

Review

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Review

# Brain Stars Take the Lead during Critical Periods of Early Postnatal Brain Development: Relevance of Astrocytes in Health and Mental Disorders

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**Abstract:** In the brain, astrocytes regulate shape and functions of the synaptic and vascular compartments through a variety of released factors and membrane-bound proteins. An imbalanced astrocyte activity can therefore have drastic negative impacts on brain development, leading to the onset of severe pathologies. Clinical and pre-clinical studies show alterations in astrocyte cell number, morphology, molecular makeup and astrocyte-dependent processes in different affected brain regions in neurodevelopmental (ND) and neuropsychiatric (NP) disorders. Astrocytes proliferate, differentiate and mature during the critical period of early postnatal brain development, a time window of elevated glia-dependent regulation of a proper balance between synapse formation/elimination, which is pivotal in refining synaptic connectivity. Therefore, any intrinsic and/or extrinsic factors altering these processes during the critical period may result in an aberrant synaptic remodelling and onset of mental disorders. The peculiar bridging position of astrocytes between synaptic and vascular compartments further allows them to “compute” the brain state and consequently secrete factors in the bloodstream, which may serve as diagnostic biomarkers of distinct healthy or disease conditions. Here, we collect recent advancements regarding astrogenesis and astrocyte-mediated regulation of neuronal network remodelling during early postnatal critical periods of brain development, focusing on synapse elimination. We then propose alternative hypotheses for an involvement of aberrancies in these processes in the onset of ND and NP disorders. In light of the well-known differential prevalence of certain brain disorders between males and females, we also discuss putative sex-dependent influences on these neurodevelopmental events. From a translational perspective, understanding age- and sex-dependent astrocyte-specific molecular and functional changes may help to identify biomarkers of distinct cellular (dys)functions in health and disease, favouring the development of diagnostic tools or the selection of tailored treatment options for male/female patients.

**Keywords:** astrocytes; critical period; synapse phagocytosis; sex differences; neuropsychiatric and neurodevelopmental disorders

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## Main Text

Astrocytes are the most abundant subtype of glial cells populating the brain and spinal cord [1]. Microscopically, they show a typical star-shaped morphology with few major processes extending from the soma, which further ramify into numerous fine branches and leaflets at more distal locations [2,3]. In the past years, several studies and excellent reviews have offered historical overviews about the discovery of astrocytes and covered their numerous physiological roles in the mammalian brain, ranging from their support of the formation/function of neuronal synapses to the development of a properly operating blood-brain barrier (BBB) [4–18].

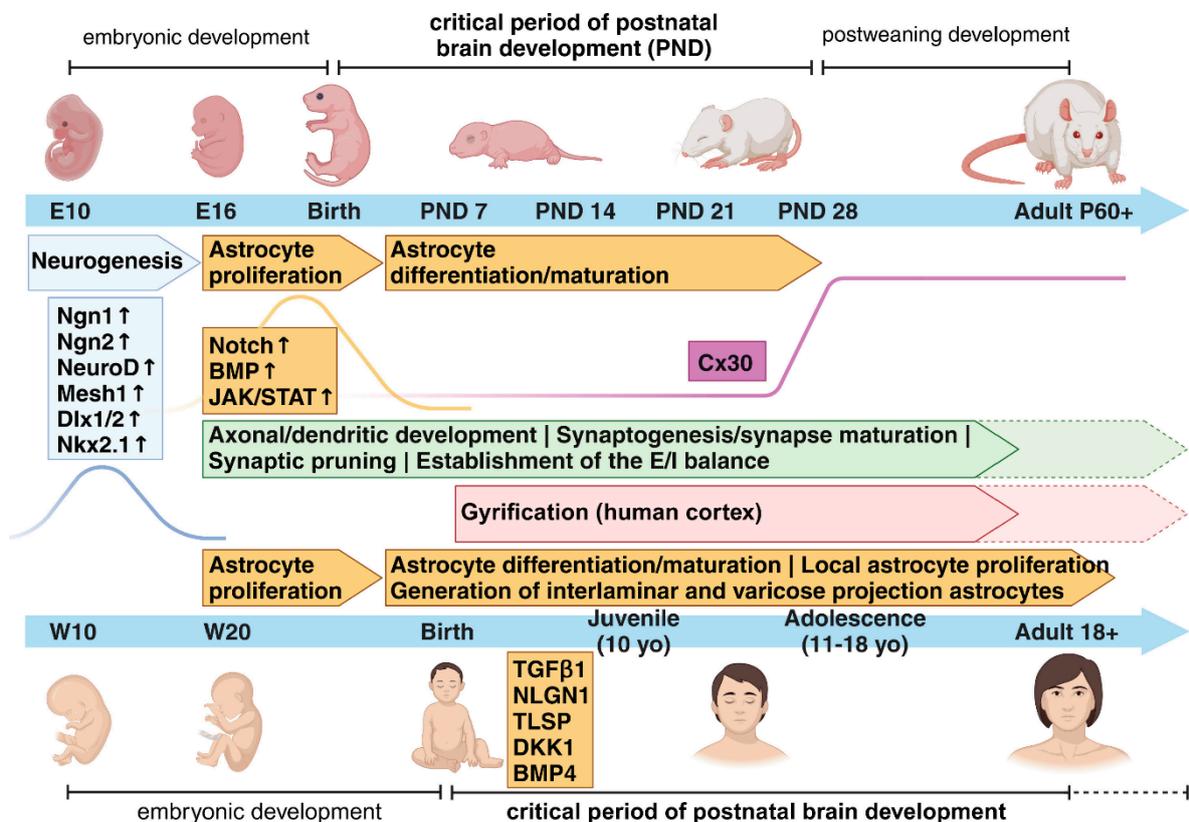
Here, we collect recent findings on astrocyte-dependent contributions to brain development during early postnatal critical periods, focusing on synapse elimination (phagocytosis) and its

putative link to the onset of neurodevelopmental (ND) and neuropsychiatric (NP) disorders. Finally, considering the asymmetry in the prevalence of mental disorders between males and females, we discuss the impact of sex differences on astrocyte properties during critical periods and propose their hypothetical causal relation to sex-skewed brain pathologies.

