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$GABA_A$ Receptor and Glycine Receptor Activation by Paracrine/Autocrine Release of Endogenous Agonists: More Than a Simple Communication Pathway

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Abstract It is a common and widely accepted assumption that glycine and GABA are the main inhibitory transmitters in the central nervous system (CNS). But, in the past 20 years, several studies have clearly demonstrated that these amino acids can also be excitatory in the immature central nervous system. In addition, it is now established that both GABA receptors (GABARs) and glycine receptors (GlyRs) can be located extrasynaptically and can be activated by paracrine release of endogenous agonists, such as GABA, glycine, and taurine. Recently, non-synaptic release of GABA, glycine, and taurine gained further attention with increasing evidence suggesting a developmental role of these neurotransmitters in neuronal network formation before and during synaptogenesis. This review summarizes recent knowledge about the non-synaptic activation of $GABA_A$Rs and GlyRs, both in developing and adult CNS. We first present studies that reveal the functional specialization of both non-synaptic $GABA_A$Rs and GlyRs and we discuss the neuronal versus non-neuronal origin of the paracrine release of $GABA_A$R and GlyR agonists. We then discuss the proposed non-synaptic release mechanisms and/or pathways for GABA, glycine, and taurine. Finally, we summarize recent data about the various roles of non-synaptic GABAergic and glycinergic systems during the development of neuronal networks and in the adult.

Keywords Tonic release · Non-vesicular · GABA · Glycine · Taurine · Gliotransmitter · Volume-sensitive channels · Development · Progenitor · Astrocyte · Neuron

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Introduction

Early and late development of the central nervous system (CNS) not only requires an accurate sequential activation of a genetic program but also depends on epigenetic signaling at specific developmental time windows. Our view of cell-to-cell communication involved in signal transmission has changed dramatically over the past few years. In addition to growth factors, there is increasing evidence that neurotransmitter systems, known to be involved in fast neurotransmission, also play a central role during CNS development as epigenetic signaling processes. During the last decade, several studies have uncovered evidence that neurotransmitter release occurs before synaptogenesis and that many receptor subtypes,

usually involved in synaptic transmission, can be expressed by developing neurons devoid of functional synapses or by non-neuronal cells. It is now well established that several neurotransmitters can be non-synaptically released via vesicular-dependent or -independent mechanisms [1–5]. From a functional point of view, non-synaptic neurotransmitter release or volume transmission (vesicular or not) can be referred to paracrine release mechanisms and/or to autocrine release mechanisms. Paracrine release refers to non-synaptic release of neurotransmitters that diffuse slowly, allowing basal and tonic activation of extrasynaptic receptors on neighboring cells. In paracrine release mechanisms, the releasing cell is insensitive to the neurotransmitter released. Conversely, in autocrine release mechanisms, only the releasing cell is affected. Both mechanisms can occur simultaneously as described for GABA release from polysialylated neural cell adhesion molecule-positive precursor cells [6]. Paracrine and autocrine release mechanisms must be dissociated from the neurotransmitter spillover described at many synapses. Spillover is characterized by a limited diffusion of the agonist from the release site. Spillover refers to neurotransmitters escaping from the synaptic cleft allowing the activation of perisynaptic receptors. Neurotransmitter diffusion in spillover mechanisms is restricted to the perisynaptic area due to the presence of membrane transporters near the synaptic boutons.

Because such non-synaptic release is a hallmark of the immature CNS and is also observed in the adult CNS, much effort has recently been devoted to characterizing the release mechanisms, to determining the cellular origin of this neurotransmitter release and to exploring their functions. Accordingly, several studies suggest that these neurotransmitters can behave as growth regulators during specific developmental periods by acting on neuronal migration, neuronal differentiation, axonal growth, and synapse formation, as well as neural progenitor proliferation and differentiation [1, 4, 7].

It is now well established that both GABA and glycine are excitatory in the immature CNS and are directly involved in the generation and regulation of electrical activity generated by developing neuronal networks [8–13]. Both $GABA_A$ receptors ($GABA_A$Rs) and glycine receptors (GlyRs) are chloride-permeable receptor channels, and their excitatory function in the immature CNS is directly related to a depolarized chloride equilibrium potential, mainly due to the delayed developmental expression of the chloride transporter KCC2 [10, 14, 15] and/or the developmental inactivation of the chloride transporter NKCC1 [16].

Paracrine release of GABA, glycine and taurine occurs before or at the onset of synapse formation during CNS development [9, 17, 18]. In recent years, non-synaptic activation of $GABA_A$Rs and GlyRs has gained increasing interest with the discovery of their possible involvement in neural development processes, including progenitor cell proliferation, neuronal migration, neurite extension, and synaptogenesis. All these observations led to the proposal that non-synaptic activation of $GABA_A$Rs and GlyRs is important for the harmonious development of the CNS. There is also increasing evidence that, in addition to taurine, both GABA and glycine can be released in the adult by glial cells via non-synaptic mechanisms, in both normal and pathological conditions.

This review summarizes recent knowledge on the function of non-synaptic activation of $GABA_A$Rs and GlyRs, both in the developing CNS and in the adult. We first discuss recent studies that reveal functional specialization for both non-synaptic $GABA_A$Rs and GlyRs. Second, we explore the possibility that non-synaptic release of $GABA_A$R and GlyR agonists (namely GABA, glycine, and taurine) can have both a neuronal and a non-neuronal origin. Third, we discuss data on different possible non-synaptic release mechanisms or release pathways. Fourth, we summarize recent knowledge of the various functions of non-synaptic GABAergic and glycinergic systems during the development of neuronal networks and in the adult.

Extrasynaptic GlyR and $GABA_A$R Subtypes Have Particular Subunit Combinations That Confer Functional Properties Adapted to Slow Neurotransmitter Release

GlyRs and $GABA_A$Rs belong together with nicotinic acetylcholine and 5-hydroxytryptamine type 3 receptors to the cysteine-loop receptor family [19]. GlyRs and $GABA_A$Rs are pentameric assemblies of subunits, each subunit being characterized by extracellular N- and C-terminals and by four transmembrane domains (TM1–TM4), the TM2 domain forming the anionic channel pore. GlyRs are composed of α (α1–α4) and β subunits which assemble to form α(2)β(3) heteromeric or α(5) homomeric receptors [20]. The agonist binding site is mainly carried by the α subunit [19]. Two highly conserved, oppositely charged residues located on adjacent α subunit interfaces, being also present on adjacent α/β subunit interfaces, are crucial for agonist binding [20]. In addition, the β subunit binds the anchoring protein gephyrin with high affinity [21]. Gephyrin belongs to the postsynaptic protein scaffold complex and the binding of the GlyR β subunit to gephyrin allows the receptor to accumulate at postsynaptic sites [19, 22]. GlyR subunit expression is developmentally regulated [19]. The α2 subunit is already detected at the onset of synaptogenesis in the developing CNS. In the embryo, α2 mRNA is expressed in the spinal cord and the brainstem [23, 24]. It is also detected at high densities in the embryonic cortex, the thalamus, and the hippocampus [24]. On the other hand, α2 subunit mRNA is still present, but at reduced densities, in the adult rat CNS [25] (see below). The expression of β, α1, and α3 subunits

progressively increases during the first postnatal weeks, while the expression of the α2 subunit declines to the basal level during the same period. Hence, it is assumed that immature GlyRs are homomeric α2 receptors and are progressively replaced by α2/β heteromeric GlyRs and then by α1/β and α3/β heteromeric GlyRs. This developmental pattern has been determined in the spinal cord and in several brainstem nuclei of rodents [26–28].

GlyR subunit diversity is increased by alternative splicing of the α1–3 subunits (α1 and α1insert; α2A and α2B; α3K and α3L) [19, 22]. In the adult, GlyR subunits are differentially expressed depending on the SNC area, leading to GlyR subtype heterogeneity. Heteromeric α1/β GlyRs are predominant and uniformly expressed in the CNS, while the expression of the α2 subunit in the adult has been mainly detected in the cortex, the olfactory bulb, the thalamus, the central gray matter, the retina, and the hippocampus [26, 29]. The expression of the α3 subunit is restricted to the ventral horn of the spinal cord, the cerebellum, the olfactory bulb, and the retina [26]. Finally, GlyRs containing α4 subunit are poorly expressed in the mammalian CNS, the α4 subunit expression being mainly restricted to the inner plexiform layer of the retina [30].

Ionotropic GABARs are composed of a large variety of different subunits. Three $GABA_CR$ subunits (ρ1–3) and 16 $GABA_ARs$ subunits have been cloned so far (α1–6, β1–3, γ1–3, δ, ε, θ, π) [31, 32]. $GABA_CR$ subunits do not co-assemble with $GABA_AR$ subunits [33, 34]. $GABA_CR$ expression is restricted to distinct brain structures including mostly the retina but also the visual cortex, the optic layers of the superior colliculus, the hippocampus, the cerebellum, and the spinal cord [33, 35, 36]. $GABA_CR$ subunit expression is also developmentally regulated [37]. In vivo, the expression of the ρ2 mRNA in the superior colliculus and of the ρ2/ρ3 mRNA in the retina increases with age. In contrast, the expression of the ρ2 mRNA in the visual cortex and of the ρ1 mRNA in the retina and superior colliculus remains constant with age [37]. In the hippocampus, both subunits are expressed with maximum staining intensity at P7 [35]. In the spinal cord, the ρ2 signal is stronger than ρ1 and appears largely in motoneurons, while at P7 and in the adult interneurons and motoneurons are similarly labeled [35].

