir, as well as putative claustrum neurons within white matter fibers and superficial to the external capsule (ec) and caudate/putamen (CP). St, striatum. Scale bars: 2000 μ m in (a) and (g); 50 μ m in (d), (f); 200 μ m in (h). Images taken from brainmaps.org Brain Map (2019) [Color figure can be viewed at wileyonlinelibrary.com]

et al. (2004), these putative claustral cells extend all the way up into the deepest neocortical sheets, including the usual location of Layer 6b/subplate.

The putative claustrum of echidna is embedded between the external and extreme capsules, deep to the insular cortex, and at the level of the anterior commissure, based on non-cytoarchitectonic features it shares with other mammals (Ashwell et al., 2004). Whether this evidence corresponds to a true claustrum is unclear and would require additional confirmation, such as the expected expression of claustrum-specific marker genes discussed below. Interestingly, a recent study from Norimoto et al. (2020) used single-cell transcriptomics to identify a putative claustrum in both the Australian bearded dragon and a species of turtle. While this study has yet to be replicated, it suggests that the claustrum may even have been present in the common ancestor of all mammals and reptiles.

The ancestral embryological origins, and potential homology of the mammalian claustrum with that of other vertebrates is currently not fully elucidated. Puelles (2014) proposed that a claustrum-insular homolog was already present in ancestors of all modern amniotes, such that in chicken it corresponds to the lateropallial mesopallium of the dorsal ventricular ridge (DVR). Puelles assigned the embryological origin of both the claustrum and insula to the lateral pallium (first conceived for the mouse in Puelles, 2014). This implies that this complex should not be considered neocortical, as it does not originate from DPall. A similar opinion was held by Holmgren (1925), who considered the claustrum as a pallial structure not derived from the neocortex.

Butler and Molnár postulated the *collopallial field hypothesis*, which examined the differences in the claustrum-amygdala formation between birds, reptiles, and mammals (Molnár & Butler, 2002). The hypothesis held that in mammals, the thalamo-recipient collopallium differentiates into deep (claustrum-amygdala) and superficial (neocortical) components, whereas in sauropsids, and perhaps in platypus, this split may not occur. The original collopallial field hypothesis was based on a handful of gene expression patterns (Molnár & Butler, 2002), and it was formulated before the modern tetrapartite pallium model that is now commonly used. The modern concept of VPall (olfactory), LPall (claustrum-insular), and DPall (neocortical) pallial sectors (Puelles, 2014, 2017) seems to have rendered obsolete these notions articulated in Puelles et al. (2000) and Molnár and Butler (2002). Altogether, the general presence of the claustrum in all extant mammalian taxa (excluding the uncertainty in monotremes), together with shared histochemical, molecular, cytoarchitectural, and hodological features, suggests that the claustrum was already present in the now extinct pan-mammalian common ancestor (Montiel et al., 2011; Puelles, 2017; Suárez et al., 2018).

1.4 | Evolutionary origin of the subplate

The six-layered cerebral neocortex and the claustrum-insula complex have been widely reported in therians, marsupials, and placentals, but the existence of a cortical subplate in marsupials has been the subject of controversy (Montiel et al., 2011; Pearce, James, & Mark, 2000).

Subplate markers identified in the mouse are expressed in the opossum *Monodelphis domestica*, but are much more dispersed than in eutherians (Montiel et al., 2011; Puzzolo & Mallamaci, 2010; Wang et al., 2011). Moreover, thalamic cortical projections, thought to also represent subplate afferents, have a different spatiotemporal pattern in their distribution and arrival to the cortex in marsupials as compared to rodents or carnivores (Molnár, Knott, Blakemore, & Saunders, 1998). This pattern is compatible with the distribution of the early generated and subplate enriched gene expression in *M. domestica* (Molnár et al., 1998; Montiel et al., 2011). Subplate enriched genes are also expressed in the developing reptilian and avian cortices (Montiel et al., 2011).

There are three current hypotheses about the phylogenetic origin of the subplate (Hoerder-Suabedissen & Molnár, 2015; Montiel et al., 2011): (a) the *ancestral origin hypothesis* states that subplate cells were already present in the common ancestor of mammals and sauropsids (Aboitiz, Montiel, & García, 2005; Marin-Padilla, 1978), (b) the *derived hypothesis* states that the subplate is a novel character exclusive to mammals with complex and larger brains, whose emergence supported the development of expanded cortico-cortical connectivity (Kostović & Rakic, 1990; Molnár et al., 2006; Supér & Uylings, 2001), and (c) the *dual origin hypothesis* suggests that there are both ancestral elements (so-called “presubplate” cells) (Kostović & Rakic, 1990; Meyer, 2007; Meyer, Castro, Soria, & Fairén, 2000) and newly emerged cell populations in the mammalian subplate, with additional populations of subplate cells being added to the embryonic subplate as the neocortex became more complex (Aboitiz, 1999; Kennedy & Dehay, 2012; Meyer, 2007; Montiel et al., 2011; Suárez-Solá et al., 2009; Wang et al., 2011). These hypotheses could be evaluated by further researching birth dates, tracking cell origin and lineage, and examining somatodendritic morphology, physiological properties, and single cell transcriptomics, as well as markers for differential properties among ancestral and newly developed subplate cells in various species (Hoerder-Suabedissen & Molnár, 2015). Understanding how the subplate has been altered in distinct mammalian lineages would help to understand which of these hypotheses is correct. The available evidence, however, suggests that the human subplate contains an increased number of both ancestral and derived subplate neurons (Judaš et al., 2013; Meyer, 2007; Suárez-Solá et al., 2009). Moreover, any such analysis is further complicated by the fact that the subplate compartment in development contains many radially and tangentially migrating cells that are just temporarily passing through.

1.5 | Gene expression studies in the claustrum and cerebral cortex

The upper and deeper layers of the cerebral cortex (layers 2/3 vs. 5/6 in neocortex) have distinct neurogenic programs regulated by differential transcriptional networks in mammals (Britanova et al., 2008; Molyneaux et al., 2009; Molyneaux, Arlotta, Menezes, & Macklis, 2007; Paolino, Fenlon, Suárez, & Richards, 2018). Some of these programs specify the deeper layer neurons with extracortical

projections, the upper layer neurons with mostly intracortical connectivity, while the granular layer 4 in between them receives most of the thalamic input. Importantly, the differential expression of supragranular (upper layers) and infragranular (deeper layers) cortical genes *Satb2* and *Fezf2* in the presumptive claustrum and amygdaloid nuclei of mice at E15.5, suggest that they may have different developmental origins (Figure 3a; Paolino et al., 2018). Interestingly, claustrum

and subplate/Layer 6b (L6b) neurons also express canonical markers of cortico-cortical neuronal types, such as the callosal determinant *Satb2* seen on coronal sections of developing and adult mice (arrowheads and arrows, respectively, Figure 3b–d). Similarly, additional transcription factors associated with supragranular cortico-cortical neuronal fate (Paolino et al., 2018), such as *Cux2*, *Neurod6*, *Neurod2*, *Pou3f3*, *Pou3f2*, and *Lmo4*, also show expression in the presumptive

