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RESEARCH TOPICS

BUILDING UP THE INHIBITORY SYNAPSE

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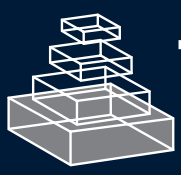

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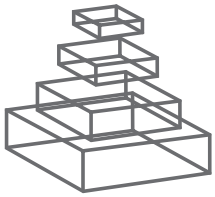

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BUILDING UP THE INHIBITORY SYNAPSE

Topic Editor:
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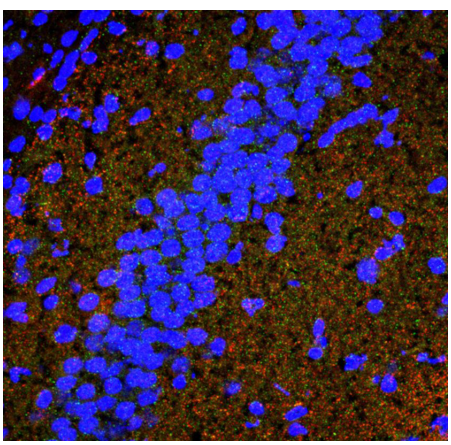

Confocal image of an hippocampal section from the brain of a juvenile mouse showing cells in stratum pyramidale and stratum radiatum of the CA1 region (DAPI staining) immunolabeled for endogenous gephrin (red) and neuroligin2 (green). In yellow overlapping distribution of the two postsynaptic components at inhibitory synapses. Image by Paola Zacchi and Enrico Cherubini.

Fast inhibitory transmission exerts a powerful control on neuronal excitability and network oscillations thought to be associated with high cognitive functions. An alteration of inhibitory signaling is associated with major neurological and psychiatric disorders including epilepsy. Once released from presynaptic nerve terminals, GABA and glycine cross the synaptic cleft and bind to postsynaptic receptors localized in precise apposition to presynaptic release sites. The functional organization of inhibitory synapses consists in a dynamic process which relies on a number of highly specialized proteins that ensure the correct targeting, clustering, stabilization and subsequent fate of synaptic receptors. Among the proteins involved in this task, the tubulin-binding protein gephyrin plays a crucial role. Through its self-oligomerization properties, this protein forms hexagonal lattices that trap GABAA and glycine receptors and link them to the cytoskeleton. By directly interacting with cell-adhesion molecules of the neuroligin-neurexin families that connect presynaptic and postsynaptic neurons at synapses, gephyrin ensures a backward control of presynaptic signaling. In addition, changes in clusters size is dynamically regulated by lateral diffusion of neurotransmitter receptors between the synaptic and extrasynaptic compartments and by their interaction with synaptic scaffold proteins.

The aim of this Research Topic (research articles and reviews) is to bring together experts on the cellular and molecular mechanisms regulating the appropriate assembly, location and function of pre and postsynaptic specializations at inhibitory synapses. A particular emphasis will be on the role of receptor trafficking in synaptic stabilization and plasticity.

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EDITORIAL
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Building up the inhibitory synapse

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"Building up the inhibitory synapse" is a complex phenomenon involving a variety of dynamically regulated molecular and cellular processes whose nature is still largely unknown. Understanding the role of different proteins in controlling synapses formation and stabilization may help elucidating, at the network level, the mechanisms by which inhibitory transmission controls network excitability and oscillatory behavior, crucial for information processing in the brain.

Aim of this e-book is to highlight recent advances in these processes, bringing together leading experts in the field, who have made major contributions to our understanding of the cellular and molecular mechanisms regulating the appropriate assembly, location, and function of pre and postsynaptic specializations at inhibitory synapses.

This e-book comprises nine reviews, one perspective and three research articles organized in a logic way following the information flow from the pre to the postsynaptic site.

In the first article, Jovanovic and Thomson (2011) (School of Pharmacy, UCL) review the developmental processes determining the tangential migration of GABAergic interneurons from the ganglionic eminence to the neocortex where the formation of appropriate synapses seems to be facilitated by cell–cell recognition, most probably *via* protein–protein interactions across the synaptic cleft.

Grantyn et al. (2011) (Institute of Neurophysiology Charité, Berlin) discuss how, during postnatal development, GABAergic synapses are characterized by a high release probability, often multivesicular (ballistic) and asynchronous. The ballistic mode of operation of immature synaptic terminals might be instrumental for recruiting and stabilizing receptors *via* postsynaptic calcium signals, triggered by the depolarizing action of GABA and activation of voltage-dependent calcium channels.

Early in postnatal life, the depolarizing action of GABA is crucial for the construction of neuronal circuits. This process depends on intracellular chloride homeostasis which is under control of the cation-chloride co-transporter KCC2. In addition, by interacting with actin cytoskeleton, KCC2 exerts, independently of its role on chloride homeostasis, a crucial role on spines morphogenesis. Chamma et al. (2012) (INSERM UMR-839, Paris), summarize the functional impact that structurally different forms of KCC2 have on inhibitory and excitatory transmission, highlighting the cellular and molecular mechanisms by which neuronal activity regulates its action *via* transcriptional and post-translational modifications.

In the following research paper, Bhumbra et al. (2012) (Department of Neuroscience, Physiology and Pharmacology, UCL), convincingly demonstrate that, in contrast to neonates, in the spinal cord of juvenile animals inhibitory postsynaptic currents are entirely glycinergic, raising the possibility that the co-release of GABA and glycine is developmentally regulated.

Once released from presynaptic terminals, GABA diffuses in the synaptic cleft and binds to postsynaptic receptors. This process is very fast and occurs in non-equilibrium conditions. Barberis et al. (2011) (Department of Neuroscience, IIT and Department of Biophysics, Wroclaw University) discuss how GABA transient in the cleft influences the shape of synaptic currents. The authors describe the methods used to estimate the neurotransmitter transient using tools enabling to indirectly infer with its time course, including low affinity competitive $GABA_A$ receptor antagonists or gating modifiers. GABA transient may limit the activation of postsynaptic receptors and their binding reaction to mono-ligand state, promoting low probability channel opening and fast deactivation kinetics.

The intriguing hypothesis that GABA *transporters* may exert a homeostatic control on GABA release is put forward in a perspective article by Conti et al. (2011) (Department of Neuroscience, Università Politecnica delle Marche). On the basis of the literature and recent findings the authors suggest that, in physiological conditions, vesicle filling is dominated by the GABA synthesizing enzyme GAD and by the GABA transporter GAT-1. This may be relevant for activity-dependent regulation of synaptic strength.

In the following review, Mortensen et al. (2012) (Department of Neuroscience, Physiology and Pharmacology, UCL), compare the potency measured under the same experimental conditions, of a series of $GABA_A$ receptor isoforms, physiologically relevant to phasic and tonic inhibition. The highest potency would be compatible with extrasynaptic receptors, containing the α6 subunit and exposed, during spillover of GABA from synapses, to low agonist concentrations, while the lowest potency would be compatible with synaptic $GABA_A$ receptors containing the α2/α3βγ subunits, exposed, during vesicular release, to high agonist concentrations.

Using a genetic approach, Janssen et al. (2011) (Department of Pharmacology and Physiology, Georgetown University) found that the conditional deletion of β3 subunits in D2-positive striato-pallidal medium spiny neurons led to a reduced network excitability due to a decrease in tonic $GABA_A$-mediated conductance. They suggest that the β3 subunit may be an important pharmacological target for the treatment of striatal disorders.

Next, Shrivastava et al. (2011) (Department of Biochemistry and Molecular Biology, Vienna University and Institut de Biologie, Ecole Normale Superieure, Paris), provide an overview

on the mechanisms regulating the cross talk between $GABA_A$ receptors and other ligand-gated ionic channels or G-protein coupled receptors co-localized on the postsynaptic membrane. The interaction may occur *via* a direct coupling between two receptors or *via* the activation of intracellular signaling pathways. Usually the interaction results in a reduced GABAergic inhibition with consequent disinhibition. A similar receptor–receptor interaction may occur also at extrasynaptic sites to regulate tonic $GABA_A$-mediated inhibition.

Sassoè-Pognetto et al. (2011) (Department of Anatomy, Pharmacology and Forensic Medicine, University Torino), provide an overview of the high degree of molecular diversity of GABAergic synapses which contrasts with the apparent simplicity of their ultra-structural organization. The authors propose to develop "cataloguing tools" that should help uncovering how different proteins impact on the functional properties of the synapses and how synaptic organization changes during development.

The complex and still "enigmatic" role of the scaffolding molecule gephyrin at GABAergic synapses is exquisitely reviewed by Tretter et al. (2012) (Department of Biochemistry and Molecular Biology, Vienna University and School of Medicine, Tufts University, Boston). Gephyrin is dissected as a structural molecule responsible for trapping and concentrating $GABA_A$ receptors in front of presynaptic releasing sites, by directly interacting with several types of α-containing subunits. Its heterogeneity, obtained through alternative splicing and post-translational modifications, associated with complex biophysical properties of its domains leads to the construction of diverse structural scaffolds that can accommodate different $GABA_A$ receptor subtypes. Gephyrin is also recognized as a crucial hub for multiple signal transduction pathways and trans-synaptic signaling that ultimately impact on synaptic dynamics and synaptic plasticity.

Papadopoulos and Soykan (2011) (Max-Plank Institute of Experimental Medicine, Göttingen), summarize recent advances in the role played by the GDP/GTP-exchange factor collybistin in selective gephyrin transport, clustering, and maintenance at GABAergic synapses. One of the most interesting issues raised by the authors regard a comprehensive description of the putative circuit-specific role played by collybistin isoforms in selected region of the mammalian forebrain.

Finally, Jin et al. (2012) (Department of Neuroscience, Uppsala University), report changes in $GABA_A$ receptors subunits mRNA in the hippocampus and cortex of *post mortem* brains of individual suffering from alcohol dependence. Interestingly, the observed changes refer mainly to extrasynaptic $GABA_A$ receptors, which mediate tonic inhibition responsible for regulating basic neuronal excitability.

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Development of cortical GABAergic innervation

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The mature neocortex contains many different classes of GABAergic inhibitory interneurons, distributed, with some degree of selectivity, through six layers, and through many different regions. Some of the events in the early lives of these neurones that may determine their ultimate destination, their maturation and their selective innervation of targets appropriate for each subtype, are discussed. Both time and place of birth influence the class of interneuron that an early post-mitotic interneuronal precursor will become, driven by the selective expression of different combinations of transcription factors in different regions of their birth places in the ganglionic eminence and ventricular zone. The long distance migration of these precursors along tangential routes in marginal, subventricular, and intermediate zones and their final radial movement, into the developing cortex, is regulated by chemical cues, both attractant and repellent. Once they arrive at their final destination, they must integrate into the developing circuitry. As they mature within the cortex, their axons grow and branch in highly specific patterns that may be partially determined by the genetic blueprint for each interneuronal class and partly by the environment in which they find themselves. Finally, as each interneuron class begins to form synapses with only certain postsynaptic targets, cell–cell recognition, most probably via protein–protein interactions across the synaptic cleft, facilitate the formation of appropriate synapses.

Keywords: synapse, GABA, inhibition, development, interneuron, cortex, synaptogenesis, ganglionic eminence

WHAT CAN MATURE NEURONAL STRUCTURE SUGGEST ABOUT DEVELOPMENT?

Anyone who has spent hours at the microscope, reconstructing cortical axons at high power, will be aware of the immense complexity of these axonal arbors and their huge diversity. A number of anatomical properties become apparent very rapidly. Firstly that pyramidal cell axons (with the exception of the drum stick like branches of layer 6 cortico-thalamic pyramidal axons in layer 4, Zhang and Deschênes, 1997), run almost straight through the neuropil, deviating significantly only as they encounter large obstacles, like blood vessels. They may have a gross structure that is peculiar to just one particular class of pyramidal cell, innervating only certain layers for example, but whatever the pattern of their branches, they run more or less straight through the cortex. They do not therefore give the impression of axons in search of a target. Pyramidal dendrites, on the other hand, are far from straight. Not only are they studded with spines of different lengths and shapes, which project in all possible directions from the shaft, but the dendritic shafts themselves take tortuous routes through the neuropil. The impression gained is that each class of pyramidal cell may have a preordained shape, in terms of the number, length, and diameter of its dendrites, whether or not it has well developed oblique dendrites, or an apical tuft, but that within these constraints, the dendritic spines and even the shafts modify this basic plan to seek appropriate excitatory axonal contacts.

In striking contrast, the axons of many classes of interneurons are often extremely convoluted, as though they were seeking targets, rather than waiting to be found. Moreover, each class of inhibitory interneuron has its own branching pattern, some having branches that exit almost at right angles, some branching at acute-, and others branching at oblique-angles. The following section explores the possibility that these differences result in part from the order in which different types of neurones arrive in the neocortex; spiny excitatory cells typically arriving before the GABAergic inhibitory interneurons that are assigned, at their birth, to the same layer (Miller, 1986a,b; Rymar and Sadikot, 2007).