As for GlyRs, the diversity of $GABA_AR$ subunits is increased by alternative splicing [32]. Despite this subunit diversity, only a dozen subunit combinations have been detected so far [32]. The most abundantly expressed $GABA_ARs$ in the adult CNS have a stoichiometry of two α1 subunits, two β subunits, and one γ subunit [38, 39]. As for GlyRs, the agonist-binding site is carried mainly by the α subunits, while the γ subunits are responsible for linking $GABA_ARs$ to the postsynaptic cytoskeleton. Like GlyRs, $GABA_AR$ subtype expression varies with CNS area [40–44]. In the adult brain, the α1β2γ2 combination is widely expressed [45, 46]. Most other subunits are more rarely expressed and/or have a restricted regional distribution. For example, the α4 subunit is highly expressed in layers II and III of the cortex, in the pyriform cortex in the hippocampus, in the thalamus, and in the hypothalamus [43], the α5 subunit is highly expressed in the granule cell of the olfactory bulb, in the hippocampus, and in the spinal trigeminal nucleus [41, 43], while the α6 subunit is predominantly expressed in hippocampal pyramidal cells [40] and in the cerebellar granule cells [43]. α2, α3, β3, and γ2 subunits are the main $GABA_AR$ subunits expressed in the spinal cord, α1, α4 α5, β1, β2, γ1, and γ3 being poorly expressed [42]. Developmental expression of $GABA_AR$ subunits follows different patterns in the brain and spinal cord [42, 47–49]. The α2, α3, and β3 subunits are likely to compose the immature form of $GABA_AR$ in the brain since they are predominantly expressed in the fetal brain and their expression declines after birth [44]. In the embryonic rat spinal cord, $GABA_AR$ subunit mRNAs exhibit three different patterns [42]. α4, β1, and γ1 subunit expression is restricted to progenitors located in the ventricular zone. They are first detected at embryonic day 13 (E13) and their expression declines after E17, α4, β1, and γ1 mRNAs being barely detected in the adult. Because of the location of these $GABA_AR$ subunits in the progenitor zone, it is supposed that these subunits can play roles in proliferation, differentiation, and migration of progenitor cells [42]. α5, β2, and γ3 mRNAs are also expressed transiently during development. They are first detected at E13, but in the mantle zone, and are almost absent in the adult. By contrast, α2, α3, β3, and γ2 mRNAs are first detected between E12 and E13, and their expression persists in the adult [42].

All postsynaptic GlyRs and $GABA_ARs$ have fast activation and deactivation kinetics allowing these receptors to respond to synaptic vesicular release on the millisecond time scale [22, 32]. This feature is essential because at central synapses the synaptic cleft is small and characterized by fast neurotransmitter clearance that can be enhanced by specific membrane transporters, with the life-time of the neurotransmitter within the synaptic clef being <1 ms [50].

During recent years, several studies have shown that extrasynaptic GlyRs and $GABA_ARs$ have different intrinsic functional properties that differ from those of postsynaptic receptors [22, 32], allowing them to respond to slow neurotransmitter release, as observed for paracrine release. These functional differences between synaptic and extrasynaptic receptors may be explained by their particular subunit combination, this feature being specific to their membrane location. A particularity of these extrasynaptic receptors is that they cannot accumulate at postsynaptic sites because they cannot anchor to the postsynaptic scaffold protein complex. Accordingly, one can speak of truly extrasynaptic receptors in contrast to other GlyRs and $GABA_ARs$ that can temporally escape from the synaptic cleft by lateral diffusion. For

example, homomeric GlyRs composed of five α subunits, and therefore lacking the β subunit, cannot interact with gephyrin and, hence, cannot accumulate within the synaptic cleft [22]. Remarkably, the first GlyR subtype expressed during development in the embryo is a homomeric GlyR composed of five α2A subunits, which displays biophysical activation properties that strongly differ from those of GlyRs composed of α1, α2B, or α3 subunits, with or without the β subunit [51, 52]. α2A homomeric GlyRs display very slow activation kinetics and slow desensitization kinetics, which are not compatible with fast synaptic vesicular release, but are rather well adapted to slow non-vesicular release and to the continuous presence of the agonist in the extracellular space [9, 52]. Similarly, extrasynaptic $GABA_A$Rs have activation kinetics related to subunit combinations that differ from postsynaptic $GABA_A$R subtypes (αβγ $GABA_A$Rs). Like α2A homomeric GlyRs, $GABA_A$Rs containing the δ subunit (αβδ $GABA_A$R) cannot accumulate at postsynaptic sites and are exclusively extrasynaptic [53]. In addition, receptors that contain the α5 but not the δ subunit (α5βxγ2) are also predominantly extrasynaptic [32]. In hippocampal pyramidal cells, the α5 subunit shows diffuse surface labeling without detectable synaptic clustering [54, 55] and the presence of the α5 subunit seems to alter the ability of the γ2 subunit to promote receptor co-localization with gephyrin [54, 55].

Extrasynaptic αβδ $GABA_A$Rs have a higher apparent affinity for GABA and desensitize more slowly and less extensively than postsynaptic $GABA_A$Rs [56], while $GABA_A$Rs containing the α5 subunit display a reduction in their desensitization kinetics when compared with receptors containing other α subunits [57]. As a consequence, extrasynaptic $GABA_A$Rs, like extrasynaptic GlyRs, remain functional when exposed to long-lasting presence of their agonist in the extracellular space. Accordingly, the presence of these ligand-gated ionotropic receptor subtypes with kinetic properties more adapted to a paracrine neurotransmitter release reveals a functional adaptation with important implications for the relevance of non-synaptic release of GlyR and $GABA_A$R agonists on target cells.

Cellular Origins of Non-synaptic Release of $GABA_A$R and GlyR Agonists: Mechanism of Uptake and Biosynthesis

GABA

Slow activation of $GABA_A$Rs has been extensively studied and recently reviewed by Farrant and Nusser [32] and by Koch and Magnusson [58]. Paracrine–autocrine release of $GABA_A$R agonists was first characterized as spillover of GABA from synapses in response to high presynaptic activity. This spillover slowly activates perisynaptic $GABA_A$Rs and reinforces the inhibitory GABAergic transmission and the retrograde vesicular GABA release from dendrites, thereby regulating the activity of presynaptic excitatory and inhibitory terminals (see for review [58]). More generally, tonic activation of $GABA_A$Rs occurs as early as the embryonic stages before and during synaptogenesis [59], and has also been observed at more mature stages in thalamocortical neurons [60], in the hippocampal dentate gyrus [61], in cerebellar granule cells [62], in pyramidal neurons from the fifth layer of the somatosensory cortex [63], and in pyramidal cells and interneurons in the CA1 region of the hippocampus [64, 65]. Except for the spillover described above, the cellular origin of the paracrine release of GABA in the CNS remains controversial. There is, however, increasing evidence that slow GABA release originates from non-neuronal cells. For example, it has recently been shown that individual glial cells can release enough GABA to produce inhibition of nearby neurons in the somatosensory cortex [66].

Although the presence of GABA has been detected in glial cells, it was first assumed to reflect GABA uptake and that the main function of glial cells was to maintain a low level of GABA in the extracellular space. Glial cells express different membrane GABA transporters (GAT-1 and GAT-3) [67], as well as GABA transaminase (GABA-T), which catabolizes GABA [68]. This rather restrictive view was challenged by studies using immunohistochemistry, in situ hybridization, and biochemistry which have shown that, in addition to GABA, the GABA synthesis machinery (GADs) is also present in glial cells and in progenitors [69–72]. Moreover, GABA, GABA-T, or GADs were also found in tissues outside the SNC, including blood, blood vessels, spleen, heart, skeletal muscle, gastrointestinal tract, liver, pancreas, kidney, urinary bladder, male and female reproductive organs, lung, pituitary, thyroid, adrenal gland, thymus, salivary gland, skin (see for review [73]), and in the developing lens of mouse embryos [74].

Two different pathways of GABA biosynthesis exist in the brain. The first involves the activity of GAD enzymes, which catalyze the formation of GABA from glutamate, while a secondary synthetic pathway originates from putrescine metabolism (Fig. 1).

Mammalian species express two isoforms of GAD: GAD65 and GAD67. These are the products of two different genes: the GAD65 gene (GAD2) is located on chromosome 10 (10p11.23) in humans and on chromosome 2 (2 9.0 cM) in mice, while GAD67 gene (GAD1) is located on chromosome 2 (2q31) in humans and on chromosome 2 (2 43.0 cM) in mice. In addition, during mouse and rat embryonic development, two additional alternatively spliced forms are synthesized from the GAD67 gene, which encodes the truncated 25-kDa leader (GAD25) and the enzymatically active protein

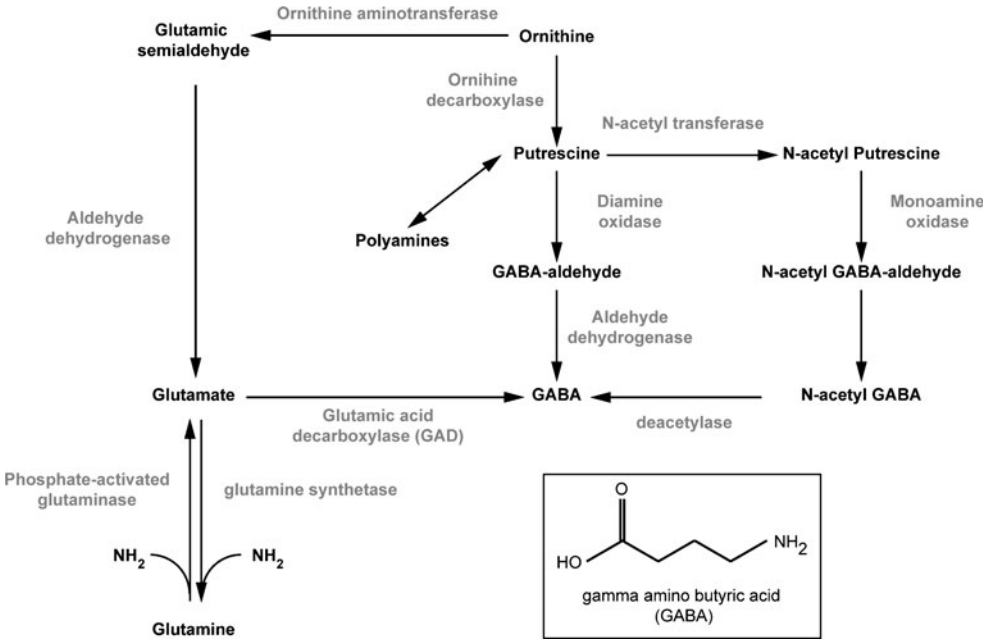

Fig. 1 Multiple biosynthesis pathways of GABA from glutamate (GAD) and from ornithine and putrescine

GAD44. GAD25 is a protein without GAD enzymatic activity and, since this truncated protein corresponds to the amino-terminal regulatory region of GAD67, GAD25 is supposed to have putative regulatory functions. This, however, remains to be determined. GAD25 and GAD44 are transiently expressed during the development of the SNC and are more abundant in proliferating progenitors [75–77]. GAD25 and GAD44 are down-regulated during neuronal differentiation concomitantly with up-regulation of GAD67 expression [78–80]. Accordingly, it has been proposed that GAD44 is predominantly involved in the synthesis of non-synaptic GABA acting as an autocrine and/or paracrine transmitter, while both GAD65 and GAD67 isoforms are assumed to produce GABA to be released by presynaptic terminals [76]. From a functional point of view, GAD44 and GAD67 differ from GAD65. GAD44 and GAD67 are constitutively active due to stable binding of the coenzyme pyridoxal 5′-phosphate (PLP) to these GADs [81], whereas GAD65 oscillates between an active holoenzyme and an inactive apoenzyme [82]. Both GAD67 and GAD65 are synthesized as soluble hydrophilic molecules, but GAD65 undergoes posttranslational hydrophobic modifications, including palmitoylation of cysteines 30 and 45 [83–85]. This results in post-Golgi targeting to cytosolic vesicles in non-neuronal transfected cells [86] and to synaptic vesicles in neurons. A dynamic palmitoylation/depalmitoylation cycle continuously shuttles GAD65 between Golgi membranes and synaptic vesicles, allowing for a rapid regulation of the enzyme level and, hence, of GABA in presynaptic terminals. The subcellular localization of GAD67 strongly varies according to cell type. Although GAD67 remains soluble in transfected non-neuronal cells [87, 88], it can also associate with membranes by heterodimerization with GAD65 [87, 89]. In addition, it has also been reported that GAD67 can anchor to membranes and be targeted to presynaptic terminals in GAD65−/− mice [89, 90]. Kanaani et al. [91] identified in the N-terminal region of GAD67 amino acid residues essential for GAD67 homodimerization and membrane targeting, reinforcing the proposal that GAD67 alone can be targeted to membranes and to synaptic vesicles independently of GAD65.