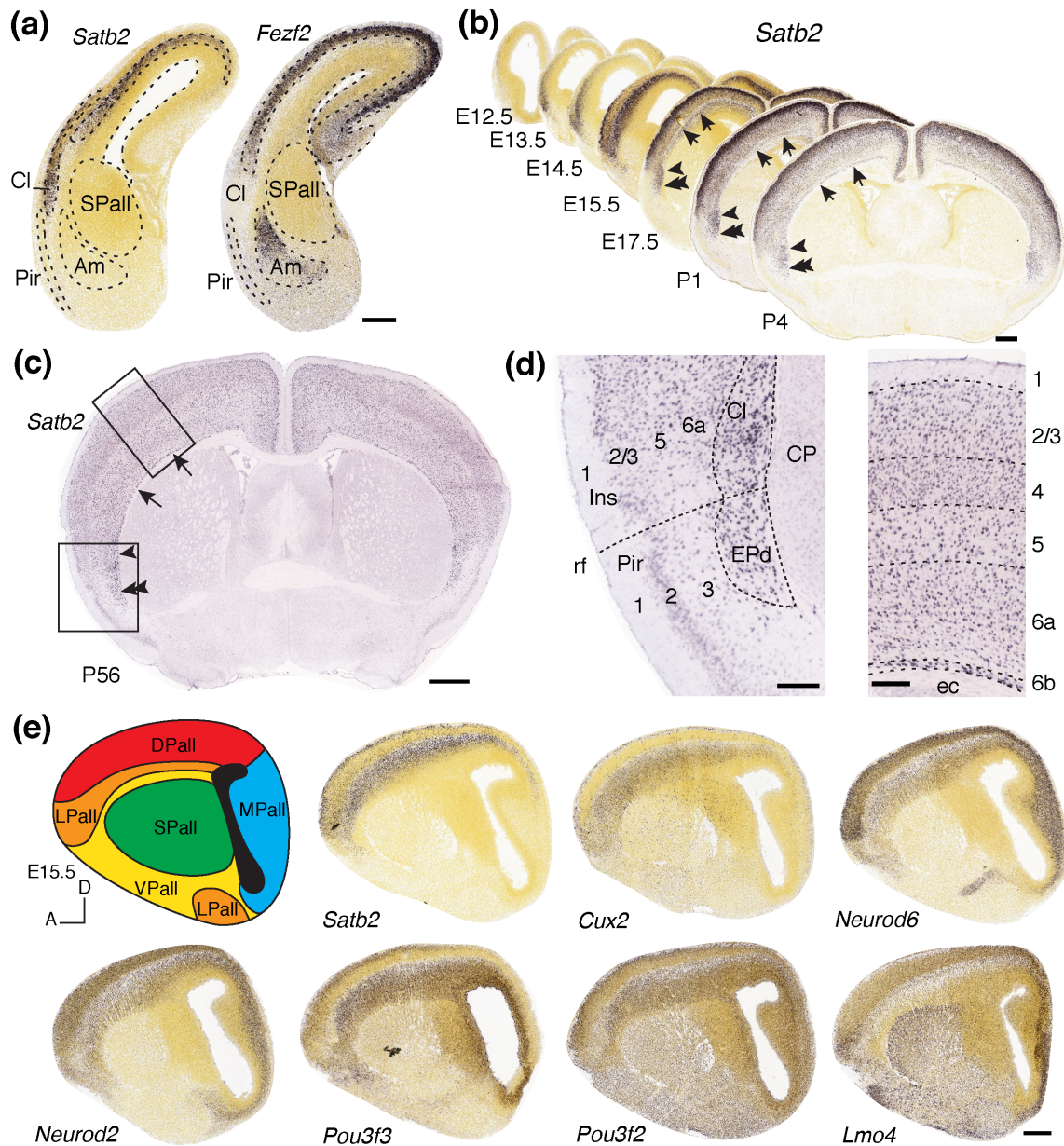


FIGURE 3 (a) Coronal sections through the developing mouse cortex at embryonic day (E) 15.5 showing expression of *Satb2* and *Fezf2* with in situ hybridization. The presumptive claustrum (Cl) and amygdala (Am) show converse patterns of expression, similar to their complementary expression in the cortex. (b) *Satb2* is expressed by E13.5 in the dorsal and lateral pallia, and can be detected in the presumptive claustrum (arrowhead), endopiriform nucleus (EPd, double arrowheads), and subplate (arrows) by E17.5. Note the presence of *Satb2*-positive neurons in subplate/Layer 6b at postnatal day (P) 4, which persists to P56 (c). Boxed areas depicted in (c) are shown in higher magnification in (d). (e) Schematic sagittal section with pallial sectors indicated with different colors and matched sections of E15.5 mouse brains, with in situ hybridization against canonical upper-layer transcription factors being expressed along the lateral pallium (LPall). DPall, dorsal pallium; MPall, medial pallium; Pir, piriform cortex; SPall, subpallium; VPall, ventral pallium. Scale bars: 300 μ m in (a) and (e), 400 μ m in (b), 800 μ m in (c), and 200 μ m in (d). Images taken from the Allen Brain Atlas Data Portal [Color figure can be viewed at wileyonlinelibrary.com]

claustrum and subplate (lateral pallium anteroventral to the DPall) as early as E15.5 (Figure 3e). Whether and how supragranular- and infragranular-specific transcriptional gene networks interact in the ontogeny and phylogeny of the claustrum and associated neocortical structures remains to be elucidated. A note of caution is needed with regard to the notion of supragranular- and infragranular-specific genes, since such classification is meant strictly for the adult pattern—at embryonic stages the same markers often distribute in a different or even inverse pattern (Puelles et al., 2016)—this is a cause of confusion in comparative studies, since non-mammals may present embryonic-like, rather than adult-like expression patterns of some markers.

One of the most salient subplate-specific genes identified is the orphan nuclear receptor *Nr4a2/Nurr1* (Arimatsu, Ishida, Kaneko, Ichinose, & Omori, 2003; Hoerder-Suabedissen et al., 2009), which is also expressed in the claustrum and EPd of various mammalian species as well as in sauropsids (Montiel et al., 2011; Puelles, 2014; Puelles et al., 2016; Wang et al., 2011). Both Wang et al. (2011) and Puelles et al. (2016) emphasized that salient markers of the adult claustrum can be observed along the lateral pallium of E15.5 mice, forming a continuous band of expression along the cortical subplate and in the claustrum. Owing to the development of new dissection methodologies combined with gene expression profiling (Wang, Oeschger, Lee, & Molnár, 2009), it became possible to identify selective gene expression patterns in cortical layers and regions including subplate and Layer 6b (Belgard et al., 2011; Hoerder-Suabedissen et al., 2009; Oeschger et al., 2011). The claustrum expresses many of the same markers that are enriched in the subplate (Montiel et al., 2011; Wang et al., 2011). However, not all genes that

are subplate-expressed in the mouse are also present in the mouse claustrum. There are important species differences in gene expression, even between closely related species such as rats and mice, both in terms of whether a particular gene is a “subplate marker” as well as when comparing subplate and claustrum gene expression (Montiel et al., 2011; Wang et al., 2011). In addition to the cortical subplate, *Nr4a2/Nurr1* is selectively expressed in the claustrum and in layers 5 and 6 of the parietal neocortex in both mice and rats (Wang et al., 2011), as well as in macaque monkeys (Watakabe et al., 2014).

Another subplate specific gene, Complexin 3 (*Cplx3*) is exclusively expressed in the subplate of both mouse and rat (Hoerder-Suabedissen et al., 2009; Wang et al., 2011). Mouse *connective tissue growth factor* (*Ctgf*) only labels the subplate component in neocortex and the deep part of the claustrum and endopiriform nucleus complex (Hoerder-Suabedissen et al., 2009). DOPA decarboxylase (*Ddc*) is expressed in the mouse subplate, while in rat, it is found in the EPd (Wang et al., 2011). *Transmembrane Protein 163* (*Tmem163*) is another subplate-specific gene in mouse and rat dorsal cortex, but additional expression in the basomedial amygdala, bed nucleus of the stria terminalis, and medial amygdala is only observed in the mouse (Wang et al., 2011). Moreover, *Monoxygenase DBH Like 1* (*Moxd1*) and thyrotropin-releasing hormone (*Trh*) are only expressed in the subplate of mouse but not in rat (Wang et al., 2011), and differ in their non-subplate expression between these two species. *Moxd1* is expressed in endopiriform nucleus, central amygdala, while *Trh* is expressed in the bed nucleus of stria terminalis in rat, but not mouse brains (Wang et al., 2011). Mathur et al. (2009) described G-protein gamma2 subunit (*Gng2*) as a novel protein that is selectively expressed in claustrum only at striatal, but not in frontal levels.

TABLE 1 Genes expressed in the claustral complex (claustrum and endopiriform nucleus) of adult mice assessed for co-expression in neocortical layers

(1) Claustrum	(2) Claustrum + EnP	(3) Claustrum + EnP + L6b	(4) Claustrum + EnP + lateral deep layers	(5) Claustrum + EnP + lateral deep layers w/o L6b	(6) Claustrum + EnP + deep layers	(7) Claustrum + EnP + Ctx w/o L6b
<i>Adamtsl2</i>	<i>Bok</i>	<i>Chst11</i>	<i>Car12</i>	<i>B3gat2</i>	<i>Fxyd6</i>	<i>Zfp804a</i>
<i>Bace1</i>	<i>LOC433093</i>	<i>Mmp16</i>	<i>Gfra1</i>	<i>BC100451</i>	<i>Galnt14</i>	
<i>Msmo1</i>	<i>Dock6</i>	<i>Pcsk5</i>	<i>Gm10413</i>	<i>Cntnap3</i>	<i>Hs6st2</i>	
<i>Nmb</i>		<i>Pdia5</i>	<i>Gnb4</i>		<i>Lrfr2</i>	
		<i>Prss12</i>	<i>Itga7</i>		<i>Lypd6b</i>	
		<i>Rai14</i>	<i>Lxn</i>		<i>Nsdhl</i>	
		<i>Rspo2</i>	<i>Plcl1</i>		<i>Slit1</i>	
		<i>Sema5b</i>	<i>Rftn1</i>			
		<i>Sulf1</i>	<i>Rtn4rl2</i>			
		<i>Thsd7b</i>	<i>Col11a1</i>			
		<i>Tmem163</i>				
		<i>Trp53i11</i>				
		<i>Usp46</i>				

Note: 44 genes with enriched claustrum expression were identified using the Allen Brain Atlas fine structure search tool. Of these, three were discarded as they showed weak and/or uniform expression. Each of the remaining 41 genes was placed into the best fitting single category (1–7). Genes in bold were independently identified as “subplate/Layer 6b markers” in one of our previous microarray or RNAseq experiments (Hoerder-Suabedissen et al., 2013). Group assignment based on RNA in situ data from the Allen Brain Atlas: Data Portal.

TABLE 2 We examined genes that were preferentially expressed in Layer 6b based on the Allen Brain “fine structure search tool” and examined in situ hybridization data from the Allen Brain Atlas: Data Portal for co-expression in other brain structures

(1) L6b (with or without expression in some other cortical layers or striatum)	(2) L6b and dEnP	(3) L6b and claustrum (and dEnP)	(4) L6b and dEnP and insula (but not claustrum)	(5) L6b, dEnP and striatum (with or without insula)	(6) L6b claustrum, dEnP and striatum	(7) Deep layers of cortex, claustrum, dEnP and striatum	(8) Deep layers of cortex, claustrum and dEnP	(9) L6b + L2/3, claustrum, dEnP, striatum
<i>Pde6g</i>	<i>Gng12</i>	<i>Hspa1l</i>	<i>Col23a1</i>	<i>Hpcal1</i>	<i>Drd1</i>	<i>Insig1</i>	<i>Lypd6b</i>	<i>Rgs20</i>
<i>Rgs1</i>	<i>Inpp4b</i>	<i>Inpp5a</i>		<i>Pde1b</i>			<i>Chst2</i>	<i>Rgs8</i>
<i>Thrsp</i>	<i>Moxd1</i>	<i>Pdia5</i>		<i>Shb</i>				
<i>Trh</i>	<i>Nxph4</i>	<i>Rai14</i>		<i>Gira2</i>				
<i>Clic5</i>	<i>Tle1</i>	<i>Sema5b</i>						
<i>Doc2b</i>	<i>Cdh18</i>	<i>Thbs2</i>						
	<i>Cplx3</i>	<i>Cdh11</i>						
	<i>Ctgf</i>							
	<i>Fam65b</i>							
	<i>Galnt10</i>							

Note: Of the 48 selected subplate/Layer 6b-enriched genes, 14 had to be discarded, as they were not L6b specific on examining the ISH signal. The remaining 34 genes were placed into the best fitting single category (1–9) based on the pattern of co-expression in additional structures. Layer 6b enriched genes were also expressed in claustrum (see Groups 3, 6, 7, 8, and 9), but there were several other expression patterns of Layer 6b genes without claustral expression as well (see Groups 1, 2, 4, and 5). Genes in bold are also present in Table 1 of “claustrum enriched” genes.