ORIGINS AND FATES OF CORTICAL NEURONES

ORIGINS AND FATES OF SPINY EXCITATORY NEURONES

The spiny glutamatergic cells of the cortex (pyramidal and spiny stellate cells) are born in the ventricular zone. After their last cell division in the ventral zone, pyramidal neurones migrate to the cortical plate (the future cortex) along a common radial glial fascicle (Rakic, 1972). The radial unit model (Rakic, 1988) proposes that the position of a neuron's precursor in the ventral zone determines its final horizontal coordinates, while its birth date determines its radial position, i.e., the layer, or sublayer, it is destined to occupy. This is the anatomical basis for the columnar structure of cortex first proposed by Mountcastle et al. (1957). Later born spiny cells that are destined for more superficial layers, must pass through the layers of older neurones, as they migrate radially to their final positions. The cortex develops "inside out," laying down the pyramidal cells of the deepest layers first. The secreted signal, Reelin, its receptors and their downstream signaling pathways are thought to control/promote first this radial migration, then its termination (Huang, 2009a; Rakic, 2009; Vitalis and Rossier, 2011, for reviews). Recent studies using *in utero* intraventricular injection of EGFP-expressing retroviruses, confirm this

hypothesis, by demonstrating that sister neurones take up radially aligned positions in the cortex, across layers. Moreover, sister cells have a stronger propensity to form chemical synapses with each other, than with neighboring cells of differing lineage (Yu et al., 2009; Costa and Hedin-Pereira, 2010). That these sister cells end up in different layers and will therefore develop different morphological and physiological characteristics and make synaptic connections with different cortical and sub-cortical targets, demonstrates that the impact of lineage, while crucial, is modified by birth date and environment.

ORIGINS AND FATES OF INHIBITORY INTERNEURONS

The extent to which interneuronal properties are modified after their arrival in the cortex has been less studied to date. The vast majority of cortical interneurons are not born in the ventricular zone, but in the medial and caudal ganglionic eminence in the ventral forebrain and, in primates, in the subventricular zone (see also Inta et al., 2008).

INFLUENCE OF BIRTH PLACE

Most of the well characterized types of inhibitory interneurons are born within the ventral telencephalone (subpallium), a region comprising distinct morphological zones referred to as lateral, medial, caudal, and septal ganglionic eminence (LGE, MGE, CGE, and SGE, respectively, **Figure 1**; Wonders and Anderson, 2006; Batista-Brito and Fishell, 2009; Vitalis and Rossier, 2011). In addition, interneurons can originate from the endopeduncular and preoptic area (Gelman et al., 2009), and from the cortical subventricular zone (Inta et al., 2008).

The identity of newborn interneurons is regulated by the overlapping expression of specific transcription factors which is coordinated both spatially and temporally. The initial commitment to the GABAergic lineage is determined by the activity of *Dlx1/2* transcription factors. The expression of these factors is however under control of a proneural gene *Mash1* (Casarosa et al., 1999; Stuhmer et al., 2002). These genes are expressed widely throughout the subpallium, and they play a crucial role in development of cortical and olfactory bulb interneurons (Anderson et al., 1997a; Bulfone et al., 1998), and the striatum (Anderson et al., 1997b).

Parvalbumin-positive interneurons originate from the ventral area of the MGE (Wonders and Anderson, 2006) and their development relies on the activity of the *Lhx6* transcription factor which is itself controlled by the *Nkx2.1* transcription factor in this region (**Figure 1**; Liodis et al., 2007; Du et al., 2008). Although under the control of the same transcription factors, the dorsal area of the MGE is somehow specialized to give rise to a different class of interneurons, those that will express somatostatin/calretinin. Cell proliferation in both regions is under the regulation of β-catenin-dependent *Wnt* signaling pathway (Gulacsi and Anderson, 2008) and under the influence of sonic hedgehog (Shh) signaling which shows a gradual decrease in expression from dorsal to ventral MGE (Wonders et al., 2008; Xu et al., 2010).

In contrast, the cortical interneurons that express calretinin in combination with vasoactive intestinal polypeptide (VIP), VIP alone, or VIP, and cholecystokinine (CCK; Yozu et al., 2004; Butt et al., 2005; Fogarty et al., 2007) are generated in the CGE areas where the *Lhx6* transcription factor is not expressed (**Figure 1**; Flames and Marin, 2005). Their lineage is postulated to be regulated by *Nkx6.2* and *CoupTF1/2*, transcription factors that are widely, but not selectively expressed in this region (Sousa et al., 2009). It is currently unknown whether the LGE gives rise to any specific class of cortical interneurons (Wichterle et al., 1999). This region is, however, the main source of GABAergic striatal projection neurones (Anderson et al., 1997b), which originate from the ventral area, and of olfactory bulb interneurons (Waclaw et al., 2006), which originate from the dorsal area of LGE. Interneuronal progenitors in the endopeduncular and preoptic area give rise to NPY-containing interneurons including neurogliaform cells, under the guidance of the transcription factor *Nkx5.1* (**Figure 1**; Gelman et al., 2009). In addition, a small population of calretinin-positive interneurons, destined for deeper cortical layers, originate from the cortical subventricular zone at postnatal stages (Inta

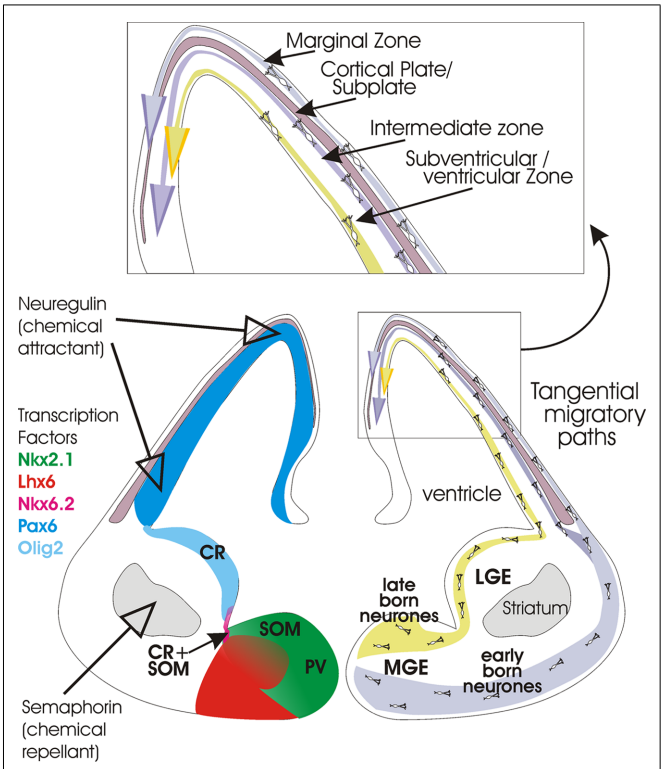

FIGURE 1 | Cartoon (lower right) summarizing the origins of cortical interneurons and the tangential migratory paths they follow to the developing neocortex (cortical plate and subplate at this stage of development). At first, early born interneurons follow a more ventral route before migrating along the marginal zone overlying the developing cortex. Once the cortical plate has developed, cells in this pathway also migrate along the intermediate zone. Later born neurones follow a more dorsal route and then migrate along the subventricular zone. See insert (top) for layers. On the left, expression patterns of some of the transcription factors that appear to play a role in differentiation and migration are indicated (see color key). The expression of two important extracellular signals, Semaphorin (3A and 3F) and Neuregulin-1 are also indicated. The birth places of somatostatin-containing (SOM), parvalbumin- containing (PV), calretinin- (CR), and calretinin- plus somatostatin-containing (CR + SOM) interneurons are also indicated. Coronal section through the brain of an embryonic mouse (approximately E14). Figure modified from Hernández-Miranda et al. (2010), Heng et al. (2007), Huang (2009b).

et al., 2008). The functional and spatial overlap in the expression levels of specific transcription factors and gradients of these factors across the ganglionic eminence, are likely to play crucial roles in determining the lineage of different types of cortical interneurons.

INFLUENCE OF BIRTH DATE

In addition to the place of birth, the temporal order of interneuronal specification is critical. It is now well established that different classes of interneurons are born at different times within the ganglionic eminence. According to the current model of neurogenic divisions in the MGE, neural progenitor division in the ventricular zone is mostly asymmetric, giving rise either to radial glia cells or to intermediate progenitor cells and neurones. The progression through the cell cycle in this zone is known to be regulated by the cyclin D1/cdk4/6 enzyme complex which is generally expressed in proliferating progenitors but also in post-mitotic neurones (Glickstein et al., 2007a).

In contrast, the intermediate progenitor cells located within the subventricular zone of the MGE are under the guidance of the cyclin D2/cdk4/6 complex and divide symmetrically to give rise to two neurones or two glia cells, which at this stage acquire the ability to migrate to the cerebral cortex or striatum (Ross, 2011). Following the temporal order of events, the first classes of interneurons born in this region between E9.5 and E15 are parvalbumin-positive and somatostatin-positive interneurons, followed by calretinin-positive interneurons which are born significantly later (between E12.4 and E15.5; Butt et al., 2007). Among these three interneuronal types however, only the future parvalbumin-positive interneurons in this region appear to be under selective regulation by the cyclin D2 complex. The deletion of cyclin D2 gene leads not only to a prominent reduction in the abundance of parvalbumin-positive interneurons in the more mature cortex, but also to a microcephalic phenotype with general impairments in cortical development (Glickstein et al., 2007b). This distinct phenotype appears to be a consequence of a decrease in the proliferation potential of cells within the subventricular zone of the MGE, leading to an increase in the number of cells exiting the cell cycle prematurely. This phenotype also suggests that the cell fate of future parvalbumin-positive interneurons is already specified at the stage of their proliferating intermediate precursors. Thus, regulation of the cell cycle of progenitors in the MGE is critical for determining the final numbers of specific interneuronal types in the cortex and may influence their final fate.

DOES GABA SIGNALING INFLUENCE THE FATE OF INTERNEURONS?

A role for GABAergic signaling in the control of proliferation of interneuronal precursors within the regions of ganglionic eminence has not been demonstrated. However, evidence from other brain regions indicates that GABA plays a potent role in determining the proliferating potential of neuronal progenitors in general. GABAergic signaling mediated by $GABA_A$ receptors was shown to inhibit proliferation of neural crest stem cells early in development (Andang et al., 2008). Proliferation of neocortical progenitors in the ventricular and subventricular zones of the developing cortex is down-regulated by GABA and glutamate, both of which can lead to depolarization of the plasma membrane and an increase in intracellular calcium. The number of progenitor cells able to synthesize DNA was reported to be reduced as a consequence of this increase in calcium (LoTurco et al., 1995). Subsequently, similar effects of GABA and glutamate on cell proliferation were reported for the subventricular zone progenitors, while the opposite was observed in the ventricular zone (Haydar et al., 2000). The effects of GABA and glutamate were mimicked by specific agonists for $GABA_A$ and NMDA/AMPA receptors, respectively, and blocked by specific receptor antagonists in both studies. Furthermore, neuronal progenitors isolated from the ventricular and subventricular zones were shown to express $GABA_A$ receptors with different functional properties; the former showing a higher affinity for GABA, slow desensitization, small currents and no evidence of any synaptic activity. In contrast to this, the later post-mitotic neurones in the subventricular zone showed spontaneous synaptic activity mediated by $GABA_A$ receptors, which was dependent on action potentials and arising from the local interneurons (Owens et al., 1999).

The extracellular concentrations of GABA in the ganglionic eminence regions may be as high as $0.5 \pm 0.1\,\mu M$ (Cuzon et al., 2006). Early interneuronal precursors and progenitors appear to be the main cellular source of this GABA, since GABA (Bellion and Metin, 2005) and GABA-synthesizing enzymes are demonstrable by immunolocalisation in the ganglionic eminence regions. At the same time, these cells may be regulated by GABA via an autocrine feed-back mechanism. By analogy with immature pyramidal neurones (Demarque et al., 2002), interneuronal precursors may be able to release GABA tonically in a calcium- and soluble NSF-attachment protein receptor (SNARE)-independent fashion, possibly from their growth cones, since the anatomically defined presynaptic elements are not yet established at this early stage (Bourgeois and Rakic, 1993; Balslev et al., 1996). It is of interest however to note that these cells express some proteins known to regulate the neurotransmitter release machinery such as NXPH1 (Batista-Brito and Fishell, 2009), a peptide that binds to α-neurexins (see below).