In the CNS, most GABAergic interneurons express both subtypes of GAD [92, 93], which are simultaneously detectable in the rat brain as early as embryonic (E) day 17 (E17) [94]. GAD67 is found in axonal regions as well as in neuronal cell bodies, whereas GAD65 is mainly associated with synaptic terminals [95] and its expression is restricted to neurons [96]. However, GAD67 was also found on glial cells, indicating that these cells can also metabolize GABA. GAD67 immunostaining was detected in glial cells of the neonatal rat brain, but its expression decreases in older animals, suggesting that GAD67 expression by glial cells could be developmentally regulated [72]. However, both GABA and GAD67 immunoreactivities were found in glial cells of the adult rat cerebellum [71], as well as in the human cerebellar cortex [69], and in astrocytes from adult human brain tissue [70]. Accordingly, these data could indicate that only a subset of glial cells is able to produce GABA, depending on the brain region, the species, and/or the developmental stages.

This image even gains complexity when taking into account the observations that some glial cell subtypes, such as the Müller cells of the retina [68], can contain GABA

without any obvious evidence of GAD expression. Of course, this could result from GABA uptake, but it was also demonstrated that, during neuronal development, cells can contain GABA before the expression of GAD or GAT, as for example in the embryonic retina [97]. It was proposed that this early GABA production results from an alternative synthetic route involving the metabolic pathway for polyamines and the oxidative deamination of putrescine [97]. In the CNS, this alternative pathway for GABA synthesis was first described in the fish brain [98], and then in the mouse brain [99] and in mouse neuroblastoma cells in culture [100]. As illustrated in Fig. 1, there are two possible pathways of GABA synthesis from putrescine. The first pathway involves oxidative deamination of putrescine catalyzed by diamine oxidase (DAO), which leads to the formation of GABA aldehyde. GABA aldehyde is then oxidized by aldehyde dehydrogenase. The second pathway of GABA formation from putrescine involves the acetylation of putrescine by *N*-acetyl transferase and acetyl-coenzyme A. Mono-acetylputrescine is then oxidized by monoamine oxidase into *N*-acetyl-γ-aminobutyraldehyde. A second oxidation step leads to *N*-acetyl-γ-aminobutyrate, which is finally converted to GABA by a deacetylase [73]. The second pathway is the most likely to occur in the SNC since DAO activity has not been detected in the brain [101, 102]. Seiler et al. first proposed a relationship between GABA production in glia and polyamine biosynthetic enzyme activity [102]. Later, Laschet et al. [103] described an accelerated formation of GABA from putrescine in cultured astrocytes obtained from epileptic mouse brain, suggesting that this GABA synthesis pathway may have particular functions in pathological conditions. In addition, the formation of GABA from putrescine could reflect an alternative GABA synthesis pathway in immature cells during SNC development. Indeed, neuroblasts of the subventricular zone synthesize GABA from putrescine [104], while GABA was also detected in cultured O2A glial progenitors of the optic nerve [105]. However, most of the experiments described above were performed in vitro and the production of GABA from putrescine in vivo remains poorly documented. It should also be pointed out that some progenitors can express GAD. PSA-NCAM+ (neurogenic polysialylated neuronal cell adhesion molecule-positive) precursor cells from the postnatal striatum express both GAD65 and GAD67, at least in vitro [6]. In addition, the GABA content of astrocytes from the optic nerve declines over the first three postnatal weeks, suggesting a developmental down-regulation of GAD expression [72].

Glycine

Unlike $GABA_A$Rs, tonic activation of GlyRs by paracrine release of glycine is poorly documented. Microdialysis studies showed an increase in the extracellular glycine concentration following calcium-dependent high potassium-mediated depolarization in the substantia nigra pars compacta of the rat [106], or after blocking either the tricarboxylic cycle with fluorocitrate or the glutamine synthetase activity with L-methionine-*S*-sulfoximine [107]. Because these products act selectively on glial cells, these authors proposed that the increase in extracellular glycine concentration resulted from glycine release by astrocytes under specific conditions [107]. A non-synaptic release of glycine in the hippocampus was first suspected by Martina et al. who observed changes in both glutamatergic transmission [108] and long-term potentiation (LTP) [109] in response to the alteration of the activity of the glycine membrane glial transporter GLYT1. This interpretation was based on the well-known increase in NMDA receptor activity when glycine binds to its allosteric binding site on this glutamate receptor [110]. The hypothesis of the presence of a significant amount of glycine in the extracellular space was strengthened by data showing that a proportion of strychnine-sensitive GlyRs are continuously active in resting CA1 pyramidal neurons of the hippocampus, creating a state of tonic inhibition that "shunts" EPSPs evoked by shocking the Schaffer collaterals [111]. Finally, microdialysis experiments also revealed a functionally significant extracellular concentration of glycine in the hippocampus during high-frequency electrical stimulations [112]. There is also evidence that Bergmann glia can release glycine [113], and we recently showed that radial cells in the embryonic spinal cord can release glycine as well, thereby modulating the early electrical activity of the developing neuronal network [9].

Like GABA, glycine is avidly taken up by both neurons and glial cells via specific membrane transporters [22]. Two membrane glycine transporters, namely GLYT1 and GLYT2, have been described so far. GLYT1 has long been considered to be exclusively expressed by glial cells, and has therefore been referred to as the "glial transporter", while the GLYT2-type is known as the "neuronal transporter", essentially localized to presynaptic terminals of glycinergic neurons [114–118]. There are, however, some exceptions to these rules. GLYT1 mRNA is present not only in glia but also in neurons [114, 115]. Accordingly, GLYT1 proteins can be detected in neurons using a specific antibody directed again the N-terminal region of this transporter [119]. GLYT1s can interact with the scaffolding protein PSD-95 and GLYT1 immunoreactivity was detected at glutamatergic synapses in the cortex and in the hippocampus of the rat [120]. In addition, GLYT1 immunostaining was also observed on glutamatergic nerve endings and on GABA-releasing nerve terminals purified from the mouse spinal cord [121, 122]. However, the functional implication of this glycine accumulation in glutamatergic synaptic terminals is still unknown.

For GLYT2, a recent report suggests that glial cells can also express this neuronal membrane glycine transporter [122]. In this work, co-expression of GAT1 and GLYT2 was found in GFAP-positive gliosomes from the mouse spinal cord using immunohistochemistry and confocal microscopy [122].

Because the presence of glycine in intracellular compartments may only result from uptake processes, it is important to determine which cell types can produce glycine. In the SNC, glycine is metabolized from serine via the L-serine hydromethyltransferase pathway (SHMT) (Fig. 2). SHMT can convert glycine to L-serine in a reversible reaction [123]. There are two isoforms of SHMT: SHMT1 encodes the cytoplasmic isozyme (SHMT1) and SHMT2 encodes the mitochondrial isozyme (SHMT2) [124]. L-Serine is biosynthesized from a glycolytic intermediate, 3-phosphoglycerate (3-PG), in a three-step process involving the following enzymes: 3-phosphoglycerate dehydrogenase (3-PGDH), phosphoserine aminotransferase (PSAT), and phosphoserine phosphatase (PSP). L-Serine can also be derived through the degradation of proteins and phospholipids as well as through dietary intake. Glycine may also be produced from threonine via the action of the enzyme threonine aldolase. Glycine is catabolized by the glycine cleavage system (GCS) in addition to SHMT. As illustrated in Fig. 3, GCS consists of four proteins referred to as P-protein, H-protein, T-protein, and L-protein [125]. GCS is considered to be the major pathway for the catabolism of glycine not only in mammals but also in birds, reptiles, amphibians, and fishes [125, 126]. GCS catalyzes the direct cleavage of glycine to form one molecule of 5,10-methylenetetrahydrofolate, one molecule of carbon dioxide, and one molecule of ammonia (Fig. 2).

There is now strong evidence that the metabolism of L-serine and of L-serine-derived glycine is mainly restricted to glial cells [127–129]. The lack of 3PGDH expression in neurons [128, 130] indicates that the production of L-serine in the CNS originates mainly, if not exclusively, from non-neuronal cells. There is, however, one exception so far since 3PGDH mRNA was also detected in granular cells of the cerebellum [131]. Are neurons able to synthesize glycine from taken up L-serine? There is only one report showing that neurons can express SHMT. However, this was demonstrated in culture only [132]. What about in vivo? The limiting factor for neurons in synthesizing glycine would then be their ability to take up L-serine released by glial cells. This can be achieved via a specific transporter system for neutral amino acids [131]. Alternatively, neurons can take up glycine directly via the glycine transporter GlyT. In this case, glycine must be released by glial cells.