Recent single-cell transcriptomic analyses of the claustrum have aided in further defining its cell types and developmental relationships to other nearby structures (Norimoto et al., 2020; Tasic et al., 2016, 2018; Tosches et al., 2018; Wang, Koch, Luo, Peng, & Zeng, 2019). Wang et al. (2019) continued the classification of claustrum neurons first described by Tasic et al. (2018) and found that Guanine nucleotide-binding protein subunit beta-4 (*Gnb4*)-positive claustrum cells can be clustered into a subclass with deep layer cortical neurons defined by the expression of *carbonic anhydrase III* (*Car3*). These results suggest a common developmental origin of claustrum neurons and deep layer cortical neurons from agranular insular, somatosensory, and temporal cortex. In a transcriptomic study of the highly connected DVR of reptiles, Norimoto et al. (2020) discovered that it expresses markers characteristic of the mouse claustrum and was subsequently proposed as the reptilian claustral homolog. Interestingly, the next most closely related structure to the reptilian DVR is the dorsal lateral amygdala, a structure whose homolog in mice, the basolateral amygdala, was noted to be of similar developmental origin to the mouse claustrum by Puelles et al. (2000) and Molnár and Butler (2002). Collectively, these data point to the mammalian claustrum having a conserved genetic and developmental relationship to structures derived from the lateral pallium.

It is difficult to say with certainty if there is an appreciable genetic relationship between the mammalian claustrum and subplate and if the cell types found therein are developmentally analogous to one another. This uncertainty is due to transcriptomic analyses focusing on the adult claustrum, the developing subplate, and adult Layer 6b—different developmental time-points between different structures (Hoerder-Suabedissen et al., 2009; Oeschger et al., 2011; Tasic et al., 2018; Wang et al., 2019). Direct comparisons of time-matched claustral and subplate development will be necessary to determine the exact genetic and cell type relationship of these two structures.

These early studies suggested that the subplate/Layer 6b-enriched genes are also expressed in claustral complex and *vice versa*, many of the claustral complex enriched genes were also expressed in the subplate/Layer 6b. Since these studies, numerous additional genes have been identified with similar gene expression patterns during development and in the adult. This prompted our further analysis of these similarities and differences on the genes presented in Tables 1 and 2 and Figures 4 and 5.

1.6 | Claustrum- and Layer 6b-enriched genes can be classified into distinct sub-groups according to their patterns of expression in other structures

To systematically explore the similarities and differences in the patterns of gene expression between subplate/Layer 6b and claustrum in the adult mouse, we identified claustrum-enriched genes using the “fine structure search” tool on the Allen Brain Atlas: Data Portal (ABADP; <https://www.nitrc.org/projects/abaportal/>), a manually curated list of up to 50 genes with enhanced gene expression in different small brain structures. We also identified subplate/Layer 6b-enriched genes by using the “fine structure search” tool on the ABADP. The former group

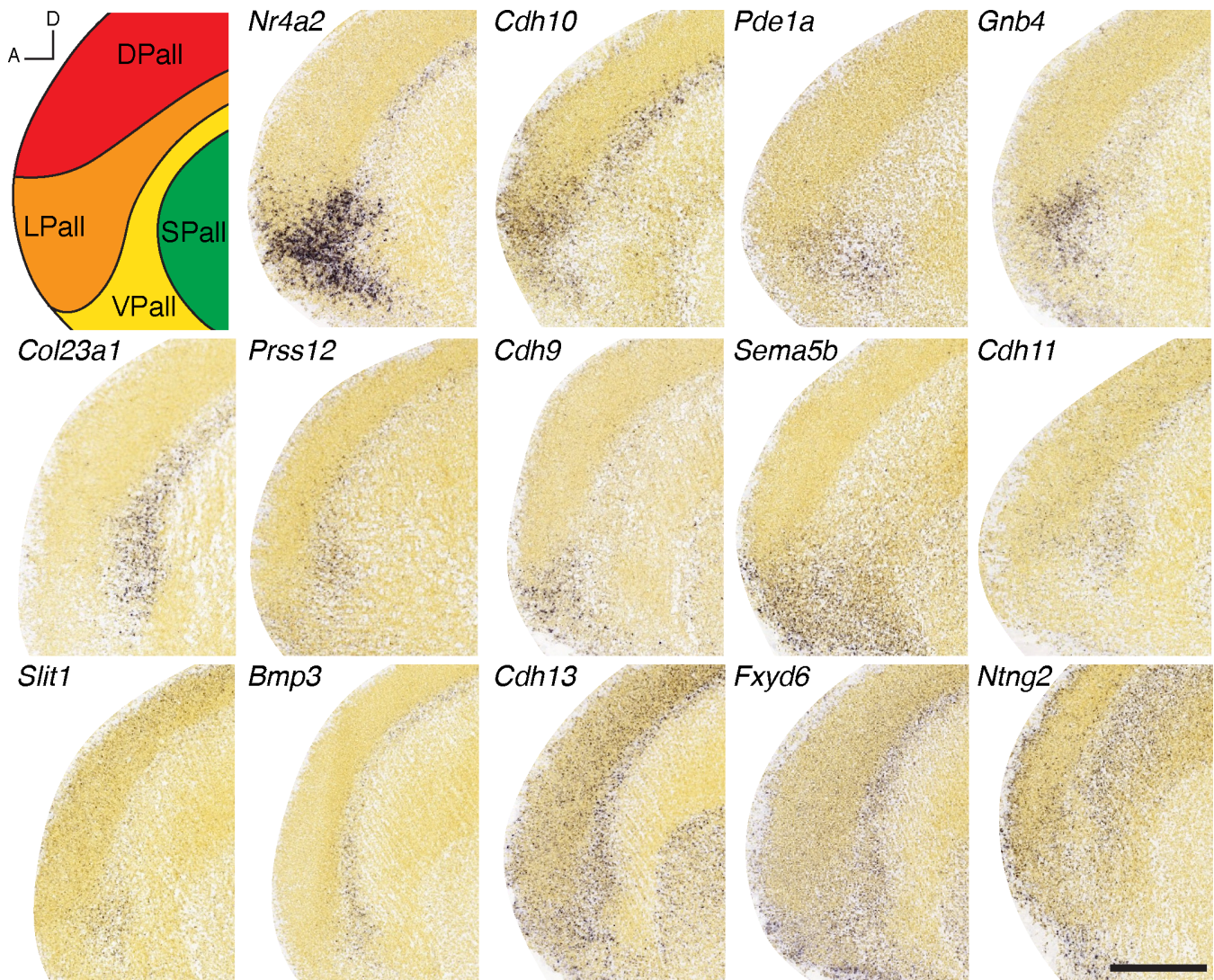


FIGURE 4 Schematic sagittal sections of E15.5 mice processed for in situ hybridization for selected markers of the adult claustrum. *Nr4a2*, also known as *Nurr1*, is one of the strongest and most exclusive markers of the lateral pallium (LPall) and putative claustrum. Note a continuum of labeled neurons into the cortical subplate is present in most cases, some stronger (e.g., *Nr4a2* and *Cdh10*), and others are much weaker if at all (*Gnb4*). Scale bar: 300 μ m. Images taken from the Allen Brain Atlas Data Portal [Color figure can be viewed at wileyonlinelibrary.com]

of genes was additionally annotated for whether the genes were identified as subplate-enriched in one of our previous microarray surveys (see raw data in Belgard et al., 2011; Hoerder-Suabedissen et al., 2009, 2013; Oeschger et al., 2011), whereas the latter table was additionally annotated to highlight the subset of genes identified by the “fine structure search” tool as both claustrum and Layer 6b enriched. Then, using the in situ hybridization data available on the Allen Institute we identified the structures these genes were expressed in, focusing specifically on the cortical layers, EPd, claustrum, and striatum. We then grouped these genes according to their common co-expression patterns. We found that the claustrum-enriched genes could be classified into one of at least seven different patterns of expression (Table 1). Similarly, subplate/Layer 6b-enriched genes could be classified into at least nine common co-labeling patterns, some of which were distinct from the claustral co-expression patterns (Table 2).

The fine-structure search tool returned 44 genes with expression enriched in the claustrum. Using RNA in situ hybridization data from the ABADP we examined their expression in other telencephalic areas such as: subplate/Layer 6b; in other cortical layers of cortical areas including putative visual, auditory, and somatosensory cortices; claustrum; and (caudal) EPd (Table 1). Three genes were not analyzed further, as we could not confirm the claustrum-enriched expression. The other 41 genes, we manually clustered into seven groups based on the combinatorial gene expressions in other areas: (a) claustrum but minimal neocortical expression; (b) claustrum and endopiriform nucleus but minimal neocortical expression (c) claustral complex and neocortical Layer 6b; (d) claustral complex and lateral deep layer cortex (e) claustral complex and lateral deep layer cortex without Layer 6b (L6b), (f) claustral complex and deep cortical layers; and (g) claustral complex and all neocortical layers without L6b.