Interneuronal precursors express functional $GABA_A$ receptors, predominantly containing the α4/β1/γ1 or γ2 subunits, GABA transporters VIAAT and GAT1, as well as chloride transporters NKCC1 and KCC2 (Laurie et al., 1992; Ma and Barker, 1995; Batista-Brito and Fishell, 2009). GABAergic markers GAD65/67 and $GABA_B$ receptors are also expressed at the earliest stages of neuronal lineage progression, and the autocrine activity of GABA appears to regulate the neurite outgrowth in cells cultured from these regions (Maric et al., 2001). Although GABA transporters are expressed they are suggested to be non-functional at this stage (Demarque et al., 2002), as the excitatory GABAergic currents recorded are slow tonic currents, unaffected by the presence of GABA transporter inhibitors.

Thus, interneuronal precursors within the proliferating zones of the ganglionic eminence not only synthesize and probably release GABA, but also express all the components of the molecular machinery necessary to respond to the secreted GABA. By analogy with the well established regulation of both embryonic (LoTurco et al., 1995; Haydar et al., 2000) and adult neuronal

progenitors (Ge et al., 2007), a modulatory role for GABAergic signaling at the early stages of interneuron proliferation can be proposed. Additional direct evidence is, however, required.

MIGRATION OF INTERNEURONS

Following their birth and early specialization in the ganglionic eminence, post-mitotic cortical interneuronal precursors embark on a long journey to their final destinations within the cortex. With a remarkable precision, they first follow tangential routes along the cortical marginal, subventricular, or intermediate zones (**Figure 1**). They then migrate into the growing cortical plate in a radial direction (Huang, 2009b). Their progression along these defined routes is regulated by a number of chemical cues which, in a highly coordinated fashion, either attract or repel the migrating interneurons. One such cue is semaphorine (Sema 3A and 3F), a factor expressed in the lateral ganglionic eminence which inhibits the entry of the migrating interneurons into this region (Marín et al., 2001). Another example of factors influencing the directionality of migrating interneurons are *Slit* proteins and their *Robo* (Roundabout) receptors expressed throughout the ventricular and subventricular zone of the ganglionic eminence. Although the exact function of these proteins is unclear, knock out mice studies have revealed miss-localization of calbindin-positive interneurons in the striatum and the embryonic cortex, and altered morphology of these cells (Andrews et al., 2008).

Ephrin EphA5/EphA4 receptors are expressed in the ventricular zone and are chemo-repellent for MGE-derived neurones (Zimmer et al., 2008). The other powerful cues include attractant neuregulin-1 (Yau et al., 2003; Flames et al., 2004), hepatocyte growth factor/scatter factor (Powell et al., 2001), and chemokine stromal-derived factor 1 (SDF-1; also known as CXCL12) which was shown to influence tangential migration in the subventricular/intermediate zone and integration of interneurons into their appropriate cortical layers (Stumm et al., 2003; Tiveron et al., 2006; Li et al., 2008; Lopez-Bendito et al., 2008). The migration is strongly enhanced by neurotrophins (BDNF and NT4; Polleux et al., 2002), glia-derived neurotrophic factor (GDNF; Pozas and Ibanez, 2005), and glutamate acting on AMPA receptors (Poluch et al., 2003). BDNF was also found to regulate the distribution of Cajal–Retzius cells in the medial zone and migration of interneurons within the cortex (Alcantara et al., 2006).

Cortical entry of tangentially migrating interneuronal precursors arriving from the medial ganglionic eminence is enhanced by GABA via $GABA_A$ receptors. As these cells progress toward the cortex, they also show increased sensitivity to GABA (Cuzon et al., 2006), in parallel with increased levels of expression of α1, α2, α5, γ2, and γ3 subunits of $GABA_A$ receptors (Cuzon Carlson and Yeh, 2011). Enhanced motility of interneurons is dependent on $GABA_A$ receptor-mediated depolarisation and downstream activation of L-type calcium channels (Bortone and Polleux, 2009). However, soon after interneurons enter the cortex their spontaneous intracellular calcium oscillations and their migration terminate. This was shown to be caused by an increase in the expression of KCC2 transporter which reduces the intracellular chloride concentration and terminates depolarizing activity of $GABA_A$ receptors (Bortone and Polleux, 2009). Possibly guided by intrinsic genetic cues or extrinsic environmental cues such as GABA itself (Ganguly et al., 2001; Kriegstein and Owens, 2001; Ludwig et al., 2003; Titz et al., 2003; Toyoda et al., 2003; Rivera et al., 2004; Leitch et al., 2005; Bortone and Polleux, 2009) or BDNF (Rivera et al., 2002, 2004), this change in the functional outcome of $GABA_A$ receptors expressed by migrating interneurons was suggested to play a key role in their correct positioning within the cortical layers. Once at the right place, further development of specific characteristics of some interneuronal classes, for example, those that express NPY, may also be modified by activation of $GABA_A$ receptors, as well as by BDNF released from their target neurones (Marty et al., 1996).

A DELAY IN THE MARGINAL ZONE

Thus, interneurons migrate tangentially from the regions in which they are born, through the marginal zone and along the subventricular and intermediate zones, reaching their final positions by radial migration within the cortex (Ang et al., 2003; Marín and Rubenstein, 2001, for review). There is, however, a delay *en route*. Interneurons are held in the marginal zone for a few days before entering the cortical plate (Tanaka et al., 2006). Interactions between the chemokine SDF-1 (CXCL12) and receptors expressed by the interneurons (CXCR4) delay interneuron entry into the cortex (Stumm et al., 2003; Tiveron et al., 2006; Li et al., 2008; Lopez-Bendito et al., 2008). It is proposed that changes in responsiveness to CXCL12 are responsible for controlling the timing of interneuron invasion of the cortical plate and that delayed entry is essential for the proper integration of interneurons into the cortical circuitry (Huang, 2009b, for review).

Since both pyramidal cells and at least some of the interneurons that are destined for the same layer are born at the same time (Miller, 1986a,b; Rymar and Sadikot, 2007), the delay in the marginal zone would ensure that interneurons enter each layer after the projection neurones have populated it (Pla et al., 2006). From their birthdays it would appear that parvalbumin, somatostatin, and somatostatin/calretinin-containing interneurons from the dorsal and ventral medial ganglionic eminence respectively (Butt et al., 2005; Wonders and Anderson, 2006; Wonders et al., 2008 for review) might be the first interneurons to enter the cortex, following an inside-out pattern similar to that of the pyramidal cells. Later, the VIP, calretinin/VIP, calbindin, VIP/Chat (Choline acetyltransferase) containing interneurons and NPY (neuropeptide Y) containing neurogliaform cells from the caudal ganglionic eminence (Yozu et al., 2004; Vucurovic et al., 2010; see also Batista-Brito et al., 2008; Gelman et al., 2009; Vitalis and Rossier, 2011, for review) would enter, but may not follow the conventional inside out pattern of destination. Since other interneurons may be the preferred targets of some of these later born VIP and calretinin-containing interneurons (by analogy with the CA1 region of the hippocampus, Freund and Buzsáki, 1996; Somogyi and Klausberger, 2005 for reviews), it seems reasonable to propose that interneurons like to "make an entrance," entering their final layer destination only after their preferred targets have arrived.

Interneurons do not therefore have, as it were, a blank cortical canvas upon which to organize themselves. The complexity of their axonal arbors most probably reflects their need to provide many opportunities for the formation of synaptic contacts with appropriate targets. An important recent study (Wierenga

et al., 2008, discussed in more detail below) confirms predictions that might be made from detailed examination of mature axonal and dendritic structures (see above), that inhibitory synapses do not exploit exploratory postsynaptic filopodia as many cortical excitatory synapses do, but must rely upon close physical associations between presynaptic axons and postsynaptic dendritic shafts. Whether and where such close associations occur and therefore where inhibitory synapses have opportunities to form, will be dependent upon the interneuronal axon arborisation within the context of the local target distribution (see also Stepanyants et al., 2004).

INPUTS TO INTERNEURONAL DENDRITES

Before leaving the question of target *versus* input selection, it is of interest to note that interneuronal dendrites do not typically exhibit the convoluted trajectories seen in pyramidal cells, nor are mature interneurons more than very sparsely spiny. With the exception of neurogliaform cells, each class, in each layer, appears to develop a stereotypical dendritic branching pattern, with relatively straight trajectories and few, if any spines. If (as suggested above) the excitatory axons are not seeking targets, but positioning themselves to receive the "correct" postsynaptic contacts and since the interneuronal dendrites are not convoluted or spiny, there is perhaps an anatomical explanation for the less finely tuned response properties of interneurons in primary sensory regions. Naturally, whether a synapse forms and even more importantly during development, whether it is maintained and strengthened, depends on additional factors, such as coincident activity. However, the opportunities available to interneuronal dendrites to "select" inputs, may be more limited by their own structure than those available to pyramidal dendrites.

WHERE DO INHIBITORY SYNAPSES FORM?

In postnatal hippocampal cultures, the formation of synapses can be observed over hours. Excitatory, glutamatergic connections that formed between exploratory dendritic filopodia and axons often became stable and exhibited structural and immunocytochemical properties of mature synapses after a few hours. This was not, however, the case for GABAergic synapses. Contacts did form between dendritic filopodia and inhibitory axons, but these contacts were always short-lived. Only those that formed between an axon and a dendritic shaft, where these elements were in close proximity, i.e., at cross-over points, developed into stable, mature synapses. Within 1 h, the new presynaptic bouton had accumulated VGAT, though accumulation of postsynaptic scaffold proteins such as gephyrin did not occur within this time frame (Wierenga et al., 2008).

That there is a strong recognition signal between the pre and postsynaptic components of a potential future synapse and that excitatory and inhibitory cortical synapses employ different mechanisms and signals, is demonstrated by a calcium imaging study in early postnatal hippocampal cultures. Calcium activity was much higher in dendrites whose filopodia had made successful contacts with excitatory axons. It was independent of glutamate receptor activation suggesting that other, probably protein–protein interactions across the future synaptic cleft mediate this response. Moreover, this activity occurred within the first minute of the contact forming and was higher in those contacts that subsequently became stable. Activity in dendrites whose filopodia had failed to make contacts was much lower and contact with GABAergic axons produced no calcium signal (Lohmann and Bonhoeffer, 2008). Exploratory filopodia therefore recognize appropriate excitatory axons and respond to contact with them with a strong calcium signal, which is proposed to contribute to their stabilization.

A large body of work, some of which is summarized below, has studied GABAergic synapse formation in neuronal cultures and co-cultures. These studies have provided extremely important insight, but differences between *in vivo* and *in vitro* should not be overlooked. One interesting point is the weeks it can take for innervation to mature during development *in vivo*, compared with the minutes to hours needed to form a functional (if not fully mature) synapse in culture (Ahmari et al., 2000; Bresler et al., 2004; see also Kirov et al., 1999). In addition to the many factors that promote synaptogenesis, there must be some very strong controls exerted to ensure that uncontrolled aberrant synaptogenesis does not occur, either in the temporal, or in the spatial domain (see L1CAM, PSA–NCAM below).

CELL ADHESION MOLECULES

The first critical step in the formation of a mature inhibitory synapse in cortical structures appears, therefore, to be close proximity of pre and postsynaptic elements and does not involve postsynaptic filopodia. It has yet to be determined whether these opportunities occur near randomly and are successful only at "appropriate" cross-over points, i.e., when the class of interneuron coincides with the appropriate postsynaptic compartment, or whether each class of axonal arborisation forms these cross-over points preferentially with appropriate potential targets. Further, if the latter is true, is it the fine structure of the axonal arbor predetermined genetically, or is it the environment in which it finds itself, that dominates, or it is a combination of the two?

THE L1 FAMILY OF IMMUNOGLOBULIN CELL ADHESION MOLECULES

Cell adhesion molecules can play an important role in determining the direction of inhibitory axon outgrowth (Panicker et al., 2003, for review). This has been demonstrated in the developing cerebellum. Basket cell axons first make contact with Purkinje cell somata. Whether they then extend processes along the axon initial segment to form *pinceau* synapses there, or make aberrant synapses elsewhere, depends on a subcellular gradient of neurofascin186 (NF186). This is a member of the L1 family of immunoglobulin cell adhesion molecules (L1CAM), which is recruited to the initial segment by ankyrin G, a membrane adaptor protein that is restricted to the axon initial segment (Ango et al., 2004). More recently, interactions between L1CAM and ankyrin have also been shown to be important for the elaboration and branching of GABAergic basket cell axons around postsynaptic somata in the prefrontal and cingulate cortex (Guan and Maness, 2010).

The L1 family of immunoglobulin cell adhesion molecules phosphorylation (on Tyr-1229) which is very high at P0, decreases postnatally, to become nearly undetectable in the adult (at P60).

This decrease in phosphorylation is correlated with increased L1CAM-ankyrin binding and coincides with periods of synaptogenesis and synapse remodeling (Guan and Maness, 2010). Another cell adhesion molecule, CHL1 (Close Homolog of L1) which is localized to cerebellar Bergmann glial cells and to stellate neurones while their axons are developing, plays a critical role in the targeting of stellate axons to Purkinje cell dendrites. Without CHL1, the axons show atypical branching and orientation, synaptogenesis is reduced and synapses are not maintained (Ango et al., 2008). A role for CHL1 in organizing the chaperoning of the presynaptic SNARE complex, through interactions between the intracellular domain of CHL1 and Hsc70, has also been demonstrated (Andreyeva et al., 2010).