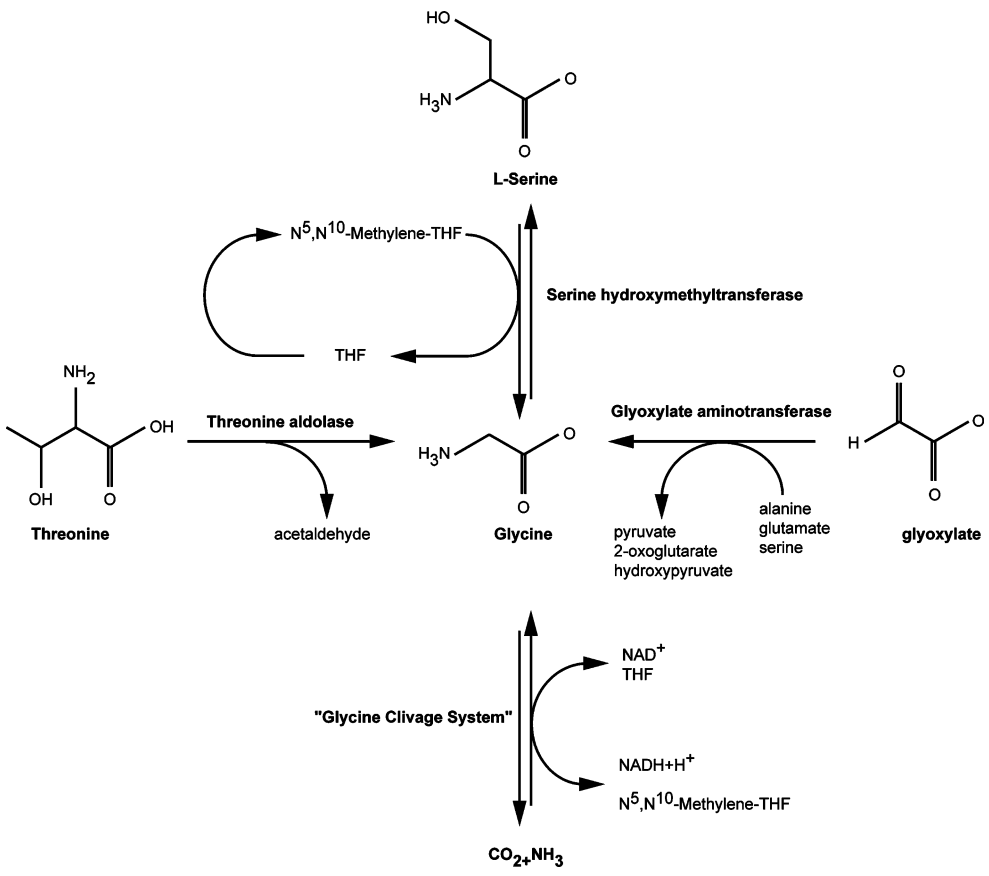

Fig. 2 Multiple biosynthesis pathways of glycine from L-serine, threonine, and glyoxylate (see Fig. 3 for detailed glycine cleavage system)

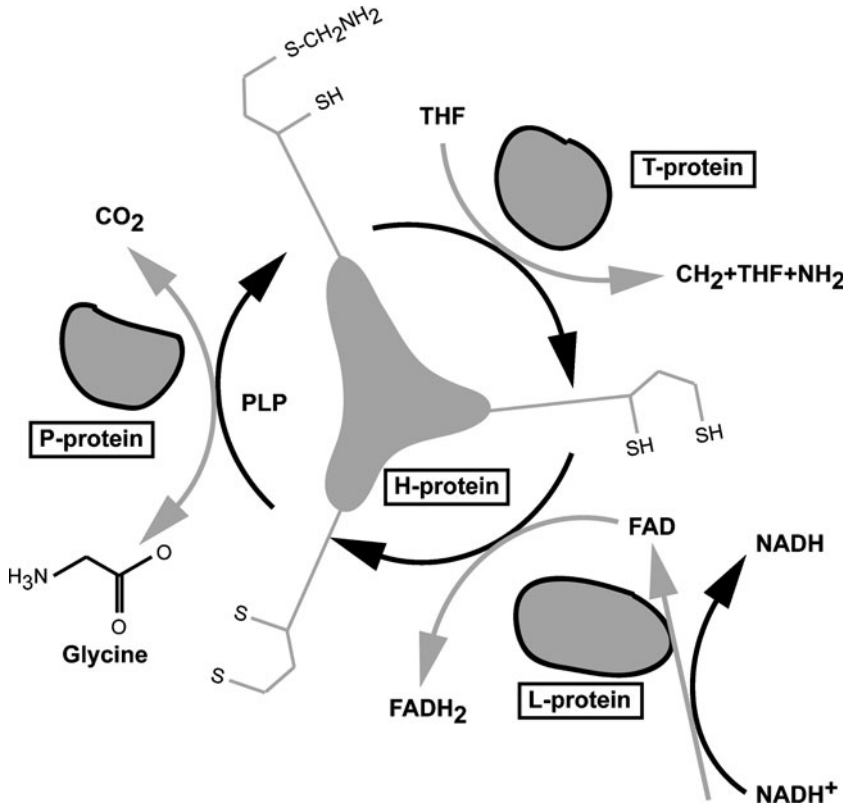

Fig. 3 Metabolism of glycine by the glycine cleavage system (GCS). Glycine enters the GCS when it is decarboxylated by the P-protein (glycine decarboxylase)

Because astrocytes express a high amount of the GLYT1 transporter, it was first supposed that their main function is to clear glycine from the extracellular space. Accordingly, it has been suggested that astrocytes are the main cells capable of catabolizing glycine via the GCS pathway [132]. However, a careful in situ hybridization analysis performed in vivo by Saka and coworkers [133] drew a more complex picture of the cellular expression of GCS proteins. They showed that P-protein mRNA is expressed only in glial cells of the cerebral cortex and of the hippocampus and in the Bergmann cells of the cerebellum, while T- and H-protein mRNAs are detected in both glial-like cells and neurons. Interestingly, P-, T-, and H- mRNAs were also detected in progenitor cells in the embryonic brain, before transfer only to radial glial cells [134], which are the precursors of astrocytes [135]. Surprisingly, Saka and coworkers found that only T- and H-protein mRNAs were expressed in the spinal cord, while spinal cord astrocytes expressed H-protein mRNA only. Because the heart muscle of the chicken has functional H- and T-proteins but no P-protein, and lacks GCS activities [136], it is assumed that P-protein expression, in addition to H-proteins and T-proteins, is required for proper glycine degradation. These observations imply that glycine can effectively be catabolized by glial cells in the brain, but not by astrocytes or by neurons in the spinal cord. Experimental evidence for a functional significance of such heterogeneity in GCS expression between SNC areas is still lacking. It is, however, possible that the presence of functional GCS reduces glycine production in areas in which glycinergic synaptic activity is absent or marginal, as for example in the hippocampus. It is also possible that GCS can have a regulatory function favoring L-serine production by astrocytes in areas enriched with glutamatergic synapses. Both glycine and D-serine potentiate glutamate NMDA receptor activity by acting as agonists on an allosteric binding site on this receptor, D-serine being synthesized from L-serine by serine racemase [137]. It will be interesting to determine to what extent serine racemase expression is correlated with the expression of H-proteins, T-proteins, and P-protein in particular astrocytes. The significance of the absence of functional GCS in the spinal cord remains unknown. The lack of glycine degradation could favor intracellular accumulation of glycine in glial cells of the spinal cord, which could be important for the proper functioning of inhibitory synapses in SNC areas strongly expressing glycinergic synapses. Of course, this hypothesis also supposes that glial cells can release glycine, which would then be taken up by glycinergic terminals via GLYT2s. Interestingly, radial glial cells in the embryonic mouse spinal cord are heavily stained by glycine antibody and can indeed release glycine [9]. Further experiments are, however, necessary to determine to what extent astrocytes can also accumulate and release glycine in the mature spinal cord.

Taurine

Alternatively to glycine and GABA release, tonic activation of GlyRs and $GABA_ARs$ can also result from the release of taurine. Taurine is, with glutamate, the most abundant free amino acid in the CNS [138]. Taurine is a weak agonist on GlyRs and $GABA_ARs$ [22] and is crucial to the proper development of the CNS [139]. A primary role of taurine has been attributed to its ability to regulate osmotic pressure in the brain since it can be released from various CNS areas in vivo and in vitro in response to hypo-osmotic stimuli [140–142]. In fact, the regulation of the osmotic balance by taurine release is likely to result from its ability to regulate the activity of vasopressin and oxytocin neurons in the hypothalamus [142]. Taurine is a sulfonic β-amino acid and a product of cysteine catabolism by cysteine sulfate decarboxylase (Fig. 4). Taurine can accumulate in cells and its concentration gradient is maintained by the specific membrane Na^+-dependent transporters TAUT-1 and TAUT-2. TAUT-2 has been predominantly associated with glial cells, including Bergmann cells and astrocytes in the cerebellum, and astrocytes in the cortex, hippocampus, and olfactory bulb [143]. TAUT-1 has been associated with the pituicytes in the posterior pituitary, but is absent from glial cells in the intermediate and anterior lobes. Conversely, in the brain, TAUT-1 expression has been found in cerebellar Purkinje cells and, in the retina, in photoreceptors and bipolar cells. TAUT-1 is absent from the cortex, the hippocampus, and the olfactory bulb [143]. TAUT activity can be blocked by guanidinoethyl sulfonate (GES). However, GES, in addition to the blockade of taurine uptake, acts agonistically on $GABA_ARs$ [144, 145]. Hence, data attributing an increase in neuronal responses to the extracellular accumulation of taurine by assuming that GES blocks TAUT should be viewed with caution.

Immunohistochemical detection of taurine is closely related to TAUT expression. In the adult, a high level of taurine has been found in the cerebral cortex, hippocampus, caudate putamen, cerebellum, and some hypothalamic areas including the supraoptic area. Lower levels have been observed in the brain stem and spinal cord [138]. The cellular location of taurine is highly variable and depends on the brain area. Taurine is expressed by neurons in the cortex, putamen, and hippocampus [146–148] and by Purkinje cells in the cerebellum [149]. Taurine has also been detected in photoreceptors, off-bipolar cells, and glutamatergic neurons in the retina [150]. Glial cells also show taurine expression in the hippocampus [151], thalamus, hypothalamus, and brain stem nuclei [146, 148, 152, 153]. A non-synaptic hypo-osmotic- or high-potassium-sensitive taurine release was also described in the neurohypophysis [154, 155], and taurine has been detected in pituicytes, the specialized glial cells of the neurohypophysis that engulf the axonal processes and the terminals of vasopressin and oxytocin neurons. As in the hypothalamus, taurine release activates GlyRs expressed by supraoptic neurons, thereby inhibiting the neuronal release of vasopressin and of oxytocin [156]. Finally, taurine is also assumed to be the major endogenous GlyR agonist in the substantia nigra pars compacta [106, 157, 158], although there is no evidence for taurine release in slice preparations of this CNS area in the rat [144]. A recent report indicates that the taurine level in astrocytes and their TAUT expression can also depend upon their activation state. Taurine level and TAUT expression in astrocytes were increased in the hippocampus during gliosis in mice treated with kainic acid, a murine model of epilepsy [159].