During embryogenesis, in both human and rodent brains, astrocytes are generated from radial glia (RG), which self-renew and differentiate into neurons and macroglia cells, e.g. astrocytes, oligodendrocytes and Schwann cells [19–25]. Cell divisions of RGs are predominantly neurogenic at early/mid-gestation and turn into gliogenic at late-gestation/early postnatal developmental stages [23,26,27] (Figure 1). The initial steps of gliogenesis produce astrocyte precursors, which then locally proliferate in the different brain areas to increase their numbers and give rise to mature astrocytes, the majority of which consist of protoplasmic and fibrous astrocytes in the grey matter and white matter, respectively [17,20,23,28]. Several studies highlight the heterogeneity of astrocyte morphologies and functions in various regions of the central nervous system (CNS). The most specialized subtypes display very distinctive structural and functional properties such as e.g. the Bergmann glia and velate astrocytes in the cerebellum, the Müller glia cells in the retina or the pituicytes in the neurohypophysis [17,29–32]. In addition to these general features common to all mammalian brains, hominid primates exhibit evolutionary unique subtypes of astrocytes, i.e. the interlaminar and varicose projection astrocytes [15,18] (Figure 1). Interlaminar astrocytes were first observed in Golgi stained samples of the human cortex by Martinotti, Andriezen and Retzius already by the end of the 19<sup>th</sup> century and described as cells with small somata residing in the upper cortical layer and long processes extending through deeper layers [33–35]. Only later, Colombo and colleagues used glial fibrillary acidic protein (GFAP) to examine these long interlaminar astrocyte processes and proposed that they were primate-specific and putatively involved in favouring a radially acting, long distance intercellular communication across cortical layers [36–39]. A more recent study has, however, challenged the idea of interlaminar astrocytes being primate-specific, and showed their presence in rodent brains, although they appear morphologically more rudimentary than their primate counterpart [40]. Varicose projection astrocytes have been first identified by Oberheim and colleagues in 2009, after examining GFAP-labelled human cortical brain slices [15]. Located in layers 5 and 6, they morphologically resemble protoplasmic astrocytes, but possess less branched short processes and few [1–5] long projecting ones bearing varicosities and extending within single cortical layers, suggesting a tangential range of action [15,41,42]. The peculiar enrichment of interlaminar and varicose projection astrocytes in human and higher-order primates, when compared to other lower-order primates, suggests their relevance for evolutionary more refined CNS functions, but this has not been definitively proven yet. However, engraftment experiments of human glial progenitors into mouse forebrains at postnatal day 1 have shown increased synaptic plasticity and learning capabilities of the chimeric adult mice, further strengthening the hypothesis of enhanced supportive properties of human astrocytes for specialized CNS abilities [43]. In addition to these morphological differences, human astrocytes show a remarkable faster propagation of calcium waves when compared to rodents' astrocytes, in contrast to more evolutionary conserved characteristics, such as an elevated intracellular calcium level in response to ATP and glutamate [15,43]. For more detailed comparisons between human and rodent astrocytes, we recommend the excellent reviews from Vasile and colleagues [14] and Verkhratsky and Nedergaard [17].

Beside these differences in subtypes of astrocytes, in various species from worms and insects to rodents and humans, the number of glia cells and the ratio glia-to-neurons are additional factors worth of consideration, because they both increase with the complexity of the CNS [44,45], suggesting a tight link between the growing number of glial cells with increasingly advanced CNS functions. For instance, the development and evolution of mammalian higher cognitive abilities correlates with the process of gyrification in the cerebral cortex during early postnatal developmental stages [46,47]. This was shown to be associated with a fibroblast growth factor (FGF)-dependent localized expansion of astrocyte numbers in the ferret cortex, with FGF1 and FGF2 being the predominant ligands involved [48,49]. Using a targeted genetic manipulation, Shinmyo and colleagues demonstrated that inhibition

of astrocyte proliferation in the ferret cortex prevented the formation of gyri, whereas an FGF-dependent overproduction of astrocytes in the lissencephalic mouse cortex was sufficient to induce gyrus-like protrusions [48].



**Figure 1. The critical period for astrocyte development and synaptic circuit formation during postnatal brain development in rodents and humans.** The timelines show the major processes occurring from pre- and postnatal early stages through adulthood in rodents and humans to develop properly functional astrocytic and neuronal synaptic networks. A few examples of intracellular signalling molecules which determine the switch between neurogenic to gliogenic cell fates are depicted, as well as selected proteins relevant for astrocyte-specific differentiation/maturation processes (e.g. Cx30 and TGFβ/NLGN1/TLSP/DKK1/BMP4 ligands). We refer the reader to the text for details regarding experimental evidences supporting our current knowledge about each event displayed in the Figure. Cx30, connexin 30. Figure created with BioRender.com.

In general, astrocytes show a great range of heterogeneity in the acquisition of various competencies selectively matching the function(s) of neighbouring cells [2,50–52]. To gain such a high degree of intra- and inter-regional diversity, astrocytes must undergo positionally and temporally regulated developmental programmes, strictly modulated by interactions between intrinsic (cell-autonomous) and extrinsic (non cell-autonomous) factors, to ultimately guarantee their specification most aligned to the requirements of the local environment (reviewed in [26]). Among intrinsic programmes, three pathways emerged as crucial players for astrocyte development: the Notch, the BMP, and the JAK-STAT signalling pathways [51,53–55]. These pathways, alone or in combination with exogenously secreted molecules, e.g. leukemia inhibitory factor (LIF), bone morphogenetic protein (BMP), sonic hedgehog (Shh), ciliary neurotrophic factor (CNTF) or cardiotrophin-1 (CT-1), induce chromatin changes, promoting astrocyte generation and differentiation from RGs [20,54]. For example, the coordinated activity of Notch and JAK/STAT pathways induces an astrocytic fate by demethylating and thereby activating astrocytic genes, such as GFAP or S100β, with the intermediate activation of the transcription factor STAT3 and the DNA methyltransferase 1 (DNMT1) ([20,56] and reviewed in [54]). On the contrary, the activity of pro-neurogenic factors such as Neurogenin1 (Ngn1), Ngn2, NeuroD, Dlx1/2, Nkx2.1 and Mesh1 inhibits astrocyte development by directly or indirectly blocking the

JAK/STAT pathway, favouring the production of excitatory and inhibitory neurons both *in vitro* and *in vivo* [23,54,57–69]. Pro-neurogenic factors promote a temporally simultaneous generation of excitatory and inhibitory neurons during embryonic development, but exert different positional influences: excitatory neurons are produced in the dorsal pallium by the radial migration of locally born neuronal progenitors into their targeted cortical layers, whereas inhibitory neurons are generated in the ventral brain and tangentially migrate to dorsal locations, thus infiltrating the developing cortical plate and dorsal areas [70]. These differences influence each other's developmental trajectories and astrocyte differentiation, ultimately affecting neuronal circuit formation. Imbalances in this finely tuned ratio may contribute to the cognitive dysfunction and neurological abnormalities of mental disorders, such as autism spectrum disorders (ASD), Alzheimer's disease and schizophrenia (SCZ) [71–73].