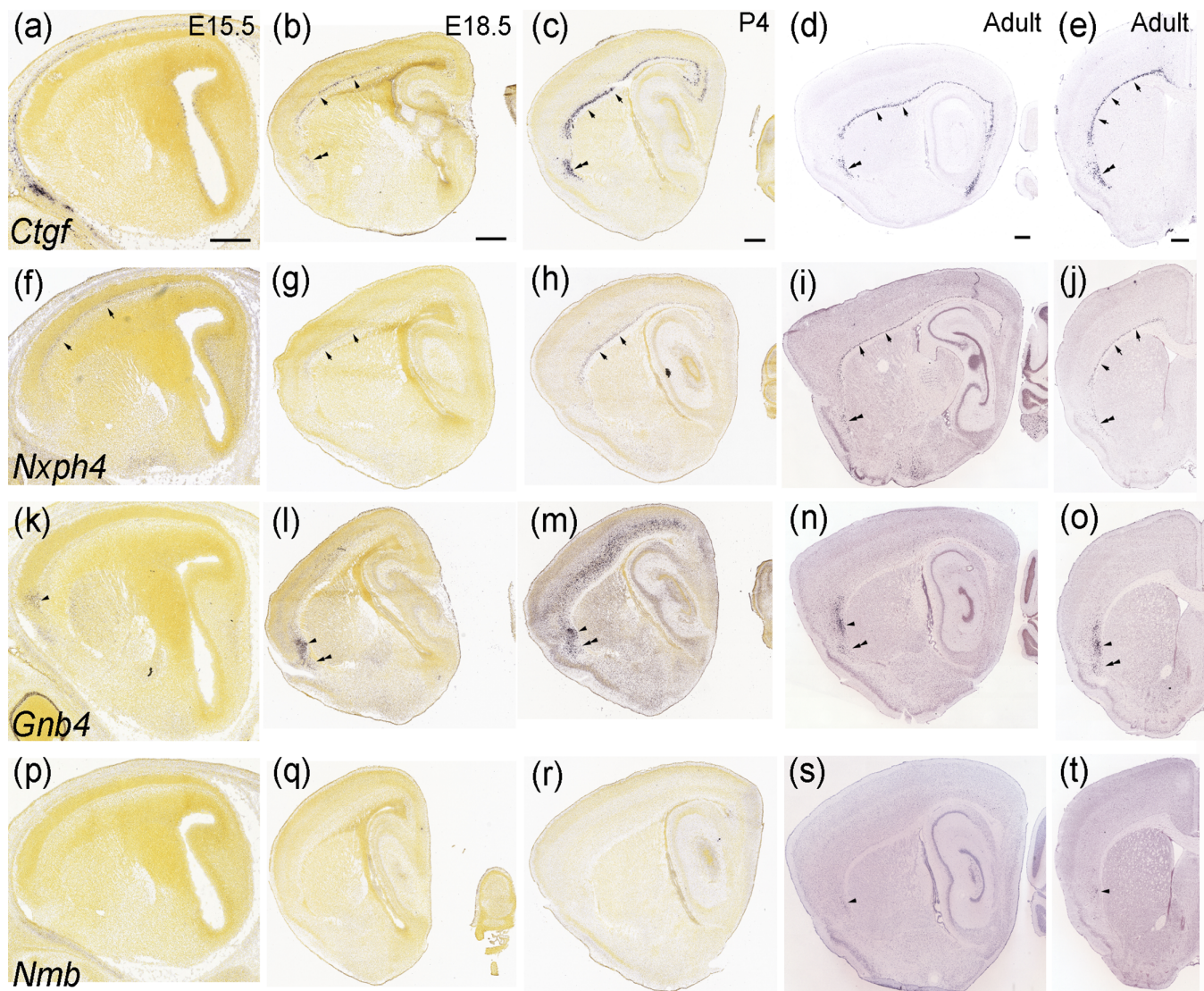


FIGURE 5 Examples for genes that were enriched in in Layer 6b and endopiriform nucleus and not claustrum (*Ctgf*, *Nxph4*, upper two rows (a)–(e) and (f)–(j)) and for genes with expression in claustrum and dorsal endopiriform nucleus but not within Layer 6b expression (*Gnb4*, *Nmb*, lower two rows (k)–(o) and (p)–(t)). This figure shows sagittal sections of embryonic (E) 15.5, 18.5, postnatal day (P) 4 and 56 (Adult) and an additional coronal section (right column) of the adult mouse brains after in situ hybridization against *Ctgf*, *Nxph4* (upper two rows) and (*Gnb4*, *Nmb*, lower two rows). Expression of *Ctgf* and *Nxph4* is evident in the subplate and subplate-derivative Layer 6b (arrows) together with the expression in the endopiriform nucleus (double arrowheads), but not in claustrum, where there is a clear gap in expression. Expression of *Gnb4* and *Nmb* is evident in the claustrum (single arrows) and dorsal endopiriform nucleus (double arrowheads). Additional genes that share similar expression patterns to *Ctgf* and *Nxph4* are listed in Group 2 of Table 2; patterns similar to *Gnb4*, *Nmb* are listed in Groups 1 and 4 of Table 1. Scale bar: 500 μm. Images were taken from the Allen Brain Atlas Data Portal [Color figure can be viewed at wileyonlinelibrary.com]

In situ hybridization signal is not necessarily absent/present in particular brain regions, but may vary in signal strength across different cell groups. Thus, genes were assigned to the single best-fitting category, but weaker or sparser expression was often present in other regions.

Interestingly, the largest group of gene co-expression was in Group 3, the neocortical Layer 6b (Table 1). For instance, there are *Sulf1*-positive cells in both the claustrum and subplate/Layer 6b forming a continuously labeled band. The vast majority of genes in Group 3 had previously been identified as “subplate/Layer 6b markers” in one of our previous microarray or RNAseq studies (Hoerder-Suabedissen et al., 2013). Overall, of the 41 genes assessed

for co-expression, 30 had at least some Layer 6b co-expression, although this did not always extend along the entire lateral-medial extent of Layer 6b. Four genes, on the other hand, were much more strongly expressed in claustrum than anywhere else (*Nmb* as an example). Insofar as some of the claustrum-enriched genes show a continuation with Layer 6b, even into very dorsal regions, it may be suggested that possibly some, but not all, Layer 6b cells might share a common origin with claustrum cells.

Conversely, we assessed the 46 genes identified as “Layer 6b enriched” by the Allen Brain Atlas fine structure search tool for co-expression in the claustral complex. Of these, 12 genes were

discarded from further analysis as the Layer 6b-enriched expression could not be verified due to weak or uniform signal strength. The remaining 34 genes were classified into nine distinct co-expression patterns, based on expression in claustrum, dorsal endopiriform cortex, other neocortical layers, and striatum. As before, genes were assigned to the single best-fitting category, but weaker or sparser expression was often present in other regions. The majority of Layer 6b enriched genes were co-expressed in the claustral complex, but a sizeable fraction of those were not expressed in the claustrum, but just in the endopiriform nucleus. Six genes showed exclusive neocortical expression (Group 1 in Table 2), 10 genes were expressed in Layer 6b and endopiriform nucleus, but not claustrum (Group 2 in Table 2, see examples of *Ctgf* and *Nxph4* in Figure 5.). Seven genes showed similar expression strength and density across all of L6b, claustrum, and EPd (Group 3, Table 2), essentially giving a continuous band of expression from dorsal Layer 6b to endopiriform nucleus. Some of the genes are not only expressed in 6b, but also in 6a and even 5 (Arimatsu et al., 2003; Hoerder-Suabedissen et al., 2009, 2013; Puelles, 2014) and they showed areal differences in expression. Insofar as some of the Layer 6b enriched genes show a continuation with the claustrum, whereas others do not, as described by Wang et al. (2011), it may be suggested that possibly some, but not all, claustrum cells might share a common origin with subplate cells.

Overall, the most prominent co-expression pattern in the adult mouse brain was Layer 6b with endopiriform nucleus (without claustrum), closely followed by continuous L6b to endopiriform nucleus (including claustrum) expression. Only a small group of genes were expressed in deep layers of cortex and the claustral complex. Overall, four genes from the “Layer 6b enriched” Table 2 are also present in Table 1 of “claustrum enriched” genes. *Nurr1/Nr4a2* was not included among the genes with “Layer 6b enriched” expression or in the “claustrum enriched expression” in the Allen Brain Atlas fine structure search tool lists. Nonetheless, the partial overlap of gene expression between Layer 6b and claustrum can be seen as partial validation of the gene-based hypothesis of Puelles (2014), which only referred to a significant, but not total, claustral contribution to the subplate, as well as to the deep layers of neighboring neocortex, as previously described by Arimatsu et al. (2003). We focused on adult gene expression, because this data set is the most comprehensive in the Allen Brain Atlas in situ hybridization image database. As Figure 5 highlights with the example of *Gnb4* in young postnatal brains, not all genes that show restricted expression in adulthood, have equally restricted expression in development. These results also highlight that although gene expression patterns can suggest the possibility of tangential migration, direct lineage tracing methodologies are required to determine lineage (see Section 1.9).

1.7 | Cell types of subplate and claustrum

The claustrum is composed of two primary cell types, previously described as Type I and Type II neurons (Braak & Braak, 1982; Crick & Koch, 2005). The claustrum contains spiny, glutamatergic excitatory neurons as well as GABAergic inhibitory neurons in roughly the same

proportions as the cerebral cortex (Mathur, 2014). The excitatory neurons of the claustrum are enriched in several genetic markers, including *Gng2*, *Gnb4*, and *Slc17a6* (Hur & Zaborsky, 2005; Mathur, 2014). These cells are responsible for the intricate and widespread connectivity of the claustrum with the cortex (Wang et al., 2017, 2019). Additionally, the claustrum contains three distinct interneuron types comprising parvalbumin-, calbindin-, or calretinin-positive interneurons (Real, Dávila, & Guirado, 2003; Reynhout & Baizer, 1999). Parvalbumin-containing interneurons are large and multipolar with smooth dendrites, whereas the calretinin-containing neurons are smaller and bipolar, with longer somata. There are multipolar and bipolar calbindin-containing neurons as well as a dense population with smaller cell bodies and convoluted dendrites. In the mouse, claustral interneurons have been shown to be *Gnb4*- and *Slc17a6*-negative, suggesting that they likely do not participate in long-range cortical signaling but instead are active in local circuits (Kim, Matney, Roth, & Brown, 2016; Real, Dávila, & Guirado, 2006; Wang et al., 2017; White & Mathur, 2018).

Neuron types in the subplate include glutamatergic projection neurons as well as local GABAergic and peptidergic interneurons (Judaš, 2011; Judaš, Sedmak, Pletikos, & Jovanov-Milošević, 2010; Judaš, Sestan, & Kostović, 1999). Subplate/Layer 6b has relatively low proportions of interneurons compared to other cortical layers in the young postnatal mouse (Boon et al., 2019). Six types of subplate neurons have been distinguished in the rodent (Hanganu, Okabe, Lessmann, & Luhmann, 2009) and human cerebral cortex (Mrzljak, Uylings, Kostović, & Van Eden, 1988); these being bitufted and monoftufted horizontal, multipolar, inverted pyramidal, polymorphous, and fusiform neuronal types. Marx et al. (2017) carried out biocytin staining of subplate neurons at 0–4 postnatal days, and Layer 6b neurons at 11–35 postnatal days and found that the subplate and Layer 6b consisted of comparable neuronal populations, each containing six spine-bearing cell types. Because of the high morphological similarity between subplate and Layer 6b neurons, it has been suggested that Layer 6b consists of persistent pyramidal and non-pyramidal neurons from the subplate (Marx et al., 2017).