During CNS development, taurine is abundantly expressed by progenitors and immature neurons. Although it has been proposed that taurine has an important function at perinatal developmental stages [139], there are few data about possible taurine release at earlier developmental stages in the embryo. So far, only one study reports a non-synaptic release of taurine, sensitive to hypo-osmotic shocks, in the immature cortex of the mouse embryo [18]. Although the release mechanism was not investigated in this study, the main source of taurine is likely to be immature cortical neurons [18]. While the concentration of taurine in the immature cortex is not known, it was estimated to be ~0.3 mM, which roughly corresponds to the EC_{50} (1.1 mM) of GlyRs expressed by these neurons [18].

Mechanisms of Paracrine/Autocrine Release of GABA, Glycine, and Taurine

Vesicular or Not Vesicular

Vesicular release of neurotransmitters as glutamate and ATP or neuromodulators as D-serine by glial cells and especially by astrocytes has been extensively discussed by Hamilton and Attwell in a recent review [3]. In this review, the authors discuss the different criteria and the limits of the different approaches used to determine to what extent the paracrine release of glutamate, ATP, or D-serine from non-neuronal cells is or is not vesicular. It is well known that astrocytes in the adult do express an exocytotic machinery that allows transmitter-containing vesicles to fuse with the plasma membrane, and that this process is promoted by the formation of SNARE complex proteins [3]. There is also evidence that some astrocytes can express the glutamate vesicular transporters VGLUTs, thus reinforcing the hypothesis that glutamate can be released via a vesicular mechanism [3]. However, this is unlikely to be the sole

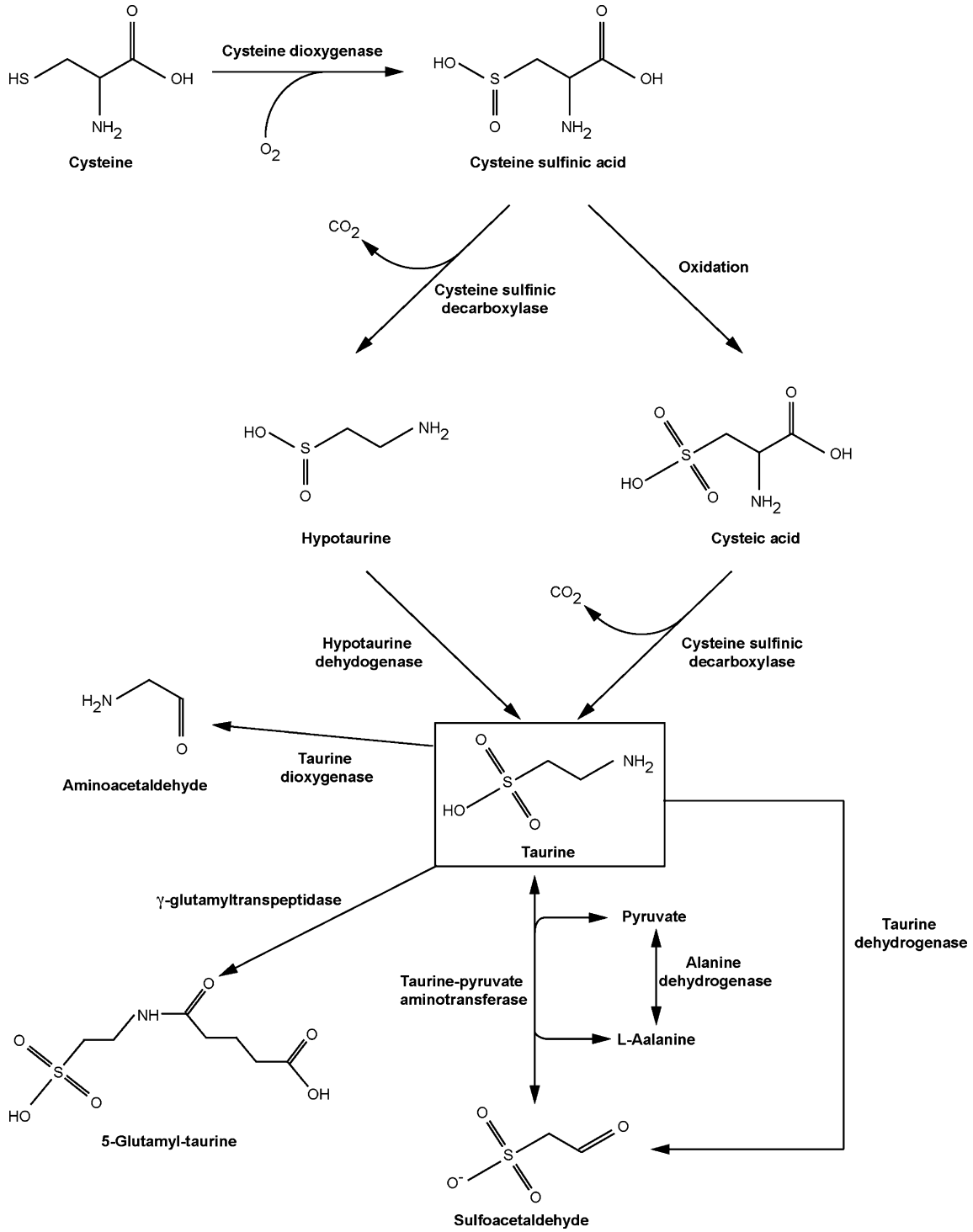

Fig. 4 Multiple biosynthesis and metabolism pathways of taurine

mechanism since the paracrine release of glutamate, ATP, and D-serine from non-neuronal cells can also be calcium-independent and is not blocked by genetic and/or pharmacological manipulations that prevent vesicle fusion to the plasma membrane, or the accumulation of transmitters in vesicles [3].

As a general rule, in order to determine to what extent the paracrine release of a neurotransmitter can be vesicular and exocytotic, several criteria have to be satisfied. Firstly, the release of neurotransmitters must be voltage- and/or calcium-dependent. For example, it must be blocked or enhanced by drugs or by genetic manipulations that prevent

or enhance calcium influx. Secondly, it must be blocked by specific toxins known to prevent vesicular fusion to the cytoplasmic membrane, as for example tetanus neurotoxin (TeNT) or botulinum B neurotoxin (BoNT) [160, 161], or by drugs preventing the accumulation of neurotransmitters within vesicles, such as specific inhibitors of the vacuolar-type H^+-ATPase like bafilomycin A1 [9, 162]. Thirdly, the neurotransmitter release has to be impeded after genetic ablation of specific proteins of the SNARE complex [17, 163]. Finally, the presence of vesicles, of the release machinery, and of vesicular transporters must be demonstrated by using immunohistochemistry, for example.

Loading of glycine and GABA within vesicles is achieved by the vesicular inhibitory amino acid transporter (VIAAT, [164]), which is also called VGAT [165] being the marker of glycinergic, GABAergic, and mixed GABA/glycine presynaptic terminals [166–168]. VIAAT expression was first described in astrocytes and microglia in cultures of rat pineal glands [169]. VIAAT expression patterns during embryogenesis were analyzed using whole mount in situ hybridization of mouse embryos between E8.5 and E12.5 [170]. VIAAT RNA was first detected in mouse embryos at E10–E10.5 in the ventral mesencephalon, hindbrain, and spinal cord. In the basal telencephalon, VIAAT expression was widespread throughout the CNS at E11.5, with the strongest expression in the medial ganglionic eminence. This developmental period corresponds to cell proliferation and migration and is concomitant with the onset of GAD67 and GAD65 gene activation (Gad1 and Gad2 genes) in the CNS [79]. There is no evidence yet that VIAAT is functional at these developmental stages, but these observations may at least suggest that GABA vesicular release could occur before synaptogenesis in the embryo.

Vesicular release is, however, unlikely to be the only mechanism for GABA release in the immature CNS. Indeed, non-synaptic GABA release in embryonic and early postnatal hippocampal slices from rats has been shown to be calcium- and SNARE-independent, suggesting that it is non-vesicular [17]. In the adult, most glial cells likely release GABA and/or glycine in a vesicular-independent way. Accordingly, GABA release from astrocytes is independent of neuronal activity in slice preparations of the olfactory bulb. Moreover, GABA release can be evoked by mechanical stimulations and is insensitive to the vacuolar-type H^+-ATPase inhibitor [171]. Basal GABA release in goldfish retinal slices is insensitive to the inhibition of vesicular release by concanamycin A or by tetanus toxin and is independent of the extracellular calcium concentration [172]. A non-vesicular GABA release has also been observed in Bergmann glia in slices [173]. However, non-vesicular release of GABA can also occur from neurons [174–176]. GABA released in response to neuronal action potential firing is not always prevented by blocking vesicular release. In such cases, GABA release is likely due to the reversal of membrane GABA transporters (see below). However, potential additional sources of non-vesicular GABA release that are independent of GAT-1 and GAT-3 were also observed in hippocampal slices [176]. The cellular origin of GABA in this case and the release mechanisms are unknown.

Paracrine release of glycine is less documented than for GABA. An increase in the extracellular glycine concentration measured by microdialysis has been described in the substantia nigra pars compacta of the rat following calcium-dependent high potassium-mediated depolarization [106]), as well as following the application of high-frequency electrical stimulations in the hippocampus [112]. These experiments suggest that the fluctuations of the extracellular levels of glycine in these areas depend on the electrical activity of the network and could be calcium-dependent. The cellular origin and the mechanisms of this glycine release remain unknown. In contrast, the release of glycine by Bergmann glia in mouse cerebellar slices [113] and by radial cells in an isolated spinal cord preparation of the mouse embryo [9] is unlikely to be vesicular. Bergmann glial cells release glycine in a calcium-independent manner [113] and glycine release by radial cells is insensitive to the vacuolar-type H^+-ATPase inhibitor bafilomycin A1, while these cells do not express VIAAT [9].

Taurine release is usually reported to be mechano- or volume-sensitive, independent of extracellular calcium, of extracellular sodium and of temperature and insensitive to the non-specific transporter blocker ouabain [177–181]. These characteristics suggest that taurine release is unlikely to be vesicular. Recently, two mechanisms of taurine release have been proposed in the rat hippocampus: an osmosensitive mechanism and an osmoresistant and partially calcium-dependent mechanism [182]. Interestingly, the osmoresistant release was partially enhanced by the activation of NMDA receptors in response to glutamate release. This could reinforce the hypothesis of a calcium-dependent release mechanism for taurine in the hippocampus, which might in turn indicate a vesicular nature. This hypothesis has yet to be demonstrated.