Beyond intrinsic factors, evidence that also extrinsic signals guide astrogenesis initially came from co-culture experiments of mouse embryonic RG with cortical slices, which produced neurons when co-cultured on embryonic slices but shifted to a glial fate when co-cultured on postnatal slices [27]. Interestingly, the postnatal release of cytokines such as the gliogenic CT-1 from young neurons promote astrocyte differentiation and their depletion can severely impair astrogenesis [26,27,74]. Similarly, in the cerebellum, neuronal Shh diversifies molecular and functional features of Bergmann glia and relate astrocytes [75]. More generally, a coordinated expression of cell-type-specific ligands and receptors is required to favour selected cell-cell interactions and support the appropriate activation of intracellular pathways to drive an astrocyte heterogeneous specification. Very recently, Voss and colleagues demonstrated that the synergistic and combinatorial activity of five ligand–receptor pairs, driven by the ligands TGF $\beta$ 2, NLGN1, TSLP, DKK1 and BMP4, guides astrogenesis in human cortical organoids and primary fetal tissue. In this work, they additionally identified a time frame of effective responsiveness to gliogenic signals, which corresponds to the initial postnatal developmental stages [76] (Figure 1).

In summary, a tight regulation and coordination of all intracellular and extracellular stimuli along with cell-cell interactions is implicated in controlling astrocyte proliferation and maturation during perinatal and early postnatal developmental periods. This goes hand in hand with neuronal development and is critical for establishing the appropriate subtypes and numbers of astrocytes in any distinct brain region. The balanced relative number of all different cell types, their appropriate functional diversification and the correct formation of elaborated neuronal networks guide the specification of high-order morphological modifications of cortical areas, e.g. gyrification of the cerebral cortex [48,53,77]. These are in turn critical for the proper acquisition of increasingly more complex CNS functions, such as human mental and cognitive abilities [1,44].

Any disruption in these delicate processes might affect the formation and functions of astrocytic and neuronal networks, leading to the onset of severe brain disorders.

### **Astrocytes and the Critical Period of Early Postnatal Brain Development in Health and Mental Disorders**

All phases of astrogenesis (generation/proliferation, differentiation and maturation) take place with distinct time courses in various areas of the CNS during the so-called “critical periods” of postnatal brain development. These periods may vary both in length and cell types involved in different species, depending on the functions that must be refined [78–81]. Critical periods are also sometimes referred to as “sensitive” periods, although it is still debated whether critical and sensitive periods temporally overlap or should be distinguished [81]. These are time frames when brain plasticity and differentiation/maturation processes are strongly dependent on experience and environmental cues to customize neural circuits and connectivity to the needs of each individual. Therefore, any interaction between intrinsic molecular/biochemical programmes with external factors becomes crucial during critical periods in shaping neuronal circuits to respond with the most adapted behaviours in juvenile, adolescent and adult life [82,83]. As described by Knudsen [81], during these developmental phases structural changes such as axon elaboration and synaptic

modifications are instructed by life experiences across brain areas in various species (reviewed in [84]) (Figure 1).

In general, identifying the beginning and end of critical periods, as well as isolating the main factors shaping their opening and closing, might support investigations of time frame(s) and mechanisms relevant for an optimal acquisition of selected cognitive abilities and general mental competences, ultimately contributing to build an individual's personality. From a translational perspective, a better knowledge of the neurobiological, genetic and environmental determinants affecting the opening/closing of critical periods and the reactions of specific cell types upon exposure to selected physiological or detrimental triggers might be essential to identify time windows, when therapeutic strategies are more effective in re-directing aberrant brain developmental trajectories by directly acting on affected cellular mechanisms.

In rodents, astrogenesis occurs during late-gestation and proceeds over the first three weeks of postnatal CNS development, concomitantly with synaptogenesis, both being key processes for the generation of fully functional neuronal networks [84] (Figure 1). Many astrocyte-derived molecules have been identified so far, playing crucial roles for the formation/functions of excitatory/inhibitory synapses during these developmental time frames. Tan and colleagues have recently reviewed the current knowledge about the cellular and molecular mechanisms by which astrocytes instruct synapse formation/functions through various proteins such as e.g glypicans, TNF $\alpha$ , thrombospondins, Hevin/SPARC ([85–93] further reviewed in [5,6,13,94,95]). Additionally, a growing body of evidence is featuring the central role of astrocytes in selectively eliminating synapses during developmental windows [96].

In humans, it is still debated whether critical periods are closed after the juvenile developmental stage (approximately corresponding to the first decade of life) or they further extend into adolescence and early adulthood [79,97]. The initial idea was that the closure of the critical period at the end of puberty marks the end for astrogenesis and synaptic spine formation/elimination in the human cortex. However, this hypothesis mostly relies on the first publications of Huttenlocher and colleagues [98,99], which contained only one brain specimen to support the claim. In contrast, more recent brain scan studies suggest that several dynamic changes in cortical gray matter density and remodelling extend into the third decade of life [79,97,100,101]. Insofar as dendritic growth and branching are mainly limited to early childhood stages, it has been postulated that this supplementary reorganization of neuronal circuitries is centred on synaptic modifications to favour the acquisition of higher brain functions, such as modulation of emotions, cognitive flexibility, socialization skills and others. In this line, investigating how a proper excitatory/inhibitory (E/I) balance instructs the opening/closing of critical periods may support our understanding of changes in plasticity related to synaptic modifications. Intriguingly, however, in disease states an E/I imbalance might seem to emerge more as a homeostatic compensatory mechanism to abnormal circuit activity, rather than a primary defective mechanism caused by e.g an inherited genetic background [102]. The E/I balance is in fact also heavily affected by neighbouring cells, including astrocytes. For example, the deletion of glutamate transporters GLAST and GLT1 in mice results in pathological repetitive behaviours commonly observed in ASD, obsessive-compulsive disorder, and Tourette's syndrome [103]. Moreover, one study showed that astrocyte reactivity reduces inhibitory currents as a consequence of diminished glutamine and GABA availability [104], although astrocyte reactivity may not always homogeneously respond to disease conditions [105].

Besides these evidences, other theories to explain late-onset neurological or NP disorders suggest defective pruning mechanisms of the initially supernumerary spine synapses among the causes of disease conditions [106–109]. An over-abundance of unpruned weak synaptic inputs appears to negatively affect the synchronized development of E/I inputs, ultimately disrupting the connectivity of brain circuits in the limbic system [13,110,111].

However, due to the limitations of human neuroimaging tools and the ethical issues associated with studies involving children and adolescents, understanding the cellular mechanisms behind these developmental periods and how perceived adverse events may become neurobiologically

embedded in brain circuits and result in ND and NP disorders, requires the continuous development of experimental models recapitulating the human conditions.