NEURONAL CELL ADHESION MOLECULES

Neuronal cell adhesion molecules (NCAMs) have also been shown to influence neurite outgrowth, axon branching, and GABAergic synaptogenesis. Cell densities were not altered in a mouse schizophrenia model in which the shedding of soluble extracellular domains (ectodomains) of NCAM (NCAM-EC) is enhanced. There were, however, abnormalities in cortical GABAergic interneurons and reduced puncta immuno-positive for the presynaptic GABAergic markers, GAD65, GAD67 and GAT1 (GABA transporter 1), suggesting that fewer GABAergic synapses had formed (Pillai-Nair et al., 2005). In a study that focused on cortical basket cells, over-expression of NCAM-EC (which would block interactions with membrane-bound NCAM) disrupted neurite arborisation at the time when maximum growth is expected. This resulted in a reduction in the numbers of perisomatic synapses *in vivo*, while in cortical neurone cultures, soluble NCAM-EC acted as a dominant inhibitor of NCAM-dependent neurite branching and outgrowth (Brennaman and Maness, 2008). In support of the suggestion that NCAM promotes neurite development and synaptogenesis, while its soluble ectodomain inhibits these processes, inhibition of the shedding of NCAM-EC in cortical neurone cultures promoted neurite outgrowth and branching (Hinkle et al., 2006).

Polysialic acid (PSA) masks NCAM function; a control that appears critical for normal brain development (Weinhold et al., 2005). Disruption of this control results in a severe phenotype, including progressive hydrocephalus, postnatal growth retardation, and precocious death, despite the presence of NCAM. In addition, wiring defects included accumulation of cells in the anterior subventricular zone and rostral migratory stream. PSA concentrations fall just after eye opening in the rodent, a decline that does not occur if the eyes remain closed. This change in PSA-masking of NCAM is therefore proposed to be activity-dependent and to promote perisomatic inhibitory synaptogenesis and the onset of ocular dominance plasticity. It is therefore proposed that a major function of PSA is to down regulate NCAM function at specific stages of development (Di Cristo et al., 2007).

GABA

Widespread remodeling of cortical circuitry during certain critical periods, such as that which follows eye opening in carnivores and rodents, is essential for the establishment of mature function. Activity in the circuit, driven by sensory input, is an essential contributor to this developmental plasticity. For example, although the targeting of specific postsynaptic subcellular compartments by different classes of interneurons occurs in the absence of sensory input (Di Cristo et al., 2004), visual deprivation retards the maturation of perisomatic inhibitory innervation of pyramidal cells (Chattopadhyaya et al., 2004) and the normal threefold increase in GABAergic input, during the critical period, is prevented (Morales et al., 2002). Moreover, this is a two way process. Evidence is growing that the functional maturation of inhibitory synapses triggers activity-dependent changes during critical periods and that development of perisomatic inhibition by parvalbumin-containing basket cells acting through α1-$GABA_A$ receptors drives these changes (Hensch, 2005, for review).

Knock out of one isoform of glutamic acid decarboxylase (GAD65) prevented the loss of visual responsiveness that is the normal consequence of eye closure during the critical period. This function was restored by infusion of the positive $GABA_A$ receptor modulator Diazepam into the visual cortex (Hensch et al., 1998). GABA itself, or its interactions with $GABA_A$ receptors also appear to influence axonal branching and synapse formation. In adolescent visual cortex, conditional knock down of GAD67 (but not of GAD65) in parvalbumin-containing interneurons, resulted in axonal branching defects in these neurones and a reduction in inhibitory innervation of pyramidal somata. Maintenance of those synapses that did form was not, however affected. Over-expression of GAD67 in these cells accelerated the maturation of somatic innervation. Since the knock down affected only PV-cells and since a less severe modification (deletion of only one *GAD1* allele), also resulted in reduced somatic innervation, it appears that "spill-over" of GABA from neighboring synapses is insufficient to promote axonal branching and synaptogenesis. It does, however appear, that GABA itself is an essential component of the signal, since somatic innervation could be rescued by blocking GABA-uptake or by adding Diazepam. Moreover, activation of $GABA_B$ receptors also appears to contribute, since addition of baclofen rescued somatic innervation, at least in part, although terminal branching remained reduced (Chattopadhyaya et al., 2007).

IMPORTANCE OF $GABA_A$ RECEPTORS IN SYNAPSE FORMATION AND STABILIZATION

There is a general consensus that cortical synaptic $GABA_A$ receptors contain two α-subunits, two β-subunits, and one γ-subunit. While the β-subunits are required for transport of the pentomeric receptors to the cell surface and may confer dendritic *versus* somatic targeting of $GABA_A$ receptors and α-subunits may determine their localisation at specific synaptic subtypes (Thomson and Jovanovic, 2010, for review), the γ-subunit is thought to be essential for synaptic *versus* extrasynaptic localisation. When the expression of γ-subunits is suppressed, the clustering of $GABA_A$ receptors becomes disrupted and the GABAergic innervation of neurones lacking γ2-receptors is profoundly reduced (Schweizer et al., 2003; Li et al., 2005). That it is the clustering of $GABA_A$ receptors at potential synaptic sites that is important for establishing GABAergic innervation, was also indicated by experiments in which neurones were transfected with dominant negative GODZ [Golgi-specific DHHC (Asp-His-His-Cys) zinc finger protein], a

member of the DHHC palmitoyl acyltransferase family. Without the palmitoylation of the γ2 subunit by GODZ, trafficking of $GABA_A$ receptors to the cell surface is disrupted and GABAergic innervation of transfected cells is reduced (Fang et al., 2006).

Clusters of $GABA_A$ receptors appear, therefore, to promote the stabilization and maturation of presynaptic GABAergic terminals. The converse is also true. Extrasynaptic $GABA_A$ receptors exhibit much greater lateral mobility in the plasma membrane than synaptic receptors. The presence of a presynaptic terminal reduces lateral mobility, enhancing the stabilization of $GABA_A$ receptor clusters at synapses. Although it is unclear whether gephyrin controls the formation or the stability of receptor clusters, or in some way regulates the numbers expressed on the cell surface, an important role for gephyrin in facilitating the accumulation of $GABA_A$ receptors at inhibitory synapses was indicated by the reduction in receptor clusters and greater mobility of those that existed when gephyrin was knocked down (Jacob et al., 2005).

INTERACTIONS BETWEEN NEUROLIGIN (NL) AND NEUREXIN (NX)

Clearly, interactions between the presynaptic axon and postsynaptic cell are required for GABAergic synapses to form, mature and survive. There has recently been intense interest in the interactions between presynaptic neurexins (Nx) and postsynaptic neuroligins (NL); interactions that are thought to be fundamental to the formation of synapses. Of the four neuroligins, NL1, NL3, and NL4 are found primarily at excitatory and NL2 at inhibitory synapses (Graf et al., 2004; Chih et al., 2005). In immature neurones, NL2 aggregates with $GABA_A$ receptors that do not (yet) face a GABAergic nerve terminal, suggesting a role for NL2 in determining the future locations of GABAergic synapses (Varoqueaux et al., 2004; see also Ziv and Garner, 2004; Gerrow et al., 2006).

In sufficiently reduced systems, NL2 appears to play a near essential role in the formation of functional GABAergic synapse-like contacts. In neurone-HEK293 cell co-cultures, the transfected HEK293 cells that express NL2 and $GABA_A$ receptors acquired functional GABAergic axonal contacts, while those expressing only $GABA_A$ receptors, or NL1 and $GABA_A$Rs, did not (Dong et al., 2007). Indeed, NL2 alone, expressed in non-neuronal cells, or attached to beads (Graf et al., 2004), appears able to promote the formation of presynaptic boutons. NL2 does not, however, appear to be an absolute requirement for GABAergic synapse-formation *in vivo* (Varoqueaux et al., 2006; Chubykin et al., 2007). In triple NL-knock out mice (NL1, NL2, NL3), the number of synapses appeared normal, but mismatches between pre and postsynaptic proteins developed. For example, VGlut was found co-localized with gephyrin and VIAAT with PSD95. Moreover, a range of excitatory, and inhibitory synaptic vesicle markers were reduced in these triple knock outs, e.g., the soluble SNARE regulators complexin 2 and (SNAP, as well as KCC2 were reduced. However, the presynaptic active zone proteins Munc13-1 and RIM1/2, SNAP-25, and calbindin appeared unchanged, as did postsynaptic gephyrin, β-dystroglycan and α1, and β2/3 $GABA_A$R subunits (Varoqueaux et al., 2006).

Neurorexins appear to recruit a number of important proteins to the active zone, including presynaptic Ca^{2+}-channels (O'Connor et al., 1993). Indeed, in the non-viable triple α-neurexin knock out, N and P/Q Ca^{2+}-channels do not cluster at active zones and action potential-triggered release fails (Missler et al., 2003; Zhang et al., 2005). The stabilization of Nxs, particularly Nx-1β, at the active zone is also regulated by its interactions with postsynaptic proteins (like NLs) and the rate at which these proteins turnover is reduced by neuronal activity and by presynaptic $GABA_B$ receptors (Fu and Huang, 2010). NLs interact with and possibly localize and stabilize Nxs, these cross-cleft interactions may therefore promote and modify a range of synaptic properties. Again, a role for specifying the location and types of synapse and more subtle aspects of their function, rather than an absolute requirement for their presence or absence, is suggested for the NL–Nx interactions.

ALTERNATIVE SPLICING AND SYNAPSE SPECIFICITY

Presynaptic Neurexins (1α, 2α, 3α, 1β, 2β, 3β) exhibit extensive alternative splicing (>2000 potential variants, Missler and Sudhof, 1998; Tabuchi and Sudhof, 2002) particularly within their laminin neurexin sex (LNS) hormone binding protein domains. Six LNS domains in α-neurexin and one in β-neurexin exhibit Ca^{2+}-dependent binding to extracellular domains of NLs, dystroglycan, and neurexophilin and provide a high affinity α-latrotoxin binding site (Craig and Kang, 2007, for review). By altering Ca^{2+}-binding affinity, splice insertions at these sites alter their interactions with other proteins (Sheckler et al., 2006).

Alternative splicing of NLs is less extensive, but powerfully influences their interactions with other proteins. Commensurate with its localisation at GABAergic synapses, NL2 is not normally alternatively spliced at splice site B. Splice insertion at this site restricts the activity of neuroligins to glutamatergic synapses and negates binding to the β-neurexin isoform that contains an insertion in site 4 [Nx-1β4(+)]. The selectivity imposed by the B insertion requires the N-glycosylation site in B (Boucard et al., 2005). Neuroligin lacking the B-site insert also interacts with Nx-1α, with or without a neurexin-site 4 (S4) insertion (Chih et al., 2005). When expressed in COS cells, Nx-1β4(+) and Nx-1α4(−) induced the inclusion of gephyrin and NL2, but not of PSD95 in postsynaptic densities in co-cultured neurones. Recombinant Nx-1β4(+)-Fc, designed to block extracellular interactions with NL, selectively reduced the density of VGAT-positive terminals with no effect on the density of vGlut1-positive puncta (Chih et al., 2006). Thus the S4 insert in Nx-1β permits binding to NL2, which typically does not include the B-site insert, and promotes clustering of postsynaptic GABAergic proteins. The S4 insert in Nx-1β also decreases the ability of Nx-1β to cluster NL1, NL3, and NL4 and postsynaptic glutamatergic scaffold proteins (Graf et al., 2006). A developmental and possibly activity-driven decrease in the inclusion of the S4 insert correlates with glutamatergic *versus* GABAergic synaptogenesis (Kang et al., 2008).

Finally, the impact of deleting NL2 in the adult appears to vary with the type of synapse involved. In adult CA1, gephyrin- and $GABA_A$ receptor-positive puncta were significantly reduced in *stratum pyramidale* (primarily basket cell synapses on pyramidal somata), but not in *stratum radiatum* (innervation from a range of dendrite-preferring interneuronal subtypes). This was despite the continuing presence of VIAAT immuno-labeling, suggesting that inhibitory innervation of *stratum pyramidale*

was maintained, but with reduced postsynaptic specialization (Poulopoulos et al., 2009).

FINAL REMARKS

From the early proliferating precursors within the ganglionic eminence to morphologically and functionally diverse, and synaptically connected interneurons within the cortex, interneurons undergo a process of remarkable transformation in time and space guided by the activity of transcription factors, but shaped by continuous signaling to and from their surrounding environment mediated by specific protein–protein interactions at the cell surface. Although the main outline of this journey is increasingly clear, a number of key steps toward the final complex phenotype of interneurons remain uncharacterized. One of the least known, yet perhaps the most critical of these, is the outgrowth and the coordination of developing axonal and dendritic arbors of interneurons once they have reached their final destinations in the cortex. This step remains challenging to tackle experimentally and will require systematic *in vivo* structural and functional analysis. The step that follows this, during which interneurons approach their synaptic partners with a remarkable selectivity and form morphologically and functionally highly specialized synapses is equally challenging. A number of cell adhesion proteins discussed here have been implicated in this step. However, the molecular key to the synaptic specificity remains to be uncovered.