The Reversal of Membrane Transporters: A Release Pathway for Paracrine Release of GABA, Glycine, and Taurine

As briefly mentioned above, GABA can also be released via the reversal of its membrane transporters, the GATs [174]. Indeed, GATs, which usually remove GABA from the extracellular space, could function in a reverse manner depending on the sodium gradient in the vicinity, thereby providing ambient GABA responsible for continuous $GABA_AR$ activation [174]. This could also be the case for glycine via the reversal of the glial glycine transporter

GLYT1 [183]. Both GATs and GLYT1 share a common stoichiometry of one neurotransmitter molecule for two Na^+ and one Cl^-. Both types of transporters can reverse at physiological membrane potential in response to an increased intracellular Na^+ concentration, which can be evoked for example during action potential firing, or when the extracellular concentration of the concerned neurotransmitter falls below the value at which the transporter is at equilibrium, as proposed for GAT-1 [175, 183]. The rate of GABA reuptake by GAT-1 is determined by the GABA driving force. This driving force will become smaller as extracellular GABA concentration decreases during reuptake. If the ambient GABA concentration falls below equilibrium values according to intracellular GABA concentration, GAT-1 reverses to raise ambient GABA concentration to equilibrium values. Such a mechanism could explain why GAT-1 will not reduce the extracellular GABA concentration below a level that is often high enough for tonic inhibition to occur [175]. However, to occur such a mechanism requires that the cytosolic concentration of GABA be high in neurons. This is apparently the case since it has been reported that the cytosolic GABA concentration ranges from 1 mM to 6 mM in mammalian neurons [184] and is close to 10 mM in horizontal cells of fish retina [185].

Because GAT-1 expression is mainly neuronal [186], GABA release via the reversal of this transporter has mainly been qualified as of neuronal origin in the CNS. There are, however, exceptions, since both Bergmann glia in the cerebellum [187, 188] and Müller glial cells in the retina [189, 190] express GAT-1 and GAT-3, GAT-3 being the glial isoform of membrane GABA transporters [186]. Baraka and Bordey proposed that GABA release via the reversal of GAT-1 can also occur in glial cells [173]. Using a cerebellum slice preparation, these authors could detect an efflux of GABA that is able to activate $GABA_A$Rs after an intracellular injection of GABA in Bergmann cells. In the same work, they discuss an additional possible role of GAT-3 in GABA release. This is also likely to be the case in the neocortex. Indeed, inhibition of GAT-3 transporter function in the cortex results in a reduction of ambient GABA levels [191]. In the developing visual cortex, the strength of GABAergic inputs to Cajal–Retzius cells is constrained by $GABA_B$Rs that are persistently activated by ambient GABA, likely provided by GAT-3 operating in the reversed mode [192]. There are no data about GABA release via the reversal of the GABA transporter GAT-1 or GAT-3 in Müller cells of the retina. So far it has been shown that Müller cells control the ambient extracellular GABA level by an uptake mechanism [189].

Although the glycine glial transporter GLYT1 also has a stoichiometry of one neurotransmitter molecule for two Na^+ and one Cl^-, predicting that it can theoretically reverse in response to an increase in cytosolic glycine or Na^+ concentration [183], there are few data showing that such a glycine release occurs in the adult or in the developing CNS. The only evidence of glycine efflux due to the reversal of GLYT1 was obtained in Bergman glial cells in cerebellum slices [113]. Using paired recordings, the authors showed that depolarization of Bergmann glia induces the activation of GlyRs in outside-out patches from granule cells. Because the depolarization was quite high (up to +20 mV), the authors concluded that GLYT1 probably mediates glycine efflux, but only under pathological conditions [113]. In contrast, blockade of GLYT1 in the embryonic spinal cord does not prevent, but enhances, glycine release from radial cells, indicating that GLYT1 in the spinal cord controls the extracellular glycine concentration by an uptake mechanism only [9].

For taurine, there is merely one report suggesting its release by the reversal of its membrane transporters TAUT. Using whole-cell patch-clamp recordings from Bergmann glia in the rat, Baraka et al. showed that taurine transporters can work in reverse mode and mediate taurine efflux at least under pathological conditions [173].

Ionic Channels: Another Release Pathway

There is now increasing evidence that paracrine release of GABA, glycine, and taurine can be evoked by deformations of the cell membrane in response to mechanical stimuli or to a decrease in osmotic pressure. This release mechanism is very unlikely to be vesicular and does not involve membrane transporters. Although many studies show that neurotransmitters as ATP and glutamate can be released via the opening of hemi-channels formed by pannexins or connexins [193, 194], via receptor channels like the purinergic receptors P2X7 [195] or via transient receptor potential (TRP) channels such as TRPV1 [196], there is no evidence yet for the involvement of such release pathways in the paracrine release of GABA and glycine, and only suspicions have been reported for their involvement in the paracrine release of taurine [197, 198]. It is also important to note that most of the molecules used to block hemi-channels or TRPs are rather unspecific. Hence, their use does not allow definite conclusions to be drawn regarding the involvement of hemi-channels or TRPs in a pathway for the paracrine release of neurotransmitters [198].

Alternatively, chloride channels, known as volume-activated anion channels (VAACs), are also strong candidates for the pathways involved in GABA, taurine, and glycine release in response to mechanical stimuli. Some of these channels are permeable to ATP and MgATP, which can be released together with water to restore normal cell volume after cell swelling evoked by hypo-osmotic stimuli [199–201]. There is no standard classification of these chloride channels [202]. VAACs include the maxi-anion

channel and the volume-regulated ion channel (VRAC), also known as the volume-sensitive organic osmolyte-anion channel (VSOAC), the volume-regulated channel (VRC), the volume-sensitive outwardly rectifying (VSOR), and the intermediate-conductance anion channels [203–205], also known as the volume expansion sensing channel [202]. The molecular identity of VAACs has not yet been determined [202]. There are some indications that VRACs could be related to proteins encoded by the nine-member ClC gene family of Cl^-–H^+ exchangers and of voltage-dependent anion channels, or bestrophins and anoctamins [206]. Genes encoding VSORs remain unknown and the VDAC1 gene proposed to code for maxi-anion channels is still under debate [207, 208].

VRACs, maxi-anion channels, and VSORs can be distinguished by their activation properties and, to some extent, by their pharmacological profiles. VRACs can be activated both by decreasing the osmotic pressure and by the application of a high potassium solution [209]. VRACs are blocked by NPPB, IAA-94, and tamoxifen and are not permeable to Lucifer yellow [198]. Maxi-anion channels, which are identified by their sensitivity to gadolinium, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), and by their insensitivity to phloretin [201], have a large pore radius of 1.3 nm, allowing ATP (0.6-nm radius) release and are permeable to Lucifer yellow [210]. Maxi-anion channels constitute a prominent pathway for ATP release from astrocytes [211]. VSORs are insensitive to gadolinium [212], are blocked by DCPIB or phloretin [211, 212], and are activated by the application of hypo-osmotic solutions, by increasing the intracellular calcium concentration, by the activation of phospholipase C1 [213], by hydrogen peroxide [214], or by the activation of protein kinase C and of calmodulin kinase II [215]. VSORs have a small pore radius (0.63 nm), allowing efflux of small amino acids only [216], as for example glycine (0.28 nm radius) [9]. This could explain why these channels are less permeable to ATP (0.6 nm radius) [200].

It should, however, be stressed that pharmacological experiments using blockers alone are not sufficient to determine the presence of functional VAACs in preserved tissue. Indeed, the blockers classically used to block VAACs are also good blockers of other channels. For example, the typical VRAC blockers, NPPB, IAA-94, and tamoxifen, also block hemi-channels [198], whereas the VSOR blockers, DCPIB and phloretin, can block glycine receptors [9]. Moreover, gadolinium is also claimed to block TRP channels [217, 218].

Accordingly, multiple approaches are necessary to prove that a neurotransmitter is released through VAACs. Such a multiple approach should include: Lucifer yellow or neurobiotin loading experiments [9], the analysis of the activation mechanism(s) of the putative channel(s) supposed to be involved in the release pathway, and the use of several channel blockers and of receptor agonists to discriminate between hemi-channels, TRP channels, P2X receptors, and VAAC subtypes. However, because the protein structure and gene sequence of VAACs are still unidentified, it remains impossible to disclose their localization using specific antibodies. Also, because it is not possible to manipulate their expression in neural tissue, identifying the type of VAACs involved in the paracrine release of GABA, glycine, or taurine is precluded.

Although it has been shown that GABA can be released by mechanical stimuli in the olfactory bulb, its release pathway has not yet been explored [171]. However, a recent report made a significant contribution to the elucidation of the release pathway that might be involved in the paracrine release of GABA. The authors demonstrated, using a multiple approach, that the main release pathway for paracrine GABA release from Bergmann glia of the cerebellum is likely to be bestrophin 1 (Best1) [219]. Best1 is also regulated by membrane stretch stimuli and belongs to a family of proteins that can function both as chloride channels and as regulators of voltage-gated calcium channels [220]. This discovery opens a new field of research on the regulation and physiological roles of volume-sensitive release of neurotransmitters both in the adult and during CNS development.

Taurine can be released in response to hypo-osmotic stimuli. This has been demonstrated by inducing the swelling of both neurons and glial cells in culture [178, 181, 199, 221–223], in slice preparation [224], and in vivo [214, 225]. Taurine permeability of volume-activated chloride channels has been demonstrated in astrocytes and neurons [226–228]. The pharmacology of taurine release has been extensively studied. It is antagonized by chloride channel blockers such as NPPB, DIDS, SITS, niflumic acid, tamoxifen, dideoxyforskolin, and DCPIB [178, 224, 226, 229–231], suggesting that VAACs (probably VRACs) are likely the main release pathway for taurine.

The only example of glycine release through VAACs comes from the spinal cord of the mouse embryo [9]. At the onset of the synaptogenesis, glycine is mainly released by radial cells in a vesicular-independent manner (insensitive to bafilomycin A1). This glycine release is proposed to be mediated by the opening of VSOR channels, rather than by hemi-channels, P2X receptors, TRP channels, VRACs, or maxi-anion channels [9]. This glycine release can be evoked by hypo-osmotic shocks or by mechanical stimuli. It is slightly decreased in the absence of external calcium and is not evoked by P2X or TRP agonists. In addition, it is poorly sensitive to gadolinium and is blocked by phloretin and DIDS. Finally, radial cells do not take up Lucifer yellow or neurobiotin, even in the presence of hypo-osmotic solutions.

Physiological Roles of Paracrine/Autocrine Release of GABA, Glycine, and Taurine

Physiological Roles of Paracrine/Autocrine Release of GABA

Besides transcription and growth factors, neurotransmitters, including GABA, glycine, and taurine, are nowadays also considered as agents that influence neurogenesis in the CNS both during development and in the adult [1]. Most of the effects of GABA, glycine, and taurine are mediated by the activation of $GABA_AR$ and GlyR, which evokes cell depolarization rather that hyperpolarization, due to the high intracellular chloride content of neural progenitor cells (NPCs), as a consequence of their low expression of KCC2 [15, 232]. $GABA_AR$ and GlyR expression is not restricted to true NPCs, but is present in progenitors isolated from different sources and displaying a "neuron-like" phenotype, as, for example, mesenchymal stem cells [233].