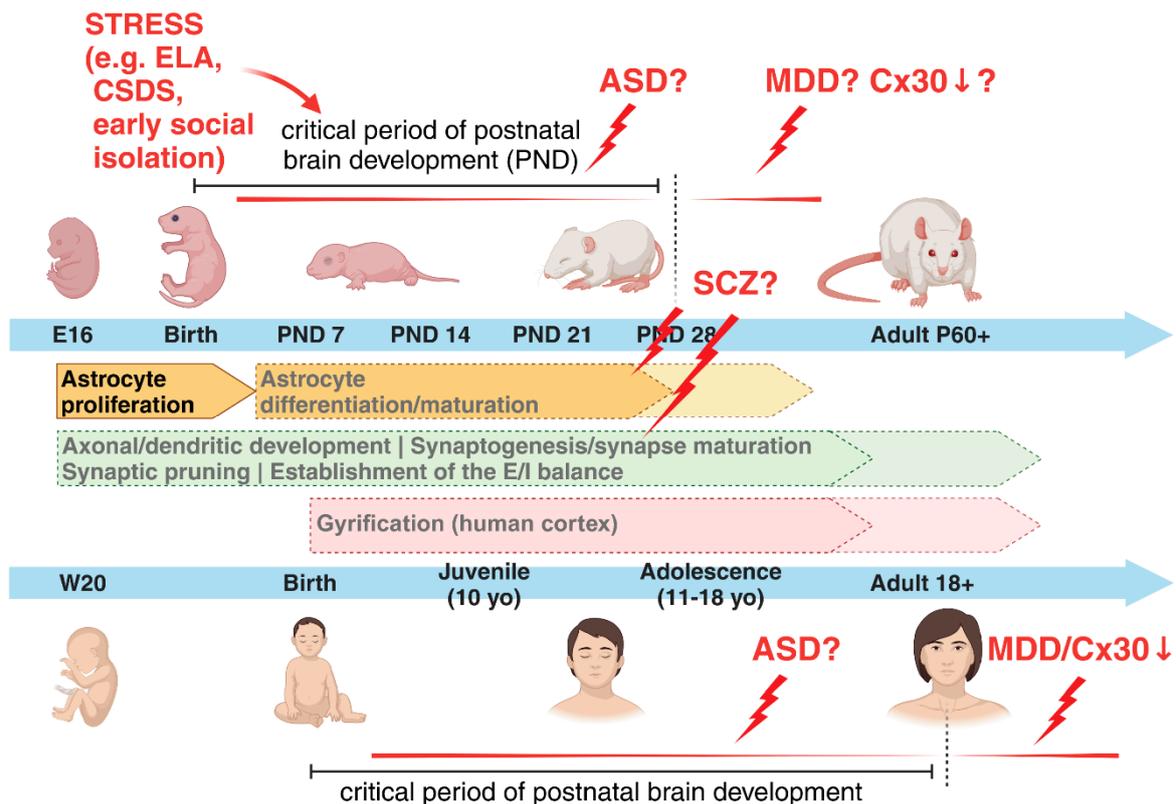
The use of animal models has been pivotal in revealing essential physiological underpinnings of critical periods and unravelling molecular pathways that respond to the simultaneous application of pharmacological treatments together with behavioural interventions, to ultimately rewire altered neuronal circuits [112–114]. Few seminal studies examined changes in the plasticity of brain circuits in various brain regions, after the application of effectors aimed at reopening critical period-like states. Specifically in two of them, the chronic administration of the antidepressant fluoxetine showed remarkable effects on the reopening of critical period-like neuronal responses, further associated with phenotypical alterations. Interestingly, in a first study, fluoxetine reinstated an ocular dominance plasticity in adult mice, promoting the recovery of visual capabilities in amblyopic animals, accompanied by a reduction of intracortical inhibition [113]. A later work showed how fluoxetine treatment combined with behavioural extinction training induced the erasure of amygdala-dependent fear memories, after reactivating a juvenile-like circuit plasticity associated with a disruption of extracellular matrix structures called perineuronal nets (PNNs) around parvalbumin-positive interneurons [112].

Although both studies evidenced a “rejuvenalization” of brain circuits with changes in the inhibitory system concurrent to the reopening of a critical period-like plasticity, neither of them proposed a cellular or molecular mechanisms possibly involved in the observed disruption of PNNs. Only very recently, the study of Ribot, Breton and colleagues has highlighted the role of astrocyte to close the critical period for visual plasticity in the mouse, favouring a remodelling of the extracellular matrix and associated maturation of the inhibitory system in the visual cortex, although the excitatory system was also, even if minorly, modulated [114]. Based on the knowledge that in rodents astrogenesis occurs during the early postnatal developmental stages and that it is for the most part completed by the end of the critical period around postnatal day (P) 28, the authors postulated that the maturation of astrocytes and its molecular regulators might be relevant for the closure of this period. In line with this, a previous study showed that transplanting immature astrocytes in the visual cortex of adult cats reactivated a period of high brain flexibility, with associated re-induction of ocular-dominance (OD) plasticity in adult animals, similar to what observed upon fluoxetine treatment [115]. In their study, Ribot, Breton and colleagues demonstrated that a molecular switch from proliferative to mature astrocytes corresponded to the closure of the critical period and the astrocyte-specific gap-junction channel subunit connexin 30 (Cx30), an important modulator of hippocampal astrocyte maturation [116], was a pivotal player in these events (Figure 1). Its lower expression in immature astrocytes and higher expression in mature astrocytes correlated with different stages of neuronal network maturation and plasticity in the visual cortex. Furthermore, the astrocyte-specific knockdown of Cx30 favoured a heightened ocular dominance plasticity until P50, confirming the astrocytic Cx30 as a factor necessary to mark the closing of the critical period [114]. It would be intriguing to investigate whether and how immature astrocytes might characterize ND or NP disorders and potentially identify underlying molecular determinants, which might trigger the onset of astrocyte-dependent brain disorders. In humans, early-life adversity (ELA) has been associated with the appearance of severe neurological and mental symptoms later in life, as well as with aberrant astrocyte functions ([82,117] and reviewed in [84]). Besides ELA, other chronic stress paradigms are accompanied with reductions in both the number of GFAP-positive astrocytes and the length of GFAP-labeled processes in the prefrontal cortex (PFC), hippocampus or other brain regions, besides changes in the expression levels of various astrocyte-specific molecular markers, suggesting a negative impact of stress on proliferation/maturation processes of astrocytes [118–124]. For example, three weeks of mild chronic unpredictable stress reduce the number of Sox9/S100 $\beta$  double positive cells in different subfields of the hippocampus [125]. Moreover, chronic social defeat stress (CSDS) hampers the expression of glucocorticoid receptors (GR) asymmetrically in astrocytes and neurons of the medial PFC. The reduced GR content in astrocytes sequentially affects Ca<sup>2+</sup> oscillations and the release of adenosine triphosphate (ATP), which in turn modulates synaptic communication [126]. Additionally CSDS decreases the expression of Cx30 and Cx43, correlating with an impaired