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Functional hallmarks of GABAergic synapse maturation and the diverse roles of neurotrophins

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Functional impairment of the adult brain can result from deficits in the ontogeny of GABAergic synaptic transmission. Gene defects underlying autism spectrum disorders, Rett's syndrome or some forms of epilepsy, but also a diverse set of syndromes accompanying perinatal trauma, hormonal imbalances, intake of sleep-inducing or mood-improving drugs or, quite common, alcohol intake during pregnancy can alter GABA signaling early in life. The search for therapeutically relevant endogenous molecules or exogenous compounds able to alleviate the consequences of dysfunction of GABAergic transmission in the embryonic or postnatal brain requires a clear understanding of its site- and state-dependent development. At the level of single synapses, it is necessary to discriminate between presynaptic and postsynaptic alterations, and to define parameters that can be regarded as both suitable and accessible for the quantification of developmental changes. Here we focus on the performance of GABAergic synapses in two brain structures, the hippocampus and the superior colliculus, describe some novel aspects of neurotrophin effects during the development of GABAergic synaptic transmission and examine the applicability of the following rules: (1) synaptic transmission starts with GABA, (2) nascent/immature GABAergic synapses operate in a ballistic mode (multivesicular release), (3) immature synaptic terminals release vesicles with higher probability than mature synapses, (4) immature GABAergic synapses are prone to paired pulse and tetanic depression, (5) synapse maturation is characterized by an increasing dominance of synchronous over asynchronous release, (6) in immature neurons GABA acts as a depolarizing transmitter, (7) synapse maturation implies inhibitory postsynaptic current shortening due to an increase in alpha1 subunit expression, (8) extrasynaptic (tonic) conductances can inhibit the development of synaptic (phasic) GABA actions.

Keywords: GABAergic synaptic transmission, quantal analysis, presynaptic function, tonic inhibition, synapse development, BDNF, NGF, excitatory–inhibitory balance

INTRODUCTION

What does the construction of the very first synapses during embryonic development and the generation of new synapses after a stroke in the aged brain have in common? What enables or constrains the formation of new synapses after transplantation of exogenous neurons into an adult brain? How could we possibly stimulate inhibitory synapse formation in an epileptic cortex – or repair the sad consequences of alcohol consumption of a pregnant mother when too many inhibitory neurons are lost in her baby's brain? – We are still far from an all-embracing answer to these questions, and more work is needed to understand the impact of synaptogenesis on the performance of each given functional system during a particular stage of development. Nonetheless we will try to delineate some basic principles of synapse development that can be regarded as common, at least in the sense that they are applicable to synaptic connections using the inhibitory γ-amino-butyric acid (GABA) as a neurotransmitter of both the hippocampus and the superior colliculus, i.e., two brain structures requiring intact GABAergic inhibition while fulfilling clearly different functions.

The hippocampus is involved in learning and memory formation, but apart from its functions, it has served as a classical model system for very many aspects of synaptic transmission and synapse development (see Ben Ari et al., 2007; McBain and Kauer, 2009). Likewise, the superior colliculus of mammals, or the optic tectum of avians, amphibians, and fish, is a structure that has become quite popular for its retina-, head-, and body-related sensory and motor maps and its role as novelty detectors (see Boehnke and

Munoz, 2008; Stein et al., 2009 for recent review). As a nearly two-dimensional projection area of retinal and visual cortical afferents it served to identify a number of molecules relevant for the formation of orderly connections (Feldheim and O'Leary, 2010). It also appears to be well suited to study inhibitory synaptogenesis since the superior colliculus is reputed to contain the largest amounts of GABA and the highest density of GABAergic synaptic terminals in the brain (see Grantyn et al., 2004 for references).

In the following we shall highlight eight principles that appear to determine the performance of GABAergic synapses during embryonic and early postnatal development. Findings from the rodent superior colliculus will be discussed and, if available, compared to results from hippocampal preparations under the common assumption that fundamental principles of synapse development and function are shared among many brain regions despite regional tailoring of synapse parameters to the specific developmental and functional requirements. The focus of the discussion will be on functional parameters of synaptic transmission at the expense of molecular determinants of developmental changes. Building on this, we explore to what extent these principles are governed by neurotrophin signaling during development and how they predict characteristics of GABAergic synapses in lesioned brain tissue or during adult neurogenesis.

FUNCTIONAL MATURATION OF GABAergic SYNAPSES

SYNAPTIC TRANSMISSION STARTS WITH GABA

The role of GABA as a "pioneer transmitter" (Ben Ari et al., 2007) implies a variety of paracrine actions of this neurotransmitter along with a lead in synaptogenesis. The rule of GABAergic lead during the formation of network activity is based on patch clamp recordings from hippocampal slices of newborn rats where AMPAR-mediated synaptic events were rare or entirely missing while GABAergic inhibitory postsynaptic currents (IPSCs) could either be induced by electrical stimulation or detected as spontaneously occurring events (Hollrigel and Soltesz, 1997; Tyzio et al., 1999).

In the superior colliculus, action potential-mediated spontaneous and unitary evoked synaptic currents did indeed occur in the absence of glutamatergic synaptic activity at E17 (Grantyn et al., 2004), i.e., at an age when inhibitory synaptic currents were first seen in the retina (Unsoeld et al., 2008). Furthermore, evaluation of double immunostaining using antibodies against synaptophysin and the vesicular GABA transporter vGAT showed that at E17 all presynaptic puncta were GABAergic (**Figure 1A**). Even at postnatal day (P) 0 the fraction of GABAergic terminals was as high as 87% (J. Walter and R. Grantyn, unpublished result).

The GABAergic lead during development has been attributed to earlier differentiation and functionality of GABAergic interneurons as opposed to glutamatergic principal cells (Gozlan and Ben Ari, 2003). As for the function of GABAergic pioneer synapses, one has to consider their depolarizing polarity (see below, part 6) and the associated capacity to generate local Ca^{2+} transients which make them a good substitute for the lack of glutamatergic inputs. Synaptic GABA release could also contribute to some initial steps of Ca^{2+}-dependent neuron differentiation, such as dendrite and axon outgrowth (see Sernagor et al., 2010), and it might assist the formation of glutamatergic afferents by producing giant depolarizing potentials The latter concept of GABAergic excitation driving maturation of the glutamatergic synaptic phenotype, also termed "mènage à trois," has attracted particular attention (Ben-Ari et al., 1997). Interestingly, GABAergic synaptic transmission could not only precede glutamatergic transmission within distinct sets of synapses, but one and the same synapse type was shown to shift from an initial GABAergic phenotype to a mixed glutamatergic/GABAergic and, later on, to a predominantly glutamatergic

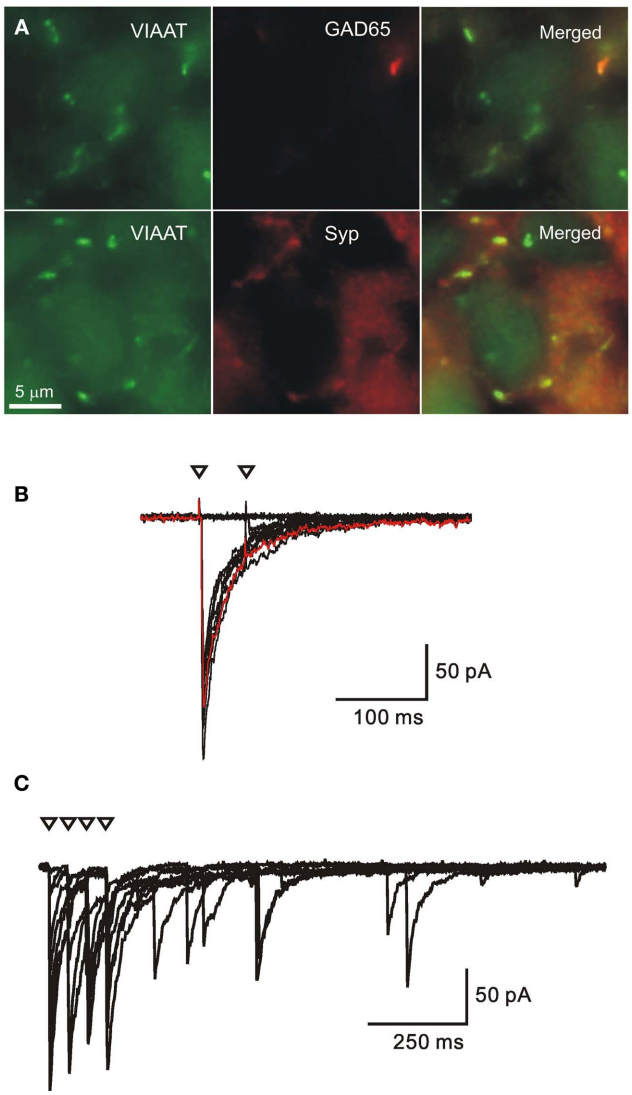

FIGURE 1 | Early onset of GABAergic synaptic transmission and synapse formation in the embryonic superior colliculus (E17). (A) Specimens from sections of the immunostained superior colliculus. Most GABAergic synaptic terminals are double-immunoreactive for the vesicular inhibitory amino acid transporter (VIAAT) and synaptophysin (Syp) but few of them already stain for GAD65. **(B,C)** Electrophysiological recording of unitary synaptic responses in acute slices from the E17 mouse superior colliculus. Minimal stimulation with two **(B)** and four **(C)** pulses at an interval of 50 ms. Triangles denote time of stimulation. Note absence of second eIPSC in A and asynchronous delayed IPSCs in **(B)**. (Modified from (Grantyn et al., 2004).

phenotype (Gutierrez, 2003; Sufiulina et al., 2010). While adult mossy fiber terminals are known to mediate a strong excitatory input to CA3 pyramidal neurons via postsynaptic AMPA-, NMDA-, and kainate receptors, at neonatal age these same terminals use GABA to induce depolarizing excitatory actions via GABA(A)Rs (Safiulina et al., 2006). Under pathophysiological conditions, such as the generation of seizures, the GABAergic phenotype can be re-expressed (Gutierrez and Heinemann, 2001).

It should be noted, however, that for many other brain areas the GABAergic lead in synaptogenesis is not yet firmly established, because recordings *in situ* were rarely performed early enough to catch the very onset of synaptogenesis and transmission at newborn synapses. In addition, different developmental scenarios are possible in the lower brainstem and spinal cord. For instance, in the rodent lateral superior olive glycinergic/GABAergic inhibitory synapses were shown to transiently use the neurotransmitter glutamate during the period of activity-dependent synapse refinement (Gillespie et al., 2005).

NASCENT/IMMATURE GABAergic SYNAPSES OPERATE IN A BALLISTIC MODE (MULTIVESICULAR RELEASE)

A distinct feature of GABAergic synaptic transmission in the embryonic mouse brain is the large amplitude of action potential-mediated spontaneous or evoked IPSCs (sIPSCs, eIPSCs, respectively; Cohen et al., 2000; Kirischuk et al., 2005) and their pronounced fatigue under condition of rapid and repetitive activation. This is illustrated in **Figure 1B**. In an E17 superior colliculus slice, the well-defined eIPSCs induced by the first stimulus preceded a complete failure after the second. When applying a stimulus train larger eIPSCs were only generated after preceding failures, and there was a tendency for asynchronous release after the stimulus trains (**Figure 1C**), suggesting a protracted elevation of presynaptic Ca^{2+} concentration (Kirischuk and Grantyn, 2003).

Were these large responses induced by one or several synaptic terminals? – According to the "one site-one vesicle"-hypothesis of synaptic transmission (Korn and Faber, 1987) one might be tempted to suggest that the connections in the embryonic colliculus must be composed of multiple contacts and/or multiple active zones per ending. However, the electron microscopy (EM) images from P1 colliculi and evaluation of immunostained synaptic terminals in E17 tecta indicate that this is rather unlikely (Lund and Lund, 1972; Juettner et al., 2005). Taking into account that under the given experimental conditions miniature IPSC (mIPSC) amplitudes amounted to 25–30 pA we concluded that 10–15 vesicles may simultaneously be released from just one site.