The relative degree of homology between cell types of these two regions has yet to be rigorously addressed. Future studies seeking to determine the morphological and genetic similarities between claustrum and subplate cell types—as well as the circuits they participate in—will need to do so in time-matched experiments in order to capture transient gene expression as these structures develop from their nascent to adult forms. Clustering cells from isolated claustrum and Layer 6b based on their single-cell transcriptomes could juxtapose the subplate and claustral cell types to further characterize their similarities and differences.

1.8 | Putative common developmental origin of claustrum and insular cortex

There was a long-standing controversy as to whether the claustrum was formed by a duplication of the suprajacent insular cortex

(Holl, 1899) or the piriform cortex (Smith, 1910), and attempts were made to link its development to cytoarchitectonic differentiation (Mathur, 2014). Sonntag and Woollard (1925) suggested that the extreme capsule separates the deepest layer of the insular cortex from the claustrum (Mathur, 2014). Brodmann (1909) considered the claustrum as a sublayer of the insular cortex split off from this cortex to generate a seventh layer. Rose (1928) deduced that in mammals with or without the extreme capsule the claustrum either represented the innermost extension of insular cortex Layer 6, or formed an independent cortical layer, Layer 8 (Mathur, 2014). However, Ramon and Cajal (1902) pointed out that claustral neurons never had apical dendrites pointing into insular layer 1; considering this an argument against the idea that claustrum was an insular layer, suggesting a hypopallial nucleus origin (Puelles, 2014). Watakabe et al. (2014) also noted that the dendrites of insular and claustral neurons do not cross the border of the two brain regions and dendrites of claustral neurons do not invade the overlying insular territory. There is also scant evidence of columnar connectivity between the claustrum and insular cortex. Modern cortical experts accordingly identified the claustrum as a separate pallial nucleus, which develops precociously within the same pallial progenitor domain as the later-born insular population (Puelles, 2014).

The common origin of claustrum and insular cortex was recently suggested on the basis of mapping of Gng2 and NtrG2 markers in the adult human brain (Pirone et al., 2012). Puelles (2014) argued that the development of the claustrum is associated with the subsequently developing insular primordium of the lateral pallium via a shared field of radial glia. The claustral population is born first and takes its position at the thin and superficial LPall mantle layer of E12.5 mouse embryos (in the mouse [Hinds & Angevine, 1965; Smart & Smart, 1977, 1982]; in the rat [Bisconte & Marty, 1975; Valverde & Santacana, 1994; Valverde, Facal-Valverde, Santacana, & Heredia, 1989; Valverde, Lopez-Mascaraque, Santacana, & De Carlos, 1995]; reviewed in Puelles [2014]). Insular cells subsequently migrate radially (inside-out) through the claustrum, similarly as standard supragranular cortical cells migrate through infragranular cortical cells, thus occupying progressively more superficial positions (Puelles, 2014). This neurogenic pattern suggests that the development of the insula is linked to the claustrum. In later-stage embryos of carnivores and primates the claustrum separates cytoarchitectonically from the insular cortex primordium with the development of the extreme capsule. However, in rodents, bats, and other cases the claustrum remains embedded within the insula, though its cells keep their molecular distinctions from insular neuronal populations (Narkiewicz & Mamos, 1990; Puelles, 2014; Puelles, Alonso, García-Calero, & Martínez-de-la-Torre, 2019).

Additional evidence suggests the existence of a pseudolamination within the mouse claustrum, namely distinguishing core and shell components, as suggested by Real et al. (2006), which cannot be related strictly to the origin of neocortical layers (excepting in case of tangential palliopallial migrations) (Binks, Watson, & Puelles, 2019). However, the existence and the validity of the entire core/shell distinction were debated by Mathur (2014). A distinct pseudolamination

nevertheless had been reported by Narkiewicz and Mamos (1990) after careful cytoarchitectonic analysis of a variety of basal mammals (their "laminar" and "principal" claustrum parts), and this pattern was later discussed by Puelles (2014) into "subplate" and "principal" claustrum parts (i.e., the "shell" or "laminar" component is a subplate-like population underneath the principal claustrum, which is continuous with the neocortical subplate). Suárez et al. (2018) similarly reported that the claustrum of marsupials is rich in *Nr4a2/Nurr1* at its core and it is surrounded by *Ctip2/Bcl11b*-positive cells. Similarly, the presence of a true EPd (concept introduced by Loo [1931]) is not cytoarchitectonically clear in humans, in contrast to most other mammalian species (Figures 1 and 2). This apparent absence might also be related to deficient or superficial anatomical analysis due to the use of outdated methods. Moreover, in species with a clear endopiriform nucleus, cytoarchitectonically distinct dorsal and ventral components have been described (Hardman & Ashwell, 2012; Paxinos, Watson, Petrides, Rosa, & Tokuno, 2012), although the exact delimitation of each remains imprecise (Smith et al., 2018). Puelles (2014) showed that EPd and the claustral primordium are both *Nr4a2/Nurr1* positive. Based on sequential developmental *Nr4a2/Nurr1* in situ hybridization patterns Puelles suggested that EPd derives by tangential migration from the equally *Nr4a2/Nurr1*-positive claustral primordium. EPd is thus lateral pallial in origin, whereas EPv is *Nr4a2/Nurr1*-negative and expresses instead *Dbx1*, as a radial ventropallial derivative (Puelles, 2014; Puelles et al., 2016). Puelles (2014) proposed a separate claustral tangential migration coursing subpially into DPall. Based on the expression of selected genes (*Nr4a2/Nurr1*, *Latexin*, *Cux2*, and *NetrinG2*), Watakabe et al. (2014) also emphasized the partial commonality between claustral neurons and a subtype of infragranular cerebral cortical neurons in the monkey. In the E110 macaque brain, Watakabe et al. (2014) described *Nr4a2/Nurr1* expressing neurons that were scattered in the white matter between the claustrum and the insular cortex. They also raised the possibility that this represented their migratory history, although the claustrum does not migrate inwards; its internalization under the insula is passive, and some claustral cells apparently remain fixed within the insula, or move actively into it (Puelles, 2014). However, these migratory patterns are yet to be demonstrated with lineage analysis or with live cell tracking time-lapse studies (see next section).

1.9 | Developmental origin of subplate and claustrum

In addition to similarities in gene expression during development and in adulthood, claustral, and subplate neurons both have diverse yet overlapping cell-types and basic circuitry, further suggesting that they likely share aspects of their developmental and evolutionary origins. Young neurons of the subplate and claustral complex are quite similar in various aspects and it is only later that they increasingly become different. Subplate and claustrum cell populations are both the earliest neuronal derivatives of their respective pallial origins (DPall vs. LPall; Smart & Smart, 1977, 1982;

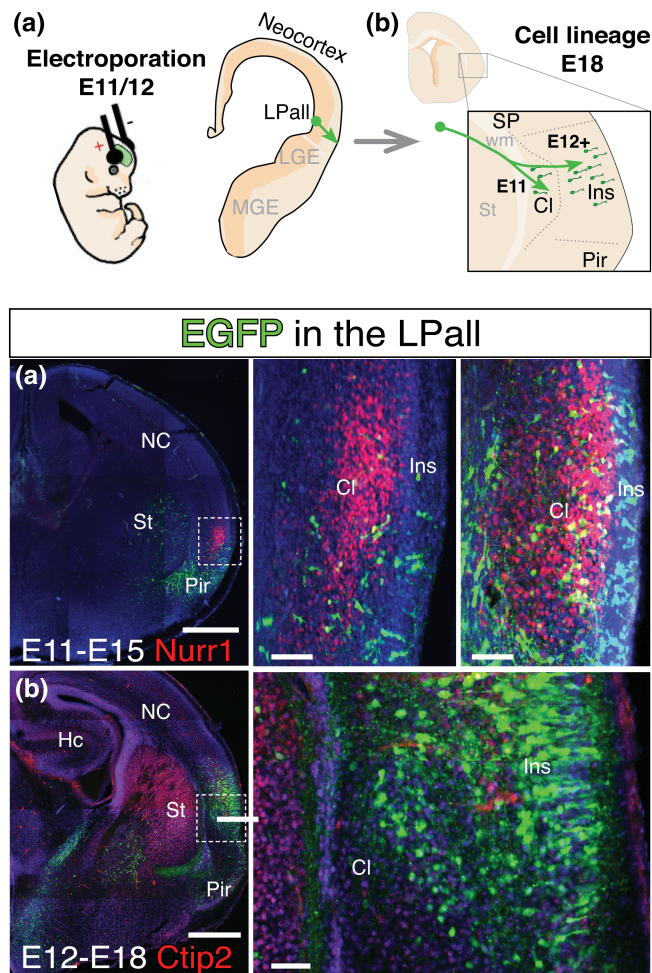


FIGURE 6 (a, b) In utero electroporation of EGFP at the approximate locus of ventral pallium (VP) and lateral pallium (LPall) at embryonic day (E) 11 labels (c) mainly piriform cortex (VPall cells), and only isolated cells under the claustrum (Cl). Some subplate neurons were labeled inside the claustrum, but only in low numbers from this particular E11 VPall and LPall electroporation. Claustrum is indicated by *Nurr1/Nr4a2* immunofluorescence (red in (c)). Note subplate cells labeled according to their topography as “insular” -Ins- most probably included populations of Cajal–Retzius neurons known to be produced at the VPall at E11.5, which then migrate subpially across the LPall into DPall (Bielle et al., 2005). In contrast, the example for the E12 electroporation was mostly restricted to LPall and less VPall (d), and labeled mostly insular cortex (Ins) with no apparent claustrum labeling. Scale bars 500 μ m for (c) and (d) and 50 μ m for high power panels on the right. For technical details see original articles by García-Moreno et al. (2018) and Rueda-Alaña et al. (2018) [Color figure can be viewed at wileyonlinelibrary.com]

Valverde et al., 1989, 1995). They both derive from very similar radial glial ventricular progenitors, which share many genes including *Pax6*, *Emx1*, *Emx2*, *Ngn2*, *Lmx* (Puelles, 2014). The neurons derived from these progenitors differentiate in the earliest mantle zone of the pallium, the primordial plexiform zone, or preplate, which is very similar in DPall and LPall at these very early stages (Marin-Padilla, 1978). The first expression of *Nr4a2/Nurr1* in the earliest-born mouse subplate cells starts at E12.5–E13.5 in the DPall, whereas earliest expression of

the same marker in the claustral primordium occurs in the lateral pallium at E12.5; it is possible that these earliest cells correspond to the subplate component of the LPall, to which other claustral and endopiriform cells are added subsequently (Hoerder-Suabedissen & Molnár, 2013; Puelles, 2014). No combined *Nr4a2/Nurr1* gene expression and birth dating study has been performed at the LPall as for subplate (Hoerder-Suabedissen & Molnár, 2013), but the observed early *Nr4a2/Nurr1* expression at E12.5 and separate mouse autoradiographic data suggest this is the earliest local wave of neurogenesis, which actually starts at E11.5 (Puelles, 2014; Smart & Smart, 1977, 1982). Thus, both the claustrum and the subplate contain some of the earliest-born neurons in the developing pallium. Most of the glutamatergic subplate neurons are usually assumed to be generated in the neocortical germinal zone (DPall) and to migrate radially into the cortex (Pedraza, Hoerder-Suabedissen, Albert-Maestro, Molnár, & De Carlos, 2014), as well as neurons that are generated separately in the rostral medial telencephalic wall (cingular mesocortex) and arrive to the neocortex through tangential migration (Pedraza et al., 2014). The GABAergic subplate neurons arrive through tangential migration from the subpallial ganglionic eminences (Anderson, 1997; Boon et al., 2019).