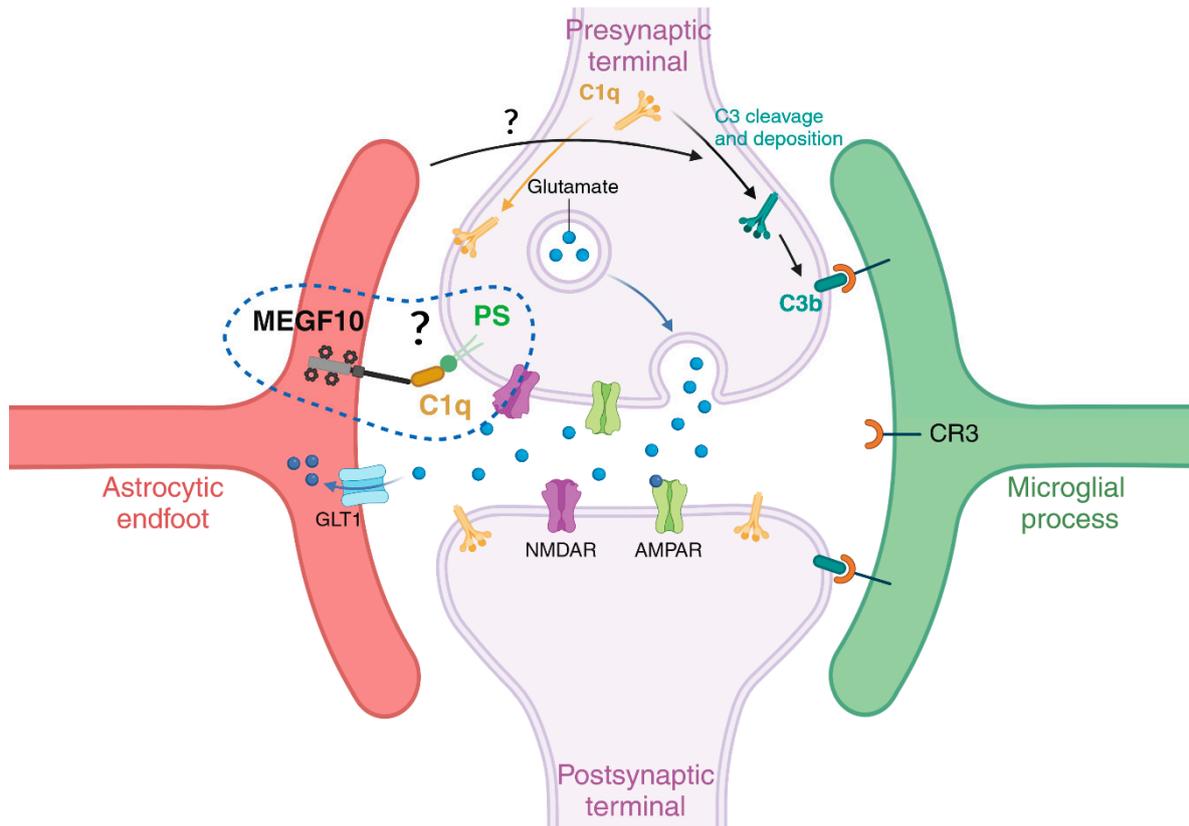
frequency of spontaneous EPSCs in slices from the mPFC and hippocampus and suggesting a reversal of the system to a functionally immature phenotype [127]. Furthermore, early social isolation in rodents induces an astrocytic GR-dependent activation of MERTK pathway, ultimately leading to an excessive phagocytosis of excitatory synapses and consequent unbalanced neuronal firing patterns [128]. Strikingly, a selectively lower expression of the astrocyte-specific protein Cx30 characterizes postmortem brain tissues of patients with major depressive disorder (MDD) [129], supporting a link between the expression of cell-type specific markers reminiscent of a critical period-like brain state and CNS pathologies. Furthermore, worth to mention is the association of several mental disorders with a reduced gyrification index (GI) in cortical areas, measured by magnetic resonance imaging (MRI) in patients, also suggestive of hypothetical deficits in astrocyte proliferation affecting cortical formation being behind certain brain diseases ([130–132] and reviewed in [133,134]).

Several studies have revealed astrocyte deficiencies in other brain disorders besides MDD, such as SCZ, bipolar disorder (BD), ASD and epilepsy [135–144]. In young MDD patients, postmortem brains present a profound reduction in the number and morphology of GFAP- and S100 $\beta$ -positive astrocytes in the prefrontal and anterior cingulate cortices and in the hippocampus, all regions heavily affected in MDD [135,138,145–148]. To validate this, it was also shown that antidepressants target astrocytes and reverse disease symptoms [136,149–151]. It remains to be clarified, however, which subtype(s) of astrocytes are affected, as far as currently available markers cannot discriminate among interlaminar, varicose projection or protoplasmic astrocytes. Conversely, in SCZ or BD, changes in astrocytes are less consistent, with different studies showing both their increased and decreased numbers in postmortem tissue of SCZ patients [140,141]. The most reproduced findings show a hypofunction of NMDA receptors (NMDAR) in SCZ. Astrocytes enhance NMDAR activity through the release of gliotransmitters, such as D-serine, which acts as co-agonist of glutamate [152]. The binding of D-serine to synaptic NMDARs additionally modulate the induction of long-term potentiation (LTP), the latter being the cellular correlate of memory formation [153]. Memory deficits are among the most common symptoms of various ND and NP disorders, thereby supporting an involvement of gliotransmission in their etiopathogenesis. In line with this hypothesis, a disrupted NMDAR function may also rely on an imbalanced postsynaptic AMPA receptor composition or missed AMPA receptor maturation due to an altered astrocyte-dependent glypican secretion or chordin-like secretion, respectively [90,154]. Related to this, the work of Caldwell and colleagues recently showed how an aberrant secretion of astrocyte-derived proteins is associated with severe ND disorders accompanied by an altered neuronal development [155]. Indeed, recent clinical work has proposed D-serine as a promising therapeutic option for the treatment of ND disorders, most notably SCZ [156,157]. Moreover, it has further been shown that mutations in Disrupted-In-Schizophrenia 1 (Disc1) reduce the stability of the D-serine synthesizing enzyme serine racemase, resulting in SCZ-like behavior [158]. Thus, in SCZ, not the number but rather the functionality of astrocyte seems to be mostly affected and influence disease onset and/or progression, principally driven by genetic factors. This might explain the inconsistent results from postmortem brain tissues, which did not show reproducible deficiencies in astrocyte counts [140,141]. Despite SCZ having been considered a disorder with a high genetic component, in reality the concordance rate among identical twins is only about 50% [159], with a substantial proportion of SCZ being of idiopathic origin. More interestingly, cortisol levels, an index of stress, measured in adolescents at-risk for SCZ were significantly elevated in subjects who transitioned to psychosis later in life, further supporting the negative impact of early life environmental factors such as stress for worsening NP disorders when encountered during critical periods of postnatal development [160,161]. Although a defective neurogenesis during the critical period has been long described in SCZ and associated with its etiopathogenesis [162], recent work has elucidated the putative role of astrocytes and their aberrant maturation paths impacting synapse formation during critical periods as a possible cause of disrupted neuronal circuit formation and disease onset [142,163]. In ASD, astrocyte involvement in its etiology has been recently investigated using patient-derived iPSCs [144,164]. Even in ASD, the essential role of the critical period has been proposed, with a shortened time frame of neuroplasticity indicated as a common cause of disease among various patients, despite their highly divergent