The possibility of multivesicular release has also been considered for inhibitory synapses in hippocampal cultures (Fedulova and Veselovsky, 2002) and hippocampal slices (Biro et al., 2006) while data from small glutamatergic synapses appears contradictory (see Oertner et al., 2002; Huang et al., 2010 vs. Chen et al., 2004). Kirischuk et al. (1999) characterized the release of GABAergic from single synaptic terminals in dissociated cultures from the embryonic rat tectum. Selective stimulation of axon terminals after loading a Ca^{2+} indicator allowed us to record single-bouton-evoked stimulus-locked IPSCs (sbIPSCs) along with the respective asynchronous delayed IPSCs (dIPSCs; **Figures 2A,B**) and presynaptic Ca^{2+} transients. An estimate of quantal size (Q) can be extracted from amplitude distributions of sbIPSCs and dIPSCs (**Figure 2C**). Dividing maximal or mean sbIPSC amplitudes by the experimentally derived values of Q (**Figures 2D,E**) one directly obtains the maximal or mean quantal content (m) (Kirischuk and Grantyn, 2002). Even under physiological conditions ($[Ca^{2+}]/[Mg^{2+}] = 2$), the mean m value was larger than 1 in 30 out of 40 tested boutons (range: 0.4–6), and the maximal m reached 8–12. At any given synapse, the mean sbIPSC amplitudes changed with the third power of the presynaptic bulk Ca^{2+} concentration (**Figure 2F**). With few exceptions, these boutons had one active zone per terminal only, which lead us to suggest that these immature GABAergic terminals had the capacity to release several vesicles from just one docking site (Kirischuk and Grantyn, 2002).

It should be mentioned that simultaneous release of several transmitter quanta can even occur spontaneously, due to random elevations of presynaptic $[Ca^{2+}]$, as observed in basket cell terminals in slices of the postnatal rat cerebellum (Llano et al., 2000b). Lowering extracellular $[Ca^{2+}]$ would decompose "giant" mIPSCs and eventually limit spontaneous synaptic activity to monoquantal events.

IMMATURE SYNAPTIC TERMINALS RELEASE VESICLES WITH HIGHER PROBABILITY THAN MATURE SYNAPSES

Presynaptically, synapse maturation is characterized by the formation of multiple release sites and the differentiation of the release machinery which encompasses a complex set of changes affecting docking, molecular and positional priming, fusion, site clearing, and several pathways for replenishment of the vesicle pool (Neher and Sakaba, 2008; Pang and Sudhof, 2010). Most commonly, changes in the release are characterized by invoking the statistical parameter "average probability of release" (Pr), often defined as the likelihood that any contact of a synaptic connection would liberate one quantum of the transmitter in response to a presynaptic action potential. In most cases Pr is not directly accessible for measurement, but determined by binomial fitting (for instance, Stricker et al., 1996), variance–mean analysis (Clements and Silver, 2000) or covariance analysis (Scheuss and Neher, 2001). In the frame of the binomial model of synaptic transmission, the unitary postsynaptic response (i.e., the response obtained by activation of just one presynaptic neuron) would reflect the product of N, Pr, and Q, N being the total number of synapses/active zones/docking sites formed by the presynaptic cell.

In synapses with multivesicular release, presynaptic differentiation can also be characterized on the basis of changes of m, the mean quantal content (Taschenberger et al., 2005). In view of the difficulties to accurately determine the number of active zones participating in release (or the structure-based "histological N") it has become acceptable to disregard the site-dependent heterogeneity of individual release sites and to conceptually merge all the vesicles to one common pool, an approach first introduced for the calyx of Held (Sakaba et al., 2002) and later extended to general models of quantal synaptic transmission (Neher and Sakaba, 2008; Pan and Zucker, 2009), where N becomes the number of vesicles in the readily releasable pool (RRP). The average probability that a given vesicle is released from that pool is p_{ves}. (Heterogeneous vesicle pools and different modes of vesicle fusion are complexities to

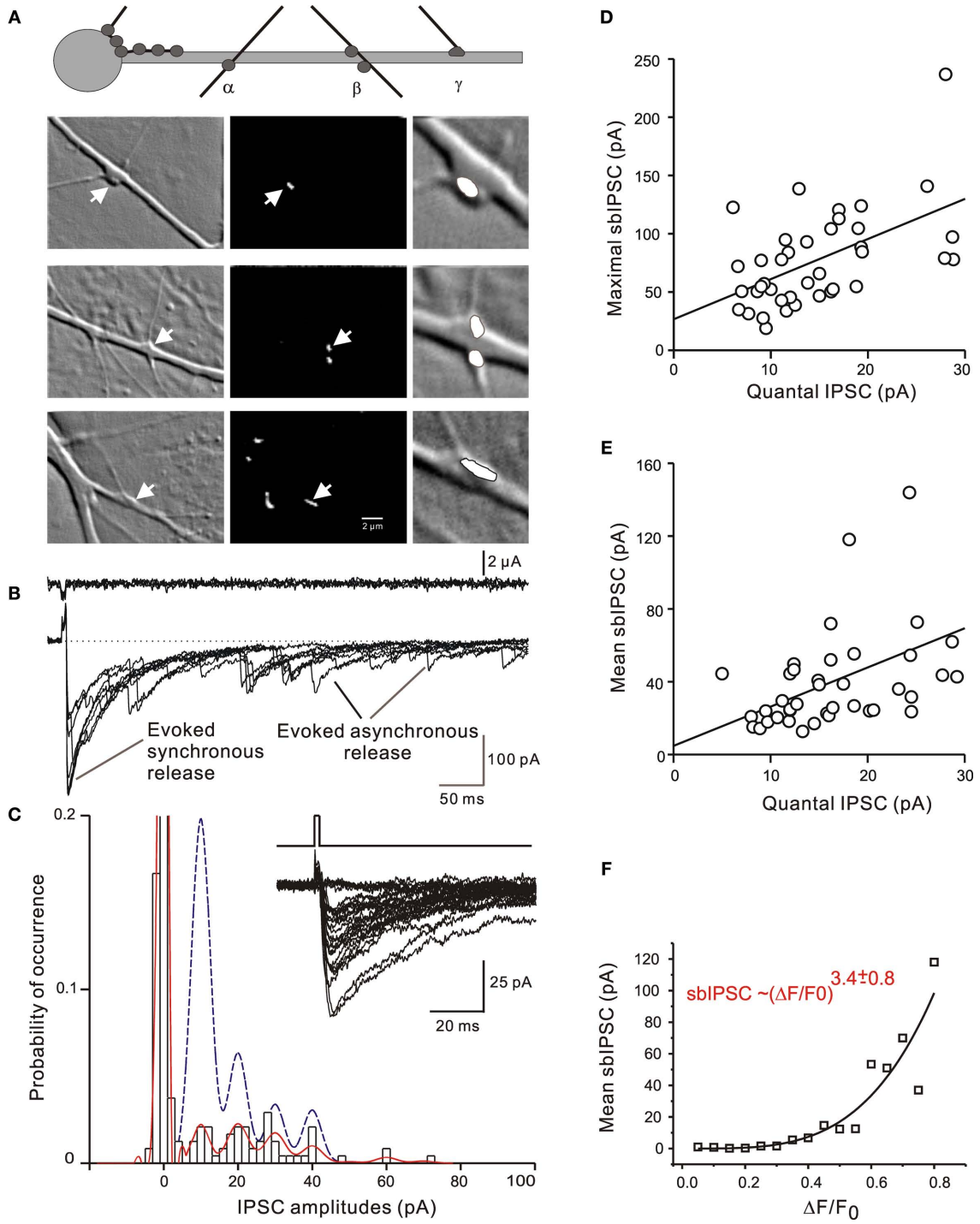

FIGURE 2 | Multivesicular release from single GABAergic boutons in cultures from the E20 rat superior colliculus. (A) Types of synapses selected for direct application of depolarizing stimuli to single presynaptic terminals in the presence of action potential block with tetrodotoxin. Left panel: Phase contrast images; middle panels: fluorescent images showing same view fields after up-take of FM1-43; right panels: magnified synaptic sites with phase contrast optics. **(B)** Specimen records of single-bouton-activated IPSCs (sbIPSCS, lower trace) and respective stimulating current (upper trace). **(C)** Amplitude distribution and binomial fitting of sbIPSCs (bars and solide line) and dIPSCs (dashed line) for the solitary bouton illustrated in the upper row images of **(A)**. dIPSCs were sampled during a period of 250–500 ms after the pulse. **(D,E)** Quantification of the results for maximal and mean sbIPSCs suggesting a quantal content >1. **(F)** Relationship between the mean sbIPSC amplitude and the maximal amplitude of the presynaptic bulk Ca^{2+} transient $[Ca^{2+}]_{pre}$ recorded from a presynaptic area delineated on the basis of vesicular staining. (Modified from Kirischuk et al., 1999).

be considered in more elaborate reflections on presynaptic vesicle release).

As a first approximation, p_{ves} can be determined using experimental protocols that deplete the RRP, for example a high-frequency stimulation (Schneggenburger et al., 1999; Kirischuk and Grantyn, 2000; Hanse and Gustafsson, 2001). The GABAergic synapses of the postnatal mouse superior colliculus frequency needs to be adjusted for any given type of synapses. For neonatal and juvenile inhibitory synapses an appropriate stimulation frequency would be 50 Hz. After few pulses, the cumulative eIPSC amplitude exhibits a linear dependency on the pulse number, and back-extrapolation to stimulus 0 provides a value of RRP*Q (**Figures 3A,B**). Dividing the cumulative eIPSC value at y intercept by Q (as derived from the amplitude distribution of dIPSCs and mIPSCs) one obtains an estimate of RRP. This value corresponds to the maximal number of quanta that could be released at rest. Accordingly p_{ves} is eIPSC/RRP*Q. The applicability of this method can be verified by comparing the theoretical coefficient of variation of eIPSC amplitudes derived from binomial statistics ($CV = [(1-p)/RRP^*p)]1/2$) with the CV of the recorded eIPSCs (SD/mean amplitude; **Figure 3C**) and the relationship between the paired pulse ratio and p_{ves}(**Figure 3D**). This relatively simple approach was then applied to screen for developmental changes of GABAergic synapses in the superior colliculus of C57Bl6 mice between P0 and P22 (Kirischuk et al., 2005).

It turned out that p_{ves} was high at P1, but dropped to a significantly lower value shortly after. The fact that p_{ves} undergoes an initial developmental *decrease* was at odds with expectations that nascent synapses release their transmitter ineffectively (Dumas and Foster, 1995; Aizenman and Cline, 2007), but in line with studies of glutamatergic connections in the hippocampus (Bolshakov and Siegelbaum, 1995; Wasling et al., 2004), where a developmental decrease of P, albeit at older age, had been described.

The RRP, too, experiences a decrease around P3, but increases again with age. Similar results on the developmental changes of RRP were reported for hippocampal synapses (Mozhayeva et al., 2002). Thus, a relatively large RRP containing high release probability vesicles is temporary replaced by a relatively small RRP of low release probability vesicles, resulting in a decrease of eIPSC amplitude. At P11–15 PPR size and eIPSC amplitudes increase, but the release of low probability vesicles is more tightly locked to the presynaptic action potential. As this coincides with IPSC shortening (see below), IPSCs are now fit for better temporal resolution which may play a role in the acquisition of pattern vision after eye opening.

This leaves us with the following question: is the multivesicular release/high probability of vesicle exocytosis at nascent GABAergic synapses merely a functional deficit to be overcome in the course of further development? Or does it serve a useful role in the development of neuronal networks? – The answer remains open, but one may speculate that a ballistic mode of operation of immature synaptic terminals could be the most effective way to recruit postsynaptic receptors and to stabilize them via a postsynaptic local Ca^{2+} signal (see below). The fact that in the developing superior colliculus the decrease of p_{ves} and the reduction of RRP were paralleled by the disappearance of depolarizing GABA action at P2–3 is at least in line with this idea (see below).

IMMATURE GABAergic SYNAPSES ARE PRONE TO PAIRED PULSE AND TETANIC DEPRESSION

As already mentioned, at immature stages stimulus-locked responses tend to undergo depression if two or more presynaptic depolarizations occur at short intervals. The mechanisms underlying the use-dependent depression in immature GABAergic synapses have been studied at some detail in our lab using single-bouton activation in the presence of TTX or Ca^{2+} channel blockers to suppress co-activation of other boutons in contact with the postsynaptic cell (Kirischuk et al., 2002). This approach has the advantage that one and the same synaptic terminal is reliably stimulated with each trial, presynaptic depolarizations can be graded and presynaptic Ca^{2+} transients could serve as indicators of presynaptic activation (Kirischuk et al., 1999). The experiments showed that at short intervals (<100 ms) different depressant mechanisms occur simultaneously but differ in their recovery kinetics.

The rapidly recovering paired pulse depression (PPD_{fast}), as seen at interstimulus intervals of 25–50 ms, is release-dependent (the amplitude of the second eIPSC being inversely proportional to the amplitude of the first one) and strongly affected by the extracellular Ca^{2+} concentration (Jensen et al., 1999; Chen et al., 2004). Developmental changes of PPD_{fast} can be expected due to the maturation of presynaptic Ca^{2+} buffering (for instance, Llano et al., 2000a) or changes in the patterns of presynaptic G-protein coupled receptor expression. Both mechanisms account for neuron-specific differences of paired pulse plasticity in the adult brain (see, for instance, Senn et al., 1998; Poncer et al., 2000) while at the onset of GABAergic synaptogenesis modulatory diversity is low. Even a GABA(B)R-mediated contribution to paired pulse plasticity is missing in immature GABAergic synapses (Wilcox and Dichter, 1994; Jensen et al., 1999; Kirischuk et al., 2002), and the predominant type of paired pulse behavior is depression.