Based on *Nr4a2/Nurr1* in situ hybridization, (Puelles, 2014, 2017; Puelles et al., 2016) argued that at least part of the subplate population may arise at the claustral-insular lateral pallium domain (insular mesocortex) and then migrates tangentially dorsalward under the cortical plate of the DPall in the plane of the subplate. In addition to cortical subplate *Nr4a2/Nurr1* is selectively expressed in the claustral core nucleus, EPd, and in a separate set of later-born lateral pallium neurons at E14.5 and in Layers 5 and 6 of the parietal neocortex in both mouse and rat (Wang et al., 2011) and in macaque monkey (Arimatsu et al., 2003; Watakabe et al., 2014). At E14.5 granular and supragranular elements are just being born and therefore prospective neocortical Layers 5 and 6 populations form the whole cortical plate. Initially, these granular and supragranular cells are largely restricted to ventral parietal cortex, though they subsequently progressively appear in other more dorsal areas.

Puelles' proposed claustral cell contingents either reside locally (LPall claustral subplate) or might migrate tangentially into the DPall subplate and do not necessarily exclude other subplate populations in the neocortex or cingulate cortex (Puelles, 2014). However, not all genes that are subplate-expressed are also present in the claustrum, and some of these genes have diverse extracortical and pallial expression patterns (see Tables 1 and 2 above) and there are also strong species differences in subplate specific or enriched gene expression even between rat and mouse (Montiel et al., 2011; Wang et al., 2011). The attractive hypothesis on the common origin of claustrum and subplate *Nr4a2/Nurr1* expressing cells would theoretically only explain the origin of one subplate population. However, no direct lineage tracing study has been performed so far confirming this hypothesis. Our preliminary focal electroporation studies examining the VPall and LPall revealed no dorsally migrating subplate neurons from VPall and LPall electroporations in the embryonic mouse (Figure 6; García-Moreno et al., 2018; Rueda-Alaña, Martínez-Garay, Encinas, Molnár, & García-Moreno, 2018;

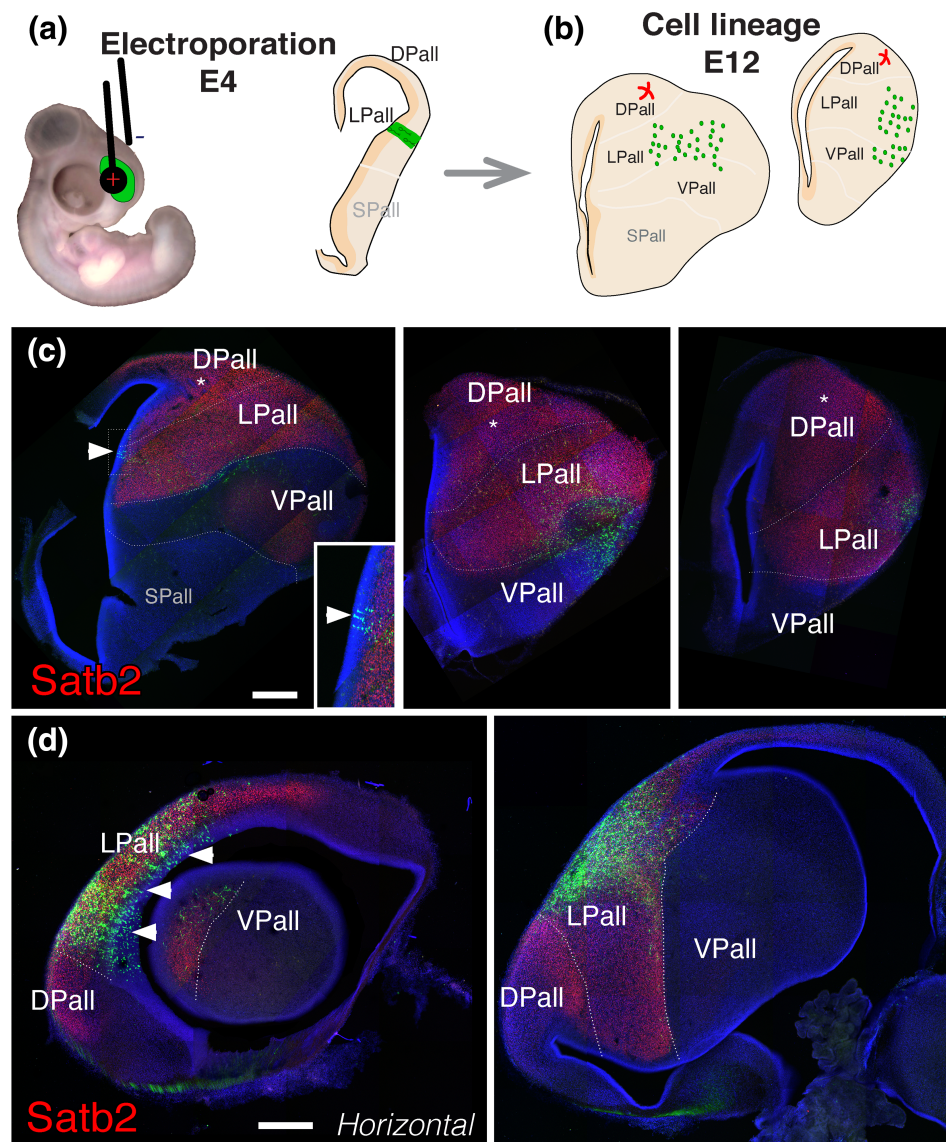


FIGURE 7 Cell lineage of the chick lateral pallium. (a, b) Schematic diagrams summarizing the electroporation of EGFP constructs in the LPall at E4 (a) and of the labeled lineage at E12 (b), following the method described in García-Moreno et al. (2018). (c) Coronal sections, medial at the left. In red, immunostaining for Satb2. Three sections (from more caudal to more rostral) of the telencephalon showing that EGFP labeled cells remain confined to the radial dimension of the LPall, and invade ventrally the nidopallium deep to the piriform cortex (expected homolog of the dorsal endopiriform nucleus, according to Puelles, 2017). Cells do not migrate dorsally toward the DPall region (marked with an asterisk). The inset shows a high-power magnification image of the very restricted locus with labeled progenitors. (d) Horizontal sections, dorsal at the left. Two representative sections, examples of long-term tracing from LPall supported with immunostainings for the pallial marker Satb2. Labeled progenitors are visible in LPall (d); (note in this section plane it cannot be ascertained whether the electroporation was restricted to LPall, or also spread into DPall; compare these domains in (c)), the progeny migrates laterally and rostrally, clearly covering the mesopallium in the lower section of (c) (LPall), whereas the upper section in c possibly shows DPall labeling (note also fluorescent axonal labeling in the septal corticomesecephalic tract, held to arise from DPall neurons). DAPI counterstain in blue. Scale bars represent 500 μ m [Color figure can be viewed at wileyonlinelibrary.com]

García-Moreno, Molnár unpublished observations). Since Puelles' hypothesis postulates a restricted *early* LPall origin of the dorsally migrating subplate neurons (Puelles, 2014), these experiments have to be repeated with selectively targeting LPall at early stages (E11). Recent time-lapse imaging studies suggest a ventral migration of subplate neurons from the dorsal cortex toward claustrum in ventral areas of the pallium (Saito et al., 2019). This is believed to be associated to a considerable expansion of the dorsal cortex with

ventral streaming of the earliest generated preplate derived neurons, including the subplate. This result, together with hypothetical lateropallial (Puelles, 2014) and cingulate elements migrating tangentially (Pedraza et al., 2014) corroborates that the subplate represents an environment where pallio-pallial tangential migrations take place (Puelles, 2011).