genetic backgrounds. In particular, the language and social skill deficits observed in ASD have been led back to a premature closure of the critical period for their development, which should typically extend into adolescence. The hypothesis is that a shorter critical period may force the brain to rely on underdeveloped neuronal networks for learning language and social skills, among other mental abilities, leading to the typical symptoms observed in ASD patients [165]. However, as of today, no work has thoroughly investigated the possibility that an earlier closure of critical periods, marked by the premature differentiation of astrocytes, might be among the neurobiological underpinnings in the etiopathogenesis of ASD. In the same line, no work has ever considered a failure in the closure of this period among possible causes for other mental disorders such as depression (Figures 2 and 3).



**Figure 2. The role of critical period for the onset of neurodevelopmental and neuropsychiatric disorders.** Intrinsic molecular programmes and environmental factors may interact with each other during the critical period of brain development to drive the proper formation of neuronal circuits. Any alterations in the sequences of events occurring during these time frames might lead to the onset of neurodevelopmental (ND) disorders such as Autism Spectrum Disorder (ASD) or neuropsychiatric (NP) disorders such as Schizophrenia (SCZ) or Major depressive disorder (MDD). Stressful triggers, such as early-life adversity (ELA), chronic social defeat stress or early social isolation, experienced during the critical period show a higher impact on the development of ND and NP disorders later in life. Molecules like Cx30 may play a role in the closure of the critical period of astrocyte development, which is necessary for the proper formation of brain circuits. Figure created with BioRender.com.



**Figure 3. Glia-mediated synaptic pruning.** Astrocytes and microglia cells both regulate the refinement of synaptic neuronal networks through the elimination of weak synapses during early postnatal developmental stages. Signalling molecules called “eat me” signals, such as C1q and phosphatidylserine (PS), recruited during these events have been identified, as well as binding partners located on the respective cells, like MEGF10 receptor on astrocytes and the receptors of the complement cascade on microglia cells. It remains unclear whether PS and C1q are directly implicated in the astrocyte-mediated synaptic elimination as well. Figure created with BioRender.com.

### Astrocytes and the Synaptic Compartments: The Relevance of an Astrocyte-Mediated Phagocytosis for Neuronal Circuit Refinement in Health and Disease

In the CNS, the peculiar position of astrocytes between synaptic and vascular compartments supports the formation of a functional unit (neurovascular, NVU) “sensing” the brain state and secreting factors in the bloodstream as a reflection of this state. In pathological conditions, such factors may serve as biomarkers of cellular (dys)functions and help to improve diagnostic/treatment options specifically adapted to the needs of individual patients. Several excellent reviews have already described secreted astrocyte-derived molecules relevant for synapse and BBB properties in health and disease [5,6,10,12,13,166–169]. We also validated and characterized astrocyte and BBB deficits in an animal model of MDD [168,170] and identified the astrocyte-derived factor GDF15 as an effector of fluoxetine to simultaneously restore the disrupted astrocyte processes and loosened tight junctions between endothelial cells [171]. Ideally, identifying cell-type specific molecular underpinnings of disease may support the discovery of equally specific diagnostic biomarkers. However, ND and NP disorders often share overlapping symptoms and underlying neurobiological alterations, making it difficult to isolate disease-specific biomarkers for personalized medicine. Therefore, only a combination of efforts and methodological approaches may offer valid guidance to reach this ambitious goal. Among less explored functions of astrocytes, we focus here on astrocyte-mediated synaptic phagocytosis during critical periods of brain development and its putative involvement in NP and ND disorders.

Astrocytes derive from the embryonic ectodermal sheet and therefore share some developmental, genetic and functional similarities with other ectodermally-derived cells, such as

neurons and oligodendrocytes, but less with other brain cells of mesodermal origin, such as microglia cells [172]. However, they might adopt analogous functions to microglia cells to cooperatively converge their efforts in the regulation of neuronal networks formation [96,173].

Together with microglia, astrocytes contribute to the formation/function of excitatory and inhibitory synapses and to the elimination of weak synapses (pruning) through released factors or membrane-bound molecules [51,169,173,174].

Early studies in the fly *Drosophila melanogaster* opened the field of glia-mediated phagocytosis, showing how this process is highly conserved across species, even when species-specific cellular and molecular mechanisms diverged along evolution to adapt to the growing complexity of selected functions [44]. In *Drosophila*, it was shown that, during its larvae-to-pupa metamorphosis, a high degree of tissue digestion occurs to reshape its body structures and nervous system, allowing the subsequent extension of adult, mature, neuronal projections [175]. The sequence of events leading to such drastic changes follows a developmentally regulated pattern, initiated by complex interactions between intrinsic and extrinsic factors, such as the neuronal hormone ecdysone. Specifically, the gene products of Draper and CED-6 were first identified to mediate the engulfment of pruned axons by glia cells during metamorphosis [176]. Accordingly, mutations in these two genes and glia cell-targeted knockdown experiments, suppressed glia-mediated axon pruning during metamorphosis. Genetic studies in *Caenorhabditis elegans* initially isolated the genes encoding for CED-1 and CED-6 as also essential mediators for the clearance of apoptotic cells [177].