With further synapse maturation the amount of PPD decreases (Juettner et al., 2001), and this change can be ascribed to a decrease in p_{ves} (Kirischuk et al., 2005). Raising the presynaptic Ca^{2+} influx by increasing extracellular Ca^{2+} levels (Senn et al., 1998), prolonging presynaptic depolarization (Kirischuk et al., 2002) or blocking G-protein mediated depression by *N*-ethylmaleimide (NEM)(Kirmse and Kirischuk, 2006) can enhance p_{ves} and turn an already established paired pulse facilitation (PPF) into PPD.

A much more slowly recovering form of PPD can be isolated at intervals of 1 s (PPD_{slow}). In contrast to PPD_{fast}, PPD_{slow} was found to be calcium- and release-independent. This type of depression has first been described for neuromuscular junctions (Betz, 1970), the squid giant synapse (Hsu et al., 1996), the calyx of Held (Bellingham and Walmsley, 1999; Borst and Sakmann, 1999), and a synapse formed by the Mauthner cell axon in the goldfish (Waldeck et al., 2000). PPD_{slow} has been incorporated in the contemporary models of transmitter release under the term "transient refractoriness" (Zucker and Regehr, 2002; Pan and Zucker, 2009) or "site clearing" (Neher and Sakaba, 2008). Despite its prominence, at least in immature synapses (see Kirischuk et al., 2002), it still awaits detailed characterization at a molecular level along with a more systematic testing for developmental changes.

It should be noted that the probability of obtaining a postsynaptic response would depend both on p_{ves} and the probability that a given site (active zone) is available for release. Therefore,

to obtain a full description of the developmental changes in the performance of a given type of synaptic connections binomial analysis or variance–mean analysis (Silver, 2003) need to be performed to determine Pr in addition to p_{ves}.

Apart from estimating the quantal parameters of synaptic transmission, the reliability or fatigability of a synaptic connection could be tested using the average eIPSC amplitude reached during the last 10 trials of a series of high-frequency pulses (50–100 Hz). This reveals the so called tetanic depression of synaptic transmission. If normalized to Q and RRP, this parameter is best suited to reflect the age-dependent changes in the release performance (Kirischuk et al., 2005). Tetanic eIPSC depression was strong at P3–6, but decreased at P11–P15. Although developmental acceleration of the RRP replenishment rate can not be excluded, the observed decrease of release probability accompanied with the increase of RRP size may underlie these changes. Interestingly, the decrease of tetanic depression coincides with the time of in-growth of cortical afferents and the massive up-regulation of glutamatergic synaptic transmission prior to eye opening at day P14–15 (see Aamodt and Constantine-Paton, 1999; Grantyn et al., 2004 for more). Experimentally, presynaptic GABA release can be modified by allowing cortical afferents to grow into tectal tissue (Henneberger et al., 2007) indicating that indeed changes in glutamatergic innervation shape the function of pre-existing GABAergic synapses.

SYNAPSE MATURATION IS CHARACTERIZED BY A DOMINANCE OF SYNCHRONOUS OVER ASYNCHRONOUS RELEASE

A developmental study in the calyx of Held has reported a changing relationship between synchronous and asynchronous release (Chuhma et al., 2001; Yang and Xu-Friedman, 2010). Delayed release might even be present when evoked release is missing, and the two modes of release display a differential dependency on the presynaptic Ca^{2+} concentration in the vesicle area ($[Ca^{2+}]_{pre}$) (Kirischuk and Grantyn, 2003; Yang and Xu-Friedman, 2010). Asynchronous IPSCs (aIPSCs) were sampled during a train of high-frequency stimulation and a synchrony index of release was defined for the period of the last 10 intervals of the high-frequency train by dividing the charge transfer of stimulus-locked eIPSCs by the charge transfer of unlocked aIPSCs. It was found that this index increased with age, showing that the relative number of vesicles released in a stimulus-locked manner increases when neurons mature. As the delayed component of asynchronous release (charge of dIPSCs) displayed a developmental decrease as well, one can conclude that asynchronous release is a characteristic feature of immature synapses, being replaced by action potential-locked transmission at older age. Again, the changes were biggest around the time of eye opening (i.e., shortly after the time of massive in-growth of glutamatergic synapses).

While asynchronous release may be more pronounced in immature GABAergic connections, its persistence at more mature stages will depend on the type of interneuron activated and the Ca^{2+}-binding proteins expressed (Daw et al., 2010).

IN IMMATURE NEURONS GABA ACTS AS DEPOLARIZING TRANSMITTER

The pioneer role of GABA at initial stages of circuit formation in the brain has much to do with its depolarizing action (Cherubini et al., 1991; Ben Ari et al., 2007). The latter has long ago been discovered in the immature rat striatum (Misgeld et al., 1982) and the immature rabbit and rat hippocampus (Mueller et al., 1984; Cherubini et al., 1990). In the superior colliculus the depolarizing/excitatory action of GABA is prominent at P0–P1 (Juettner et al., 2001), but it already disappears by P3 (Grantyn et al., 2004). In the hippocampus, the change in the polarity of GABA action occurs around P5 (Ben Ari et al., 1989) and has been attributed to the developmental up-regulation of the expression and membrane targeting of KCC2, a Cl^- exporter (Rivera et al., 1999; Ganguly et al., 2001; Hubner et al., 2001). High activity of KCC2 in relation to the activity of NKCC1, a Cl^- importer, would ensure low intracellular Cl^- concentrations and, consequently a hyperpolarizing GABA action (Blaesse et al., 2009).

However, a developmental switch from depolarizing to hyperpolarizing GABA is not observed in all developing neurons (Banke and McBain, 2006) and, where present, even parts of the somatodendritic plasma membrane can differ with regard to their local chloride gradients (Gulledge and Stuart, 2003). Whether or not at the end shunt inhibition or excitatory GABA actions will dominate the overall effect of GABA on the neuronal output will, first of all, depend on the spatio-temporal relationships of the respective chloride channels with the glutamatergic inputs (see Bracci and Panzeri, 2006).

SYNAPSE MATURATION IMPLIES IPSC SHORTENING DUE TO AN INCREASE IN ALPHA1 SUBUNIT EXPRESSION

The developmental shortening of postsynaptic currents is a widely observed phenomenon in many brain areas, including the rodent hippocampus (Cohen et al., 2000; Hutcheon et al., 2004) and superior colliculus (Juettner et al., 2001; Henneberger et al., 2005a; Kirischuk et al., 2005). The slow decay kinetics of IPSCs shortly after birth has been associated with the high expression level of the alpha3 subunit of the GABA(A)R, while the subsequent shortening of IPSCs was attributed to an up-regulation of the alpha1/alpha3 ratio.

Again, the most interesting questions concern the cause and the function of this phenomenon. We have tried to determine the timing of the developmental switch from slow to fast IPSCs in the superior colliculus and found that most of the change occurs during days P6 and 15, i.e., prior to eye opening (around P14–15 in mice, the day of birth being P0). It also coincided with the developmental peak of the NMDAR-mediated charge transfer in the glutamatergic synaptic currents and might be an activity-dependent phenomenon.

We therefore considered the possibility that glutamatergic/NMDAR-mediated activity provided the drive for the switch in the GABA(A)R subunit composition. Respective culture experiments with chronic exposure to MK-801 confirmed this suggestion, while block of mGluR receptor activity with S-MCPG had no effect on IPSC decay kinetics (Henneberger et al., 2005a).

It is particularly telling that the decrease of IPSC duration also coincided with the increase of the synchrony index and the reduction of tetanic depression of the phasic stimulus-locked release. One may speculate that by the time of onset of patterned vision GABAergic synapses assume

a new function enabling a better temporal resolution of inhibitory signals.

EXTRASYNAPTIC (TONIC) CONDUCTANCES CAN INHIBIT THE DEVELOPMENT OF SYNAPTIC (PHASIC) GABA ACTIONS

Tonic chloride conductances via extrasynaptic GABA(A) and/or glycine receptors represent a powerful means to adjust neuron excitability (see Farrant and Nusser, 2005), and is likely to happen as soon or even prior to the moment when a neuron becomes post-mitotic. Indeed, non-synaptic responses to exogenous GABA can be detected much before neurons exhibit phasic synaptic activity, an example being the E14 rat retinal ganglion cells (Rörig and Grantyn, 1994).

Associated with changes in the subunit composition there might be a cell-type-specific up- or down-regulation of tonic GABA currents [I(Tonic)GABA]. In hippocampal granule cells (Holter et al., 2010) and in D1-expressing striatal output neurons (Santhakumar et al., 2010), I(Tonic)GABA displays a robust increase with age, while D2-expressing striatal output neurons showed a decrease, which possibly reflected the general tendency of GABA(A)R α5 down-regulation with age (Laurie et al., 1992). Extrasynaptic GABA(A)Rs typically contain α5 and/or δ subunits, which makes them very sensitive to low concentrations of ambient GABA.

Considering the idea that stability of GABAergic synaptic contacts requires *local* Ca^{2+} elevations on the background of low *global* (resting) Ca^{2+} levels in dendrites and somas (see Kirsch et al., 1993; Marty and Llano, 2005), one could expect an inverse relationship between tonic extrasynaptic and phasic synaptic Cl^- conductances.

This question has recently been addressed by Meier and colleagues (Eichler et al., 2008) who transfected hippocampal neurons with a glycine receptor isoform that confers a particularly high agonist affinity to the extrasynaptic glycine receptors of hippocampal and collicular neurons (Meier et al., 2005). As expected, overexpression of the high affinity type of glycine receptor resulted in an increase of the tonic chloride conductance. Moreover, it also resulted in a massive decrease of GABAergic synapse numbers. As this effect could be reversed by transfecting hippocampal neurons with the chloride exporter KCC2, it was suggested that *global* changes of Ca^{2+} in response to depolarizing responses due to Cl^- outflux through extrasynaptic Cl^- channels and the resulting activation of voltage-gated Ca^{2+} channels in neurons with a high intracellular Cl^- concentration could mask the signals required for stabilization of GABAergic contacts. In fact, Ca^{2+} was recently shown to impact on the stability of postsynaptic gephyrin and GABA(A)Rs (Förstera et al., 2010; Tyagarajan et al., 2011). Furthermore, if neurons were no longer able to generate action potentials (due to massive shunt inhibition provided by the extrasynaptic high affinity glycine receptors) they may be afflicted by neurodegeneration, as a worst-case scenario (Tao and Poo, 2005; Legendre et al., 2009).

More studies on the relationship between tonic extrasynaptic signals and phasic GABAergic synaptic transmission are needed, since it is known that a number of pathophysiological states, including epilepsy, depression, and neurodegenerative diseases, are associated with a return to depolarizing GABA actions (see Cherubini et al., 2011).

NEUROTROPHIN EFFECTS ON INHIBITORY SYNAPSE DEVELOPMENT

EFFECTS OF BDNF

Brain-derived neurotrophic factor (BDNF) is critically involved in the activity-dependent maturation of visual structures (see Frost, 2001 for review), and it affects a number of mechanisms underlying the maturation of synaptic inhibition (Huang et al., 1999; Frost, 2001). In the following we provide a short survey of BDNF-related findings from our studies on a range of model systems and approaches, such as hippocampal and collicular cultures and acute slices from wild-type and BDNF-deficient mice, acute and chronic BDNF treatment and overexpression of BDNF by transfection.

The impact of BDNF on synapse function and development varies depending on the maturity of the synapses studied. In cultured neurons from the embryonic hippocampus, BDNF increased the number of glutamatergic synapses, but decreased the number of GABAergic, vGAT-positive, synaptic terminals (Singh et al., 2006). These changes were accompanied by an increase in the ratio between synaptic excitation and inhibition assessed by quantifying spontaneously occurring action potential-independent release events (mEPSC/mIPSC frequency; Singh et al., 2006). Our results suggest that in immature neuronal networks BDNF could promote glutamatergic synaptogenesis at the expense of GABAergic synapses.

Addition of exogenous BDNF to collicular cultures revealed two possible presynaptic targets in GABAergic synapses, vesicle loading, and asynchronous release. The former suggestion is based on the finding that BDNF treatment reduced the level of presynaptic vGAT, as judged by immunofluorescence (Henneberger et al., 2005b). No effect was found on the RRP and p_{ves} of evoked release, but it augmented asynchronous release (**Figures 3E,G**) – the characteristic feature of immature collicular synapses (see above, part 5). This could be a consequence of increased levels of the GABA-synthesizing enzyme GAD after BDNF treatment. Indeed, like hippocampal neurons (Aguado et al., 2003) collicular neurons reacted to BDNF with an increase in presynaptic GAD65 levels (Henneberger et al., 2005b), and the latter is known to facilitate asynchronous release (Tian et al., 1999). In some preparations, including hippocampal slice cultures, addition of exogenous BDNF was reported to produce a higher yield of GAD-labeled presynaptic terminals (Marty et al., 2000).