Among these neurotransmitters, GABA has been the most extensively studied (recently reviewed in [7, 234–236]) and is involved in almost all aspects of neurogenesis: proliferation, migration, and maturation (neuritogenesis/synaptogenesis). In the neonatal rat hippocampus, prior to synapse formation, a paracrine (Ca^{2+}- and SNARE-independent) release of GABA from growth cones of neurons and/or from astrocytes tonically activates synaptically silent pyramidal neurons [17]. As mentioned above, the paracrine release of GABA in the adult is likely to occur from non-neuronal cells via vesicular-independent mechanisms.

GABA has been shown to influence the proliferation of a broad range of NPCs in the nervous system, in both in vitro and in vivo studies. This effect of GABA is mainly mediated by $GABA_AR$-induced depolarization and calcium signaling [59]. Most studies point to a negative regulation of proliferation by GABA: in embryonic stem cells and peripheral neural crest cells [237, 238], in neocortical neuroblasts in vitro [239] and in vivo [59], in cerebellar granule cells [240], in postnatal striatal progenitors [6]. Some other studies indicate a positive effect on NPC proliferation: in cerebellar granule cells [241] and in prominin-positive stem cells of the ependymal zone [242]. Nevertheless, when GABA negatively influences NPC proliferation, it seems to follow a similar sequence during development and in adult-born NPCs [234].

GABA is a crucial regulator of the migration of neural progenitors both in the adult and during development (review in [243, 244]). GABA acts on different processes of migration: chemoattraction and regulation of cell motility [243, 245–247]. In the juvenile (P14–P20) subventricular zone (SVZ), neuronal precursors migrate along the rostral migratory stream towards the olfactory bulb where they differentiate into interneurons. It has been demonstrated that endogenous GABA tonically reduces the speed of migration of neuronal precursors via the activation of their $GABA_ARs$ [248]. GABA is released from the neuronal precursors themselves and taken up by surrounding astrocyte-like cells that ensheath neuronal precursors. In addition, it has been shown that neuroblasts are tonically depolarized which triggers the release of GABA via a non-synaptic SNARE-independent pathway. This ambient GABA release from neuroblasts tonically activates GFAP-expressing cells and thereby controls their own proliferation, given the fact that GFAP-expressing cells generate neuroblasts in the postnatal SVZ [249]. The mechanism of GABA action involves $GABA_AR$-induced changes in intracellular calcium concentration [234]. During the development of the neocortex, GABA influences the migration of projection neurons at different time points: activation of $GABA_BRs$ and of $GABA_CRs$ in neuroblasts promotes their migration out of the SVZ and of the IZ [250], while the activation of $GABA_ARs$ in the subplate functions as a stop signal [243, 247]. In embryonic E17–E18 rats, morphological maturation of somatosensory cortical neurons radially migrating to layer II/III requires a chloride-mediated depolarizing effect of GABA [251]. In this study, the evoked premature expression of KCC2 using KCC2 in utero electroporation did not affect the radial migration of transfected cortical progenitors, but impaired their morphology. Another study showed that, prior to synaptogenesis, ambient GABA initially stimulates the motility of cortical migrating interneurons through $GABA_AR$ activation. Once interneurons arrive in the cortex, up-regulation of KCC2 occurs and GABA reduces interneuron motility and acts as stop signal [252]. In this context, it is worth mentioning that blocking $GABA_ARs$ in vivo induces severe malformations of the cortical layers, leading to upper cortical heterotopia [243]. Finally, GABA has also been implicated in the tangential migration of cortical interneurons, probably via the activation of $GABA_BRs$ [253, 254]. As regards migration, the source of GABA has not been yet deciphered, but it is worth mentioning that $GABA_AR$ expression in cortical NPCs in vitro is closely associated with radial glia [255].

GABA release is also important for the maturation of neurons, mostly by promoting neuritogenesis (recently reviewed in [256]). In vitro, GABA has been shown to induce neuritic outgrowth in a variety of neurons: rat hippocampal pyramidal neurons, rat cerebellar granule cells [257], and chick embryonic tectal neurons [258–260]. In vivo, GABA release promotes dendritic development and synaptic integration in the hippocampus [261, 262], final maturation in the cortex [251, 263, 264], and stabilization of the dendritic arborization of olfactory bulb interneurons [265].

The role of GABA release in the development and maturation of non-neuronal cells in the developing CNS remains an open question. So far, there have been reports of

GABA and/or GABA receptor expression by NG2+ cells [266], by oligodendrocyte progenitors [267–269] and by astrocytes [270], without any clear evidence for a physiological role.

Finally, in the adult, neuroblasts derived from astrocyte-like progenitors are also able to release GABA, which acts in a negative feedback loop to inhibit the proliferation of NPCs in a $GABA_AR$-dependent fashion, hence promoting cell cycle exit and maturation of the NPCs [271]. The same maturation effect occurs in the hippocampus where GABA increases the expression of NeuroD in nestin-expressing NPCs within the subgranular zone (SGZ) [261].

Physiological Roles of Paracrine/Autocrine Release of Glycine

Although there is increasing evidence that a paracrine release of glycine can occur both in the adult and in the developing CNS, its role is less documented than for GABA. Because both glycine and taurine can activate GlyRs, one of the difficulties is to determine what type of endogenous agonist is released to account for the observed tonic activation of GlyRs. Glycine concentrations are usually thought to be low in the cortex [272], while taurine concentrations are higher, but this is still a matter of debate [273]. Nevertheless, both glycine and taurine concentration imbalances seem to influence CNS development. In non-ketotic hyperglycinemia, which is caused by a deficiency in the glycine cleavage system expressed by NPCs and then by radial glia, an accumulation of glycine is observed and is associated with CNS symptoms, such as coma, respiratory distress, seizures and brain malformations (agenesis of the corpus callosum, gyral abnormalities, and cerebellar hypoplasia) [134]. In the kitten, maternal dietary taurine deprivation leads to abnormal cortical development [274]. These phenomena are not limited to glycine and taurine since L-serine deficiency (in a model of 3-phosphoglycerate dehydrogenase knock-out mice), which is also associated with a decrease in glycine and GABA concentrations, impairs neurogenesis [275]. To gain insight into the nature of the GlyR ligand, the first step is to determine in cells the presence of glycine or taurine as well as their membrane transporters using immunohistochemistry. The second step is to prove that they are released and that the blockade of their uptake by membrane transporters affects their extracellular concentration. There are different additional approaches to measuring the release of glycine versus taurine. One of these consists in directly measuring in vivo the extracellular concentration of agonists using push–pull techniques and HPLC. Another approach, the sniffer technique [9], consists in using a specific sensor to measure fluctuations of the extracellular concentration of a given GlyR agonist. For glycine, a specific sensor can be an outside-out patch containing a GlyR subtype (Fig. 5 sniffer) that is poorly sensitive for taurine, such as the homomeric GlyR containing $\alpha 2$ subunits [9]. Unfortunately, no specific receptor for taurine exists, rendering the direct measurement of taurine release problematic. In addition, the results obtained with membrane transporter blockers for taurine and glycine must be interpreted with caution. Sarcosine is generally used to block glycine transporters, but also acts as an agonist on GlyRs [276], while the taurine transporter blocker GES is also an agonist on $GABA_ARs$ [144, 145]. Accordingly, a combination of these approaches is necessary to discriminate between taurine and glycine as the endogenous agonist involved in non-synaptic activation of GlyRs.

Overall, the role of glycine during the early development of neuronal networks is far less documented than those of GABA and taurine. Glycine begins to be expressed after GABA in the spinal interneurons during embryonic life [277]. In the zebrafish, GlyR activation during embryonic development is likely to regulate the differentiation of interneurons rather than that of motor or sensory neurons. Knocking down GlyR $\alpha 2$-subunit expression using antisense morpholino oligonucleotides strongly reduces motor network activity, synaptic activity, and the number of spinal interneurons. These effects are accompanied by a concomitant increase in the number of mitotic cells [278]. However, neither the nature of the endogenous agonist (glycine or taurine or both) nor its release mechanism was addressed in this study.

In a recent study, we demonstrated that glycine is expressed by radial cells in the embryonic mouse spinal cord (as early as E12.5) and is released from these progenitors via a mechano-sensitive mechanism [9]. We found that this non-vesicular glycine release precedes the first synaptic activity in spinal motoneurons and plays an important role in the propagation of spontaneous activity throughout the spinal cord. Preventing the uptake of endogenously released glycine by blocking the glycine transporter GLYT1 with sarcosine increases the frequency of bursting activity [8] and, interestingly enough, disrupts pool-specific axon fasciculation and path-finding of embryonic spinal motoneurons [279]. All these data indicate that the tonic excitatory action of glycine is necessary for the construction of spinal motor networks as well as for the motoneuronal projections to specific muscles. Glycine effects on maturation of neuritic outgrowth are also seen in mouse spinal cord neurons maintained in dissociated cultures, but are attributed to a Ca^{2+}-dependent and vesicular-dependent release mechanism [280].