In the mouse brain, the seminal work of Stevens [178] and Chung [174] has been key to underscore novel roles for the classical complement cascade and the MEGF10/MERTK proteins, respectively, for the refinement of neuronal synaptic networks during postnatal developmental periods. Initially, microglia were described as key players to refine synapses by engulfing presynaptic inputs during the peak retinogeniculate pruning *via* the microglial CR3/C3 phagocytic signalling pathway and under the regulatory influence of neuronal activity [179]. The involvement of microglia in synaptic pruning was simultaneously uncovered in the hippocampus and juvenile visual cortex, where the synaptic remodelling occurs through the fractalkine/CXC3CR1 signalling pathway [180]. Afterwards, numerous studies and comprehensive reviews have extensively examined the crucial role of microglia cells in the process of synapse elimination and in disease states [181–189]. Later, an additional involvement of the astrocytic MEGF10 and MERTK was highlighted for the synaptic remodelling during postnatal brain development [174] and the maintenance of hippocampal homeostasis in the adult brain [190]. Microglia and astrocytes collaborate through a synergistic and precisely orchestrated spatiotemporal coordination, ensuring efficient homeostatic phagocytosis of apoptotic cells and synaptic pruning, while respecting each other's territorial boundaries and competences: microglia exhibit an engulfing preference for large cell bodies, whereas astrocytes favour small dendritic apoptotic bodies [191]. Moreover, Lee and colleagues demonstrated how astrocytes and microglia constantly engage in phagocytosis of both excitatory and inhibitory synaptic elements within the hippocampal CA1, preferring excitatory rather than inhibitory terminals [190]. Similarly, Dejanovic and colleagues observed that astrocytes preferentially engulf excitatory synapses, while microglia tend to target inhibitory ones, revealing a divergence between astrocytes and microglia during (patho)physiological processes ([181] and reviewed in [169]). A recent study has identified a unique GABA-receptive microglia subtype that selectively remodels inhibitory synapses during mouse postnatal cortical development [182]. Furthermore, the MERTK-mediated microglial phagocytosis is crucial for eliminating inhibitory post-synapses in the juvenile brain [183]. Elucidating the complex interplay between microglia and astrocyte regulatory roles in synapse elimination is the current crucial focus of many research labs. Regarding the molecular mechanisms driving these events, the current view postulates that a balanced distribution of “eat-me signals” and “don't eat me signals” is key to determine which cells (or parts of them) should be excised/phagocytosed [51]. Among “eat-me signals”, the C1q has been described multiple times as a common tagging system shared by microglia cells and astrocytes to identify their targets. For instance, MEGF10 can mediate its phagocytic functions on apoptotic material via the recognition of C1q bound to phosphatidylserine (PS) exposed on dying cells [109]. This suggests that regulatory

mechanisms controlling C1q expression/tagging and/or its binding partners may be responsible for sorting out which cell type between microglia cells and astrocytes should be recruited to execute the job. Because of the commonly shared target(s), it has also been proposed that the selective mechanism determining which cell type acts first may rely on the specific set of receptors localized on their membranes, possibly activated alone or in combination with other molecules “on demand” [96].

From the literature, the current hypothesis is that astrocytes stimulate neurons to produce C1q, which then triggers the downstream activation of the complement cascade and C3b deposition, finally promoting synapse elimination through its microglia-specific binding partner C3 receptor (CR3) [178,179] (Figure 3). Another extensively studied “eat-me signal” in the microglia-mediated synaptic elimination is represented by PS exposure on neuronal membranes. The PS presentation on weak synapses promotes microglia-mediated synaptic pruning via the TREM2 receptor and C1q in the developing hippocampus and retinogeniculate system [184]. Beside these positive mediators of microglial engulfment, “don’t eat me signals” also modulate synaptic pruning. These signals counterbalance the effects of “eat me signals”, preventing the engulfment of viable synapses by aberrant microglial removal. Among them, CD47 was first characterized as “don’t eat me signal” because it permitted glioma cells to infiltrate the healthy brain parenchyma, after blocking their macrophage-mediated engulfment [192]. Its alternative function in synaptic pruning was uncovered later, with CD47 expressed on neuronal membranes and interacting with the microglial receptor SIRP $\alpha$  being responsible for suppressing synaptic phagocytosis. Mice lacking CD47 exhibit excess synaptic pruning and decrease synaptic connectivity [193], whereas its increased expression was found in iPSCs derived from autistic patients with enlarged brains accompanied by reduced cellular phagocytosis [194]. However, in contrast to the extensive literature describing the mechanisms of a microglia-mediated synaptic pruning in health and disease [178,179,195,196], those instructing the astrocyte-mediated synaptic elimination are still less known [96,128,173,174,190] (Figure 3).

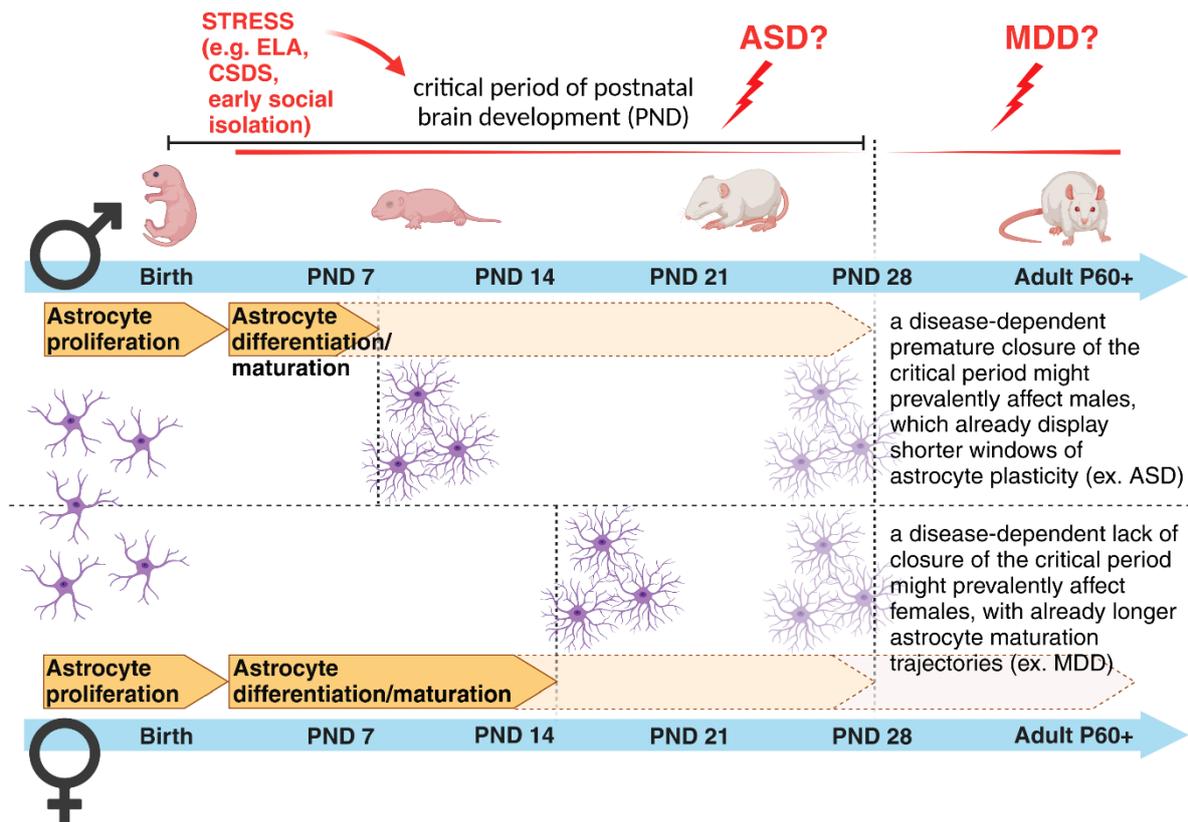
A reduced proliferation of astrocytes and/or disruption of astrocytic activity characteristic of ND and NP disorders may affect the appropriate formation of synaptic circuits in different ways. Their impaired activity in pathological conditions may for example either weaken mature synapses or prevent their maturation, thereby potentially enhancing their susceptibility to an excessive pruning. Alternatively, an aberrant astrocyte activity may negatively influence the synaptic tagging, leading to either an excessive or abrupt elimination of unwanted synapses. These possibilities further sustain the importance of the critical developmental periods, when astrocyte proliferation/maturation and synaptogenesis take place simultaneously, in sculpting brain circuits in health and disease.