In utero electroporation of the lateral pallium at E12 labels overlying neurons of the insular cortex but no cells migrating dorsally

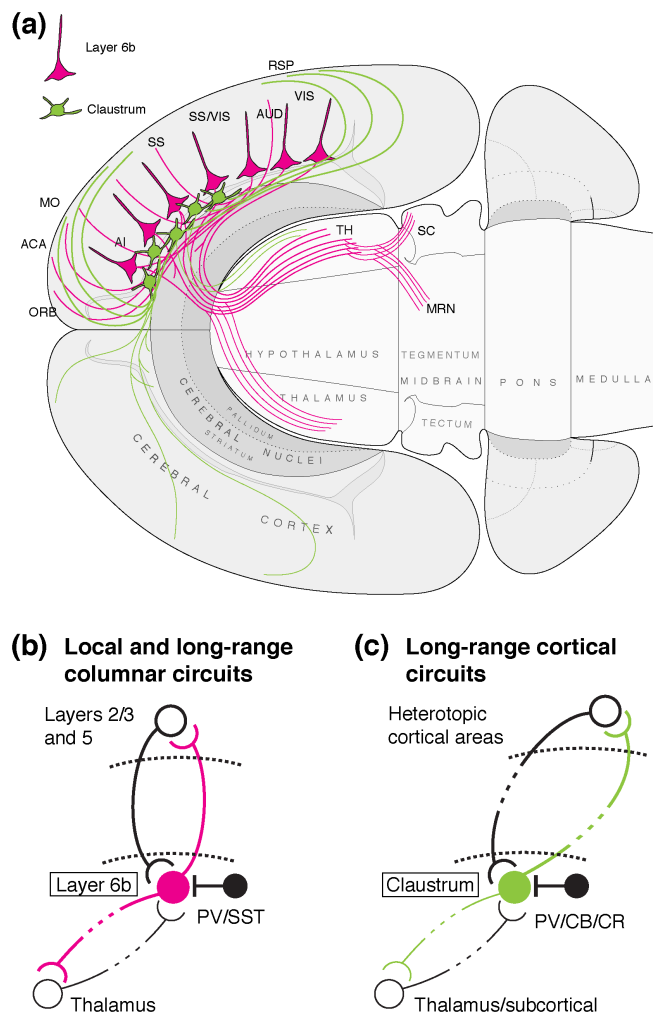


FIGURE 8 Main connectivity features are shared by the Layer 6b (purple) and claustrum (green) in the adult mouse brain. (a) The tracts are shown on a flatmap of the mouse central nervous system (Swanson, 2018), tracing data of the *Drd1a*-cre positive Layer 6b neuronal population is based on Hoerder-Suabedissen et al. (2018). (b, c) Schematic summaries of connectivity features of subplate (b) and claustrum (c). While the subplate establishes local circuits within the overlaying neocortical column, the claustrum forms long-range connections with heterotopic cortical areas of the same and contralateral hemispheres. Subplate/Layer 6b initially during development receives strong thalamic input and projects back to the thalamus. The adult claustrum neurons mostly connect to heterotopic cortical areas, but there is some much less significant thalamic input and even some minor claustrum projections back to the thalamus. Black circles represent local GABAergic interneurons [Color figure can be viewed at wileyonlinelibrary.com]

toward the neocortex and subplate (Figure 6d; Rueda-Alaíña et al., 2018). This is consistent with previous hypotheses stating that both the claustrum and EPd arise from the early lateral pallium, with the claustrum migrating first along the insular cortex pathway whereas the EPd neurons would migrate tangentially ventrally out of lateral pallium into VPall, to localize deep to the VPall-derived piriform cortex (Puelles, 2014; Rueda-Alaíña et al., 2018; Watson & Puelles, 2017).

Selected gene expression patterns suggested the existence of a claustrum homolog in non-mammalian amniotes that is relatively large and contributes to the subpial part of the mesopallial DVR and includes as well a credible EPd homolog cell population in VPall, under piriform cortex (Molnár & Butler, 2002; Montiel et al., 2011; Norimoto et al., 2020; Puelles, 2017; Puelles et al., 2000, 2016; Wang et al., 2011; Watson & Puelles, 2017). Analysis of the entire transcriptome from selected regions of the adult chick and adult mouse brains only partially supported this notion (Belgard et al., 2013), but the restricted subpial position of the possible claustrum homolog may have hindered the sampling approach. Long-term cell tracing from lateral pallium of the E4 chick brains using focal electroporation revealed in some cases labeled progeny that remained largely confined to the radial dimension of the chick lateral pallium, a lineage equivalent to that labeled at E12 from the mammalian LPall (Rueda-Alaíña et al., 2018). Such labeled cells did not migrate dorsally toward the DPall region (Figure 7d), but invaded ventrally the nidopallium (VPall) deep to the piriform cortex (Figure 7b,c).

The above presented preliminary results (Figures 6 and 7) are not quite sufficient to reach a firm conclusion with respect to the different interpretative possibilities (also see figure 6 in Puelles, 2017), nevertheless they portray a glimpse into the lineage relations that we could further explore with these approaches. Refining the precise location and timing of these electroporation experiments will hold the key to reveal possible relationships between subplate and claustrum lineages.

1.10 | Comparisons of connectivity of the claustrum and Layer 6b/subplate

The claustrum has the strongest connectivity in the adult human brain by regional volume (Torgerson, Irimia, Goh, & Van Horn, 2015), and it is considered a major hub, whereas the subplate has the most extensive precocious intracortical and extracortical connectivity during development which is held to be necessary for the guidance of various sorts of axons during development and to orchestrate intracortical and extracortical connectivity (Allendoerfer & Shatz, 1994; Kostović & Rakic, 1990; Molnár & Blakemore, 1995). During cortical development, subplate neurons receive glutamatergic input from thalamocortical axons, as well as GABAergic input from interneurons, and establish reciprocal connections with cortical neurons within the local radial column (Friauf & Shatz, 1991; Higashi, Hioki, Kurotani, Kasim, & Molnár, 2005; Higashi, Molnár, Kurotani, & Toyama, 2002; Molnár, Kurotani, Higashi, Yamamoto, & Toyama, 2003; Piñon, Jethwa, Jacobs, Campagnoni, & Molnár, 2009). Some of these connections are remodeled during development, but in the adult mouse there are extensive connections maintained from Layer 6b to local and distant cortical areas, and to thalamus (Hoerder-Suabedissen et al., 2018). Layer 6b also receives input from thalamus as well as ipsi- and contralateral cortical areas (but mostly Layer 5) in S1 (Zolnik et al., 2020) and in V1 and M1 (Szabó, Berry, Hoerder-Suabedissen, Sharott, Molnár, unpublished).

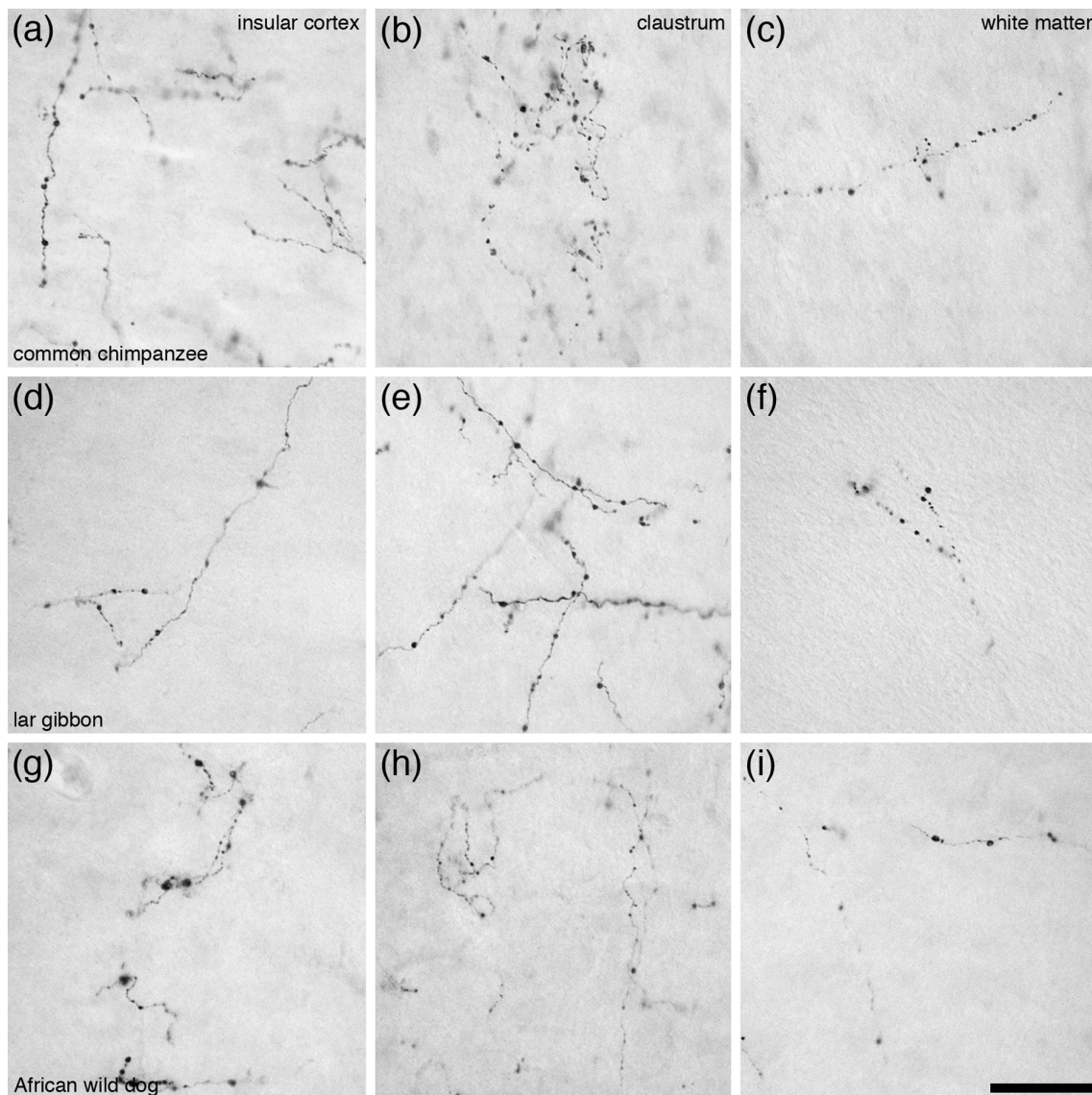


FIGURE 9 Photomicrographs of immunostained orexinergic boutons from the insular cortex (a, d, g), claustrum (b, e, h), and subcortical white matter (c, f, i) from the common chimpanzee (a–c), lar gibbon (d–f), and African wild dog (g–i), demonstrating the presence and relative innervation density of these regions of the telencephalon by the orexinergic system. Note that in all cases the orexinergic boutons are present and are comprised of both large and small boutons, as seen in other mammals (Dell et al., 2015). These boutons were revealed with the anti-orexin-A antibody (AB3704, Merck-Millipore; RRID AB_91545; raised against a synthetic peptide corresponding to the c-terminal portion of bovine orexin-A peptide). In all images dorsal is to the top and medial to the left. Scale bar in i = 50 μ m and applies to all

Similarly, the claustrum has reciprocal connections with cortical neurons, but these are largely long-range with heterotopic areas of the same and contralateral hemispheres (Atlan, Terem, Peretz-Rivlin, Groysman, & Citri, 2016; Smith & Alloway, 2014; Suárez et al., 2018; Wang et al., 2017; Figure 8). The claustrum also has some projections to the thalamus and it receives some thalamo-claustral input (Zingg, Dong, Tao, & Zhang, 2018), but these are minor compared to the cortical connections in the adult.