Finally, although GlyRs are expressed by astroglia, oligodendrocytes, and their progenitors [1, 267, 270, 281, 282], their physiological role remains unknown and there is no clear evidence for a non-synaptic release of glycine in the spinal cord and brain stem in the adult. In the adult, the only evidence of basal GlyR activation by extracellular

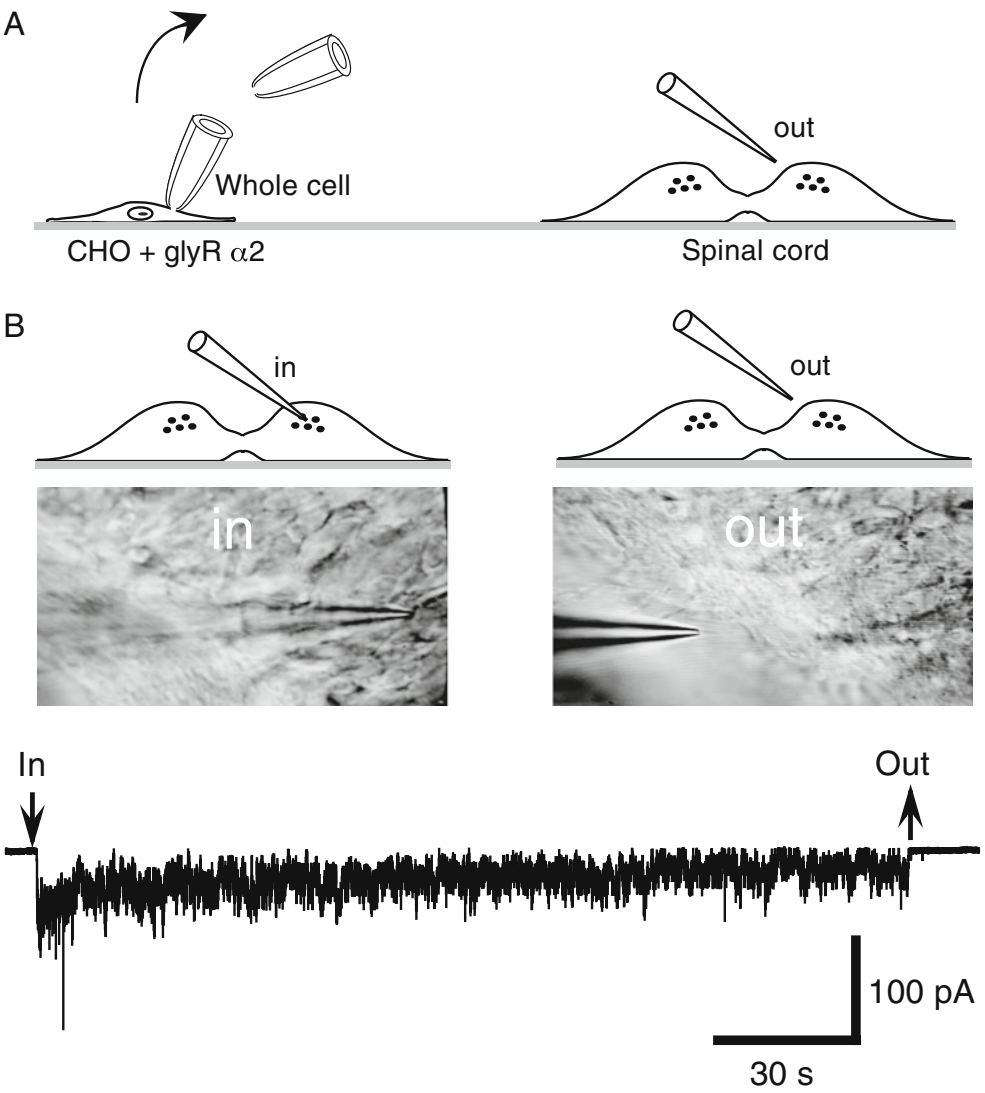

Fig. 5 Detection of non-synaptic release of endogenous glycine receptor agonists using a "sniffer" electrode in the embryonic spinal cord. A sniffer electrode makes use of an outside-out patch pulled from a transfected CHO cell line expressing glycine receptors. The electrode is gently pushed in the motoneuronal area (in) and then removed from the spinal cord (out). Lower trace: when the electrode is positioned close to motoneurons, GlyRs are activated, revealing the presence of GlyR agonist in the extracellular space (adapted from Scain et al. [9])

endogenous glycine is obtained in the hippocampus by inhibiting the glial membrane transporter GLYT1 using specific blockers [276]. Raising the endogenous glycine concentration with GLYT1 antagonists, which do not interfere with GLYT2 transporters, evokes a strychnine-sensitive depression of the excitatory synaptic activity recorded in hippocampal slice preparations [276].

Physiological Roles of Paracrine/Autocrine Release of Taurine

One of the possible roles of taurine during development is a control of neuronal migration. This is shown in vitro using dissociated cerebellar granule cells in which both taurine depletion and impairment of cell migration induced by the taurine transporter inhibitor GES are prevented by adding taurine to the culture medium [283]. Interestingly, in newborn kittens from taurine-depleted mothers, neuroblasts from the visual cortex fail to migrate and to differentiate normally, highlighting the necessary role of this amino acid in developmental processes [274]. Another study further demonstrated that postnatal P4 cortical subplate neurons and Cajal–Retzius cells expressed α2/β heteromeric receptors, indicating that the taurine effect on neuronal migration likely operates through GlyR activation [284]. This taurine effect is likely due to a depolarizing effect of GlyRs because it has been shown that taurine exerts a chloride-mediated excitatory effect on postnatal P2 neocortical mouse neurons by acting on GlyRs [285]. In immature postnatal neurons from the ventral tegmental area (VTA), taurine induces large depolarizations through non-synaptic GlyRs, but not through $GABA_A$Rs, and likely represents an important endogenous trophic factor [286]. In the immature retina, taurine potentiates neuritic fiber outgrowth [287], this effect being mediated by an interaction with intracellular zinc [288]. Taurine, via an action on GlyRα2, also

seems necessary for the production of rod photoreceptors. Actually, E16 retinal explants infected with retroviruses encoding GlyRα2 and allowed to develop in vitro upregulate photoreceptor markers, whereas GlyRα2 knockdown inhibits rod photoreceptor development [289].

Because taurine can be released in response to cell swelling and because taurine release through VAACs induces a decrease in cell volume (regulatory volume decrease—RVD), it was proposed that the major function of taurine in the adult brain is to contribute to volume regulation of the cells [290]. So far, the most convincing argument is the decrease of RVD by intracellular depletion of taurine in cultured astrocytes in the presence of GES and the blockade of RVD in the presence of a high extracellular concentration of taurine (>90 mM) [291]. However, such cell volume regulation might not be due to taurine release only, but also to the activation of VRACs themselves. In fact, it has been shown that VRACs are water-permeable channels [292], which makes them powerful candidates as cell volume regulators. A combined water and osmolyte permeation of VRACs provides a powerful combination for the protection of the osmotic thermodynamic equilibrium across the plasma membrane. In the hypothalamus of mature rats, low concentrations of taurine, released from astroglial cells by hypo-osmotic challenges, are not able tonically to activate $GABA_A$Rs located remotely from synapses in supraoptic neurons [293]. However, in the neurohypophysis, where the axons of supraoptic neurons project, taurine is also released osmo-dependently from glial cells, and unsheaths nerve terminals, activates GlyRs, and reduces vasopressin secretion, through a Ca^{2+}-dependent mechanism involving mechanoreceptors [156]. Accordingly, it has been proposed that taurine regulates the transmission of the osmotic information through its action on vasopressin secretion (reviewed in [142]).

Pharmacological applications of taurine can modulate neuronal network activity in vitro and in vivo via the activation of GlyRs, as shown for example in dissociated culture of nucleus accumbens neurons [294], in the ventral tegmental area [286], in the inferior colliculus of young rat [295], and in the rat nucleus accumbens [296]. In addition, low concentrations of taurine can activate extrasynaptic $GABA_A$Rs in the ventrobasal thalamus of adult mice [297]. However, it is unclear if endogenous taurine can be released in these CNS regions and it should be pointed out that the results of the experiments in which the taurine transporter blocker GES is used to enhance endogenous taurine release are questionable since GES can also act as an agonist on $GABA_A$Rs [144]. Conversely, there is evidence that taurine can be released under pathological conditions and may provide protection against excitotoxicity. In the brainstem, taurine release is correlated with the activation of glutamate signaling during ischemia [298] and a reduction in the taurine concentration was observed in the hypoxic–ischemic hippocampus of perinatal rats, especially when these rats were suffering from iron deficiency [299].

Conclusions and Perspectives

$GABA_A$ and glycine receptor activation have for a long time been known to mediate inhibitory transmission in the adult. Paracrine release of $GABA_A$ and of glycine receptor endogenous agonists is now well described both in culture and in ex vivo preparations. The cellular origin of these "unconventional release" mechanisms varies strongly depending on the CNS area and the developmental stage. One of the intriguing features is that the same cell subtype, as for example astrocytes and Bergmann cells in the cerebellum, could release glycine, GABA, and taurine. The functional significance of this multiple neurotransmitter release and whether it occurs in particular conditions for a given neurotransmitter remain open questions. If one glial cell can synthesize more than one neurotransmitter, it is unclear whether this cell will specifically release one neurotransmitter in response to a specific stimulus. This apparent lack of specificity is puzzling unless one supposes that it reflects cell heterogeneity in the same area and/or depending on the CNS regions, the latter being observed for taurine and for glycine. It is unlikely that the release of GABA, glycine or taurine from glial cells is vesicular, which is not always the case for glutamate [3]. Accordingly, the different non-vesicular release mechanisms described in this review could allow the cell to specifically release one neurotransmitter instead of the others depending on the physiological stimuli and on the expression of the corresponding proteins. For example, the reversal of GABA membrane transporters occurs in neurons and in glial cells in response to cell depolarization, to an increase in sodium influx or to a decrease in neurotransmitter extracellular concentration below equilibrium. Few data suggest that this could also be the case in glial cells ex vivo for glycine or taurine. Assuming that reversal of glycine or taurine membrane transporters occurs in glial cells, it is more likely to happen under pathological conditions, which could not be the case for GABA, indicating that differences in transporter kinetics might specify the type of neurotransmitter release in response to a particular stimulus. On the other hand, the release pathway involving swelling-activated anion channels appears not to be specific for one particular neurotransmitter.

Is this release pathway involving swelling-activated anion channels really functional in non-pathological conditions? The analysis of such a neurotransmitter release pathway is greatly limited by the poor specificity of their blockers and their unknown protein structures. However, there is experimental evidence that these channels can control the basal extracellular level of these neurotransmit-

ters at least ex vivo. How these channels are activated under physiological conditions remains poorly understood. Small mechanical deformations of the cell membrane in response to blood or cerebrospinal fluid flow could be one of the stimuli [9, 171]. There are also indications that intracellular second messenger cascades and some metabotropic receptors can control the activation of these channels. Accordingly, it will be important to determine if some neurotransmitters can evoke or regulate GABA or glycine release through swelling-activated anion channels, which will strengthen the hypothesis of a physiological role of this release pathway.

Because paracrine release of neurotransmitters occurs before synaptogenesis, it is likely to be the first communication pathway involving neurotransmitters in the developing CNS. But since this release mechanism remains in the adult and is also observed in differentiated cells as astrocytes, it cannot be referred to as a primary release mechanism only. Paracrine release of neurotransmitter is involved in the control of progenitor proliferation in both the embryo and adult. But this non-synaptic release is also known to regulate the excitability level of the neuronal network in both the adult and immature CNS, being important in the latter for the development of neuronal networks. It is unclear to what extent paracrine release of neurotransmitter in addition to the electrical activity of developing neurons is essential to achievement of a functional adult network.

Finally, a better understanding of the regulation of non-vesicular glial release pathways, for example by neurotransmitters released from developing neurons, will greatly enhance our knowledge of the mechanisms involved in cell–cell interactions working hand in hand with genetic programs to control the development and maturation of neuronal networks.

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