### **Sex-Dependent Differences in Astrogenesis and in Time Frames of Vulnerability to Perturbations and Disease Onset**

Sex differences have long been recognized as a variable affecting many brain disorders in terms of predisposition, rates of incidence, age of onset, symptomatology and outcome, with skewed prevalence towards one sex in different pathologies [111,119,197–199]. However, research on sex-dependent disorders and their neurobiological molecular and cellular causes is still scarce, thereby limiting the development of sex-specific diagnoses and treatments.

In the past, many human studies have often lacked sufficient sample availability to stratify results based on sex differences for any given disease category. Nowadays, the access to huge genomic, transcriptomic and proteomic datasets help to discriminate the impact of sex variables on various biological parameters [200–203]. However, our understanding of whether and how sex might affect astrogenesis, synaptogenesis and synapse elimination in humans and to which extent this contributes to the onset of brain diseases is still limited [204]. Currently, the overall idea is that mental conditions with early-onset neurodevelopmental origin, such as ASD, attention deficit/hyperactivity disorder and SCZ show a higher male prevalence. On the other hand, disorders with a higher emotional component, such as depression, anxiety disorder, and eating disorders, which usually start during puberty or later in life, show a higher female incidence [205,206]. This asymmetric development of psychopathologies has been analyzed across studies with the goal of elucidating how

and which sex-specific developmental maturation trajectories might influence it. Interestingly, regional differences in volume and tissue density were found between sexes in areas implicated in sex-biased NP conditions, suggesting a few candidate regions susceptible to sex differences in the developing brain [207]. Among those regions, the cortex has been implicated as one of the brain areas with a high sex-dependent diversification in cellular and synaptic densities [99,111,119,208]. Most of these differences have been attributed to remodelling processes occurring at the synaptic level, with an aberrant pruning of weaker synapses being recently regarded as a mechanism relevant for the onset of brain disorders [110,111]. As previously mentioned, most of these remodelling events take place during the critical periods of brain development, when astrogenesis in parallel with synaptogenesis contribute to the formation and refinement of neuronal circuits [54,78,79,209]. Mechanisms controlling the opening and closure of these developmental time windows might be essential in building healthy neuronal networks and establishing appropriate behaviours in adult life. Recent work has highlighted sex differences in the maturation processes of astrocytes during early postnatal developmental stages, showing that astrocytes in male mice reach a mature state earlier than in female mice [210]. Sex-dependent maturational trajectories are influenced by the perinatal surge in testosterone aimed at masculinizing the brain after birth and establishing sexually dimorphic brain circuitries responsible for sex-differentiated behaviours and reproductive processes [211]. Astroglia respond to circulating gonadal hormones [212], which influence their relative sex-dependent maturation rates [210]. These may in turn correlate with sex-dependent closure times for temporal windows of high plasticity necessary for astrocyte-mediated modulation of neuronal circuit development. It would be highly interesting to investigate whether in ND diseases, for example, a further earlier closure of the critical period, accompanied by a putative premature differentiation of astrocytes, may account for the observed prevalence of disorders such as ASD in male individuals. Although this hypothesis has not yet been tested, we may speculate that a shorter time window for astrocyte maturation in males might make them more vulnerable to diseases affecting the astrocyte-dependent early-onset synaptic changes. This would explain some phenotypic effects seen in ASD. In such a case, there would be a narrower time frame for male individuals carrying those affected cells to recover from (or adapt to) detrimental environmental challenges occurring during this period and to rescue impaired neuronal circuits (Figure 4). Correspondingly, slower astrocyte maturation time frames in females might trigger the onset of female-biased disorders eventually characterized by an inappropriately longer immature brain state. Similar considerations can be true for other pathologies for which a sexual dimorphism has been already recognized, opening alternative lines for their investigations [213] (Figure 4).



**Figure 4. Sex-dependent differences in astrogenesis and vulnerability to disease onset.** A sex-dependent predisposition to brain disorders has been long recognized for many brain pathologies. Differential time-shifted maturation trajectories observed in astrocytes and astrocyte-mediated processes between males and females may account for such sex-dependent biased windows of vulnerability to disease onset. Figure created with BioRender.com.

## Conclusions

In conclusion, we propose astrocytes as critical novel targets for the development of efficacious medical treatments for NP and ND disorders. These disorders could potentially result from aberrant opening/closure of critical developmental periods, accompanied by defective astrocyte proliferation/maturation processes either affecting synapse formation and gyrification and/or destabilizing neuronal circuits with E/I imbalances and/or impairing an astrocyte-dependent synapse elimination. These hypothesis point out the urge of intensifying research efforts to identify the molecular drivers of such processes and propose astrocyte-based therapeutic approaches to reverse disease phenotypes. Moreover, specific attention to early postnatal brain developmental periods, in combination with the study of sex-specific differential maturation patterns, may improve our knowledge regarding physiological and pathological processes taking place during these time spans and develop treatments personalized to the needs of female and male patients.

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