The ipsilateral claustrum-cortical projections in rodents are much stronger than contralateral projections (Wang et al., 2017; White et al., 2017). Layer 6b Drd1a-cre neurons have mostly ipsilateral projections for cortex, but have bilateral projections to the thalamus with

a strong ipsilateral dominance in the adult mouse (Hoerder-Suabedissen et al., 2018). Mathur et al. (2009) revealed claustral projections by using retrograde labeling from the mediodorsal thalamic nucleus and from lateral hypothalamus. Similarly, Hoerder-Suabedissen et al. (2018) used cre-dependent AAV tracing to reveal Layer 6b projections (Drd1a-cre populations) and demonstrated strong projections to the higher order thalamic nuclei, including the mediodorsal nucleus. That study, however, did not detect specific Layer 6b projections to lateral hypothalamus, but more recent reports suggest that Layer 6b receives direct input from the lateral hypothalamus and some of these neurons are orexin-immunoreactive (Szabó, Berry, Hoerder-Suabedissen, Sharott, Molnár, unpublished).

Orexin is a peptide that is delivered from the lateral hypothalamus via wide projections across the entire central nervous system, including to other local (hypothalamic) neurons that are important for modulating arousal, appetite, and activity of the neuroendocrine functions (Li & de Lecea, 2020). Layer 6b cortical cell populations are the only orexin sensitive cell groups in sensory regions of the cerebral cortex (Bayer et al., 2004). We have little information on the distribution of orexin receptors in the claustrum, but orexin immunoreactive boutons are present in the insular neocortex, claustrum, and subcortical white matter in common chimpanzee, lar gibbon, and African wild dog (Figure 9), demonstrating the presence and relative innervation density of these regions of the telencephalon by the orexinergic system (Manger, unpublished; Dell et al., 2015).

We hypothesize that the influence of the orexinergic lateral hypothalamic neurons on cortex is mediated through fast neurotransmitters and orexin neuropeptides on Layer 6b neurons in broad cortical areas and perhaps a similar influence is mediated through claustrum that also receives moderately dense orexin immunoreactive projections. We hypothesize that this influence of lateral hypothalamus and the sensitivity of cortex and claustrum is also present in human providing targets for potential pharmacological manipulations. Together, these investigations further suggest similarities between adult Layer 6b and claustrum connections that might underlie functional similarities (Molnár, 2019), however, we know very little about the connectivity of the developing claustrum. We do not know whether the claustrum has transient connectivity during development that is not maintained to adulthood. While some subplate connections are transient, not all subplate neurons disappear after development (the above description of inputs and outputs of Layer 6b are derived from adult mice). Figure 8 compares the adult Layer 6b projections with the adult claustrum connections, but it would be interesting to do such comparisons at various developmental stages to gain further insights into their pre- and postnatal evolution. Overall, both structures have very extensive connections, the claustrum and subplate/Layer 6b share extensive connectivity features but the relative strength of these projections differ in adult mice.

1.11 | Do claustral neurons survive in larger numbers than the subplate?

The human subplate is generally considered as a largely transient neuronal population and its postnatal remnant are interstitial neurons of the subcortical white matter (Kostović & Rakic, 1990). The subplate is the largest cortical compartment between 15 and 20 post-conceptional weeks and begins to disappear in the last third of gestation and during the early postnatal period (Judaš et al., 2013; Kostović & Rakic, 1990). Dendritic arborizations of the interstitial subplate neurons continue to grow and develop even after the disappearance of most of the subplate during the first year of life. The fate of subplate neurons during postnatal development is still a matter of debate and varies between species. In general, between 50 and 80% of subplate cells disappear by programmed cell death, as occurs, for example, in some primates (Kostović & Rakic, 1980; Meyer, Wahle, Castaneya-Perdomo, & Ferres-Torres, 1992), carnivores (Luskin &

Shatz, 1985a, 1985b; Meyer et al., 1992), and rodents (Arias, Baratta, Yu, & Robertson, 2002; Ferrer, Bernet, Soriano, del Rio, & Fonseca, 1990; Price et al., 1997); however, in cats, humans and other primates a higher proportion of subplate neurons may survive as white matter neurons (Kostović & Rakic, 1980; Meyer et al., 1992; Valverde et al., 1995; Valverde & Facal-Valverde, 1988). The quantitative differences observed may be due in part to procedural differences. Cell death has not been quantified at all in claustrum and thus the extent of cell death in the claustrum and subplate has not been compared, but an interesting possibility is that these structures may have different survival rates, with the claustrum being more permanent than the subplate. If this were the case, then a likely scenario is that different developmental dynamics may differentially affect the origin and establishment of both subsystems in amniotes. Additionally, comparing relevant embryonic processes between extant species could provide more relevant information for understanding their evolutionary history than comparing adult structures alone. Combining birth-dating, cell-death, and gene expression studies will be required to fully identify the transient and permanent neuronal populations in both structures (Hoerder-Suabedissen & Molnár, 2013).

2 | SUMMARY

Here we have reviewed evidence suggesting extensive similarities between the claustrum and the subplate. They both contain principal projection neurons that are among the earliest born in the developing pallium, as well as later-born GABAergic interneurons that arise from subpallium. There is evidence for the general presence of the claustrum in all extant mammalian taxa, but in monotremes there is some uncertainty about the cytoarchitectonic distinctions of the claustrum. The existence of a claustrum in all extant mammalian taxa in conjunction with recent evidence of a putative reptilian claustrum raises the possibility that the claustrum may already have been present in the common ancestor of all amniotes.

Almost all of the markers we examined that were expressed in the subplate/Layer 6b were also expressed in the claustral complex, but not all claustrum specific or enriched genes were expressed in subplate/Layer 6b, and vice-versa, suggesting overall similarities, but also some differences. Based on gene expression patterns we found the deep (shell or laminar) part of the claustrum the most related to the subplate. The expression patterns in other cortical layers and structures provide some support to the pallio-pallial claustral-neocortex subplate migration and subpial tangential migrations hypotheses, but also highlight the fact that confirmation of such hypotheses by direct lineage tracing methodologies is required. We presented some preliminary cell lineage studies that identified the origin of claustrum and insula, as well as the EPd, in the lateral pallium of the mouse and chick. However, to date there is no conclusive answer about the question of whether the lateral pallium produces a labeled subset of subplate cells which invades the dorsal neocortex, mixing with other subplate cells that are produced locally, or originating elsewhere. The issue is important. In one case, the observed claustrum/subplate similarities are due to shared aspects of

patterning, and similar typological and histogenetic properties of adjacent cortical progenitor areas (LPall vs. DPall). In the other case (i.e., claustral cells contributing to neocortical subplate), similarities might be due to migrated claustral elements that conserve some of their original properties. Refining the location and timing of electroporation experiments will hold the key to revealing the possible relationships between subplate and claustrum lineages, while they will also provide ways for defined experimental manipulations. There are numerous similarities between the adult connectivity patterns of Layer 6b and claustrum. In particular, both have extensive cortical connections. However, there are differences in the relative strength of their reciprocal connectivity with various other structures in the adult. Similar comparisons at various developmental stages are overdue.

Cell death comparisons between the claustrum and subplate will be required to assess whether these structures have different survival rates. Detailed comparisons through developmental stages will be critical to unravel further similarities and differences in molecular, cellular, and connectivity features between both structures, and will also provide a robust framework for similar comparisons with non-mammalian amniotes. Many questions remain about the claustrum and subplate/Layer 6b. A better understanding of the evolutionary origins; development; impact in cortical evolution; roles in brain wiring during development; and relation to higher brain functions of these structures will provide important insights. In particular, further research is needed into the neurological diseases and cognitive conditions associated with disruptions of these structures, as well as into potential strategies to design new diagnostic and therapeutic approaches.

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CONFLICT OF INTEREST

The authors have no conflicts of interest and that, no interests of any person/organization are affected by the information presented in the present manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of Figure 1 are available in Comparative Mammalian Brain Collections at (<http://brainmuseum.org>). These data were derived from their resources available in the public domain: (<http://brainmuseum.org>). The data that support the findings of Figure 2 are available in Images taken from BrainMaps.org. These data were derived from their resources available in the public domain: (<http://brainmaps.org>). The data that support the findings of Figures 3 and 4 are available in (Allen Brain Atlas Data Portal) at Mouse Brain in Situ Hybridization (ISH) Data (<http://help.brain-map.org/display/mousebrain/In+Situ+Hybridization+%28ISH%29+Data>). These data were derived from the Allen Brain Atlas Data Portal (ABADP) resources available in the public domain: (<http://help.brain-map.org/pages/viewpage.action?pageId=2424836>). The data that support the findings of Figure 5, Tables 1 and 2 were generated from the data that are available in (Allen Brain Atlas Data Portal) at Mouse Brain in Situ Hybridization (ISH) Data (<http://help.brain-map.org/display/mousebrain/In+Situ+Hybridization+%28ISH%29+Data>). These data were derived from the Allen Brain Atlas Data Portal (ABADP) resources available in the public domain: (<http://help.brain-map.org/pages/viewpage.action?pageId=2424836>). The data that support the findings of Figures 6 and 7 are based on the datasets partially published by García-Moreno et al. (2018; <https://doi.org/10.1016/j.celrep.2017.12.032>) and Rueda-Alaño et al. (2018; <https://doi.org/10.3389/fnins.2018.00792>) available from the corresponding author upon reasonable request. The data that support the findings of Figure 8 are based on the datasets published by Hoerder-Suabedissen et al. (2018; <https://doi.org/10.1093/cercor/bhy036>) and available from the corresponding author upon reasonable request. The data that support the findings of Figure 9 are based on the datasets published by Dell et al. (2015; <https://dx.doi.org/10.1016/j.jchemneu.2015.07.007>) and produced in additional species by Professor Manger's laboratory using the same methodology. The data is available from the corresponding author upon reasonable request.

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