On the postsynaptic side (**Figure 3H**), BDNF treatment of cultured collicular neurons resulted in a suppression of GABAergic synaptic transmission (Tanaka et al., 1997; Brünig et al., 2001) which could be attributed to a reduction in the number of open channels contributing to the IPSCs without affecting their single channel conductance, RRP or p_{ves} (Henneberger et al., 2005b). In principle, the depressant effect of BDNF on the postsynaptic response to GABA could reflect reduced neurotransmitter loading into vesicles and thus a smaller number of open postsynaptic receptors/channels during transmission. However, we are not aware of any direct evidence supporting this interesting alternative hypothesis and would tentatively conclude that a reduction in postsynaptic receptor number accounts for most of the BDNF-mediated inhibition of GABAergic transmission elicited by single action potentials (Brünig et al., 2001).

Similar or related results were obtained in the intact superior colliculus of *bdnf*−/− mice, however only after P13/14, i.e., at a

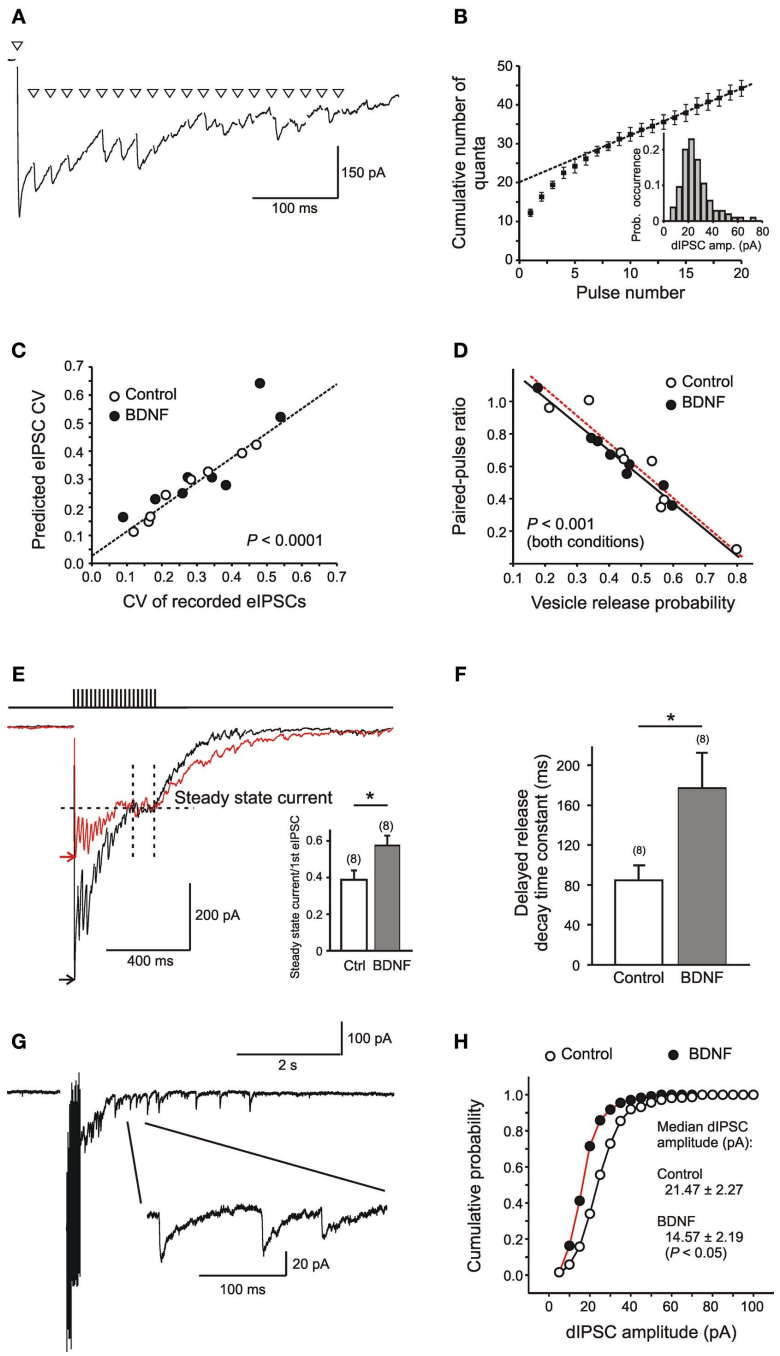

FIGURE 3 | Measurement of RRP, p_{ves}, and Q in collicular neurons, and the effects of BDNF. (A) Specimen trace of eIPSCs induced by high-frequency (HF) stimulation (20 pulses at 50 Hz) of a single GABAergic axon. For clarity stimulus artifacts are replaced by triangles. **(B)** Cumulative plot of eIPSC amplitudes vs. stimulus number. The eIPSC amplitudes were normalized to the median dIPSC amplitude of the same unitary connection (inset). Back-extrapolation to the *y* intercept indicates RRP. **(C,D)** Tests for applicability of the estimates of p_{ves}. **(E)** Sample records to illustrate BDNF effects on IPSCs induced by HF stimulation. To obtain the amplitude of the steady state current, the current integral was normalized to the total time of integration for the last five stimulus intervals. Arrow heads denote the peak level of the eIPSC after the first pulse in the train. Note that BDNF does not affect the steady state current but significantly reduces the ratio between steady state current and first eIPSC (inset). **(F)** BDNF increases the time constant of decay of the "synaptic tail current," i.e., the current produced by dIPSCs after the stimulus train, as estimated by single exponential fit. **(G)** Sample record of the postsynaptic response to HF stimulation of a single GABAergic axon. Note the presence of dIPSCs (inset: enlarged) after the end of stimulation. **(H)** Reduction of dIPSC amplitudes as evidence for a depressant postsynaptic effect of BDNF, in contrast to the absence of significant changes in the range of coefficient of variation **(C)**, paired pulse ratio, and p_{ves} **(D)**. (Modified from Henneberger et al., 2005b).

stage when glutamatergic inputs were available and active (Henneberger et al., 2002, 2005b). At a younger age (P1–5) evidence for a suppressive postsynaptic effect of BDNF was missing, although the chronic absence of BDNF delayed the switch from depolarizing to hyperpolarizing GABA action (Grantyn et al., 2004), in accord with similar findings in immature murine hippocampal slices (Aguado et al., 2003).

In summary, the actions of the neurotrophins BDNF vary with brain region and developmental stage. In the superior colliculus, BDNF is likely to accelerate the transition to hyperpolarizing GABA action and to facilitate asynchronous release during early postnatal development whereas at later stages it primarily inhibits GABAergic synaptic transmission.

EFFECTS OF NGF

That nerve growth factor (NGF) can be an influential player in the maturation and function of GABAergic synapses has only recently been recognized. Still very little is known on the possible role of NGF during *in situ* development of GABAergic synapses.

We have explored some acute effects of added NGF in hippocampal cultures and found that NGF promoted GABAergic synaptogenesis (Salama-Cohen et al., 2006). The effects of NGF included

(1) a TrkA-mediated up-regulation of vGAT expression
(2) an increase in the number of vGAT-immunopositive synaptic terminals in contact with hippocampal neurons
(3) a prominent reduction in the E/I ratio of contacting boutons.

The final outcome of exogenous NGF depended on its depressant action on the proneural gene neurogenin 3 (Ngn3), a nuclear transcription factor that is also under the control of Hes1/5.

Our results suggest that with regard to the glutamate/GABA or E/I balance of synaptic transmission and development BDNF and NGF might assume antagonistic roles. Therefore, to further explore the significance of NGF for the development and function of GABA synapses might be one of the most rewarding tasks in the near future.

ACUTE GABAergic SYNAPTOGENESIS AFTER LESION

Among the stimuli inducing inhibitory synaptogenesis are factors liberated from damaged tissue. Several labs have described "inhibitory sprouting" in the immediate environment (Mittmann and Eysel, 2001) or the terminal area (Deller et al., 1995) of lesioned cells.

Consistent with the pioneer role of GABA at initial stages of synaptogenesis (see above, part 6), our investigation of synapse formation after slicing of late embryonic or neonatal rodent superior colliculi revealed that at this age lesion preferentially facilitates the formation of new GABAergic synapses (Meier et al., 2003). The up-regulation of GABAergic synapses was inferred from the increase of vGAT- or GAD65-positive terminals, mIPSC frequency, and postsynaptic GABA(A)R immunofluorescence. GABAergic synaptogenesis could be prevented/facilitated by blocking/enhancing, respectively, PKC activation, suggesting the involvement of phosphorylation-dependent mechanisms. Manipulations assisting the return of *globally elevated* intracellular Ca^{2+} levels to resting Ca^{2+} concentrations promoted this form of reactive synaptogenesis in the neonatal superior colliculus (J. Walter, C. Henneberger, J. C. Meier and R. Grantyn, unpublished observation).

GABAergic SYNAPTOGENESIS DURING INTEGRATION OF NEWLY GENERATED NEURONS IN THE ADULT BRAIN

Adult neurogenesis represents an important response of the damaged as well as the learning brain (see Ma et al., 2010). Notably in the dentate gyrus newly formed neurons integrate into pre-existing networks, which requires the formation of new inhibitory synapses (Tozuka et al., 2005; Wang et al., 2005; Toni and Sultan, 2011). The functional properties of nascent GABAergic synapses and their subsequent development were shown to reproduce some essential features of immature synapses in the embryonic and neonatal brain: there was a GABA lead in the innervation of newborn granule cells, the initial action of GABA was depolarizing, the decay kinetic was slow, and synaptic currents were relatively insensitive to zolpidem (Overstreet-Wadiche et al., 2005; Ge et al., 2006; Karten et al., 2006). Preventing a depolarizing GABA action by knock-out of NKCC1 reduced and delayed GABAergic synaptogenesis (Ge et al., 2006).

These results validate major milestones, as defined above for the ontogeny of inhibitory synaptic connections in the brain, and suggest that the outlined developmental mechanisms may apply irrespective of the given local conditions of a neuronal network.

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Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission

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The K-Cl co-transporter KCC2 plays multiple roles in the physiology of central neurons and alterations of its function and/or expression are associated with several neurological conditions. By regulating intraneuronal chloride homeostasis, KCC2 strongly influences the efficacy and polarity of the chloride-permeable γ-aminobutyric acid (GABA) type A and glycine receptor (GlyR) mediated synaptic transmission. This appears particularly critical for the development of neuronal circuits as well as for the dynamic control of GABA and glycine signaling in mature networks. The activity of the transporter is also associated with transmembrane water fluxes which compensate solute fluxes associated with synaptic activity. Finally, KCC2 interaction with the actin cytoskeleton appears critical both for dendritic spine morphogenesis and the maintenance of glutamatergic synapses. In light of the pivotal role of KCC2 in the maturation and function of central synapses, it is of particular importance to understand the cellular and molecular mechanisms underlying its regulation. These include development and activity-dependent modifications both at the transcriptional and post-translational levels. We emphasize the importance of post-translational mechanisms such as phosphorylation and dephosphorylation, oligomerization, cell surface stability, clustering and membrane diffusion for the rapid and dynamic regulation of KCC2 function.

Keywords: KCC2, neuronal activity, excitatory and inhibitory synapses, post-translational regulation

INTRODUCTION

Fast synaptic transmission relies on ion fluxes through ligand-gated channels. Therefore, the maintenance of transmembrane ionic gradients is critical to preserve synaptic efficacy. The electroneutral KCC2 co-transporter is the major chloride (Cl^-) extruder in mature neurons (Payne et al., 1996; Williams et al., 1999; Karadsheh and Delpire, 2001; Uvarov et al., 2005). Unlike other KCC family members known to regulate cell volume in non-neuronal cells (Zeuthen and MacAulay, 2002; Zeuthen, 2010), KCC2 has been mostly studied for its role in maintaining low intracellular chloride concentration $[Cl^-]_i$ in neurons [reviewed in (Ben-Ari, 2002; Blaesse et al., 2009)]. $[Cl^-]_i$ influences the efficacy and polarity of synaptic transmission mediated by γ-aminobutyric acid (GABA) type A receptors (GABAARs) and glycine receptors (GlyRs) which both flux chloride ions. The spatio-temporal regulation of KCC2 mRNA and protein expression levels orchestrates the developmental shift in synaptic glycinergic and GABAergic transmission from depolarizing to hyperpolarizing or shunting in many but not all species (Rivera et al., 1999; Vanhatalo et al., 2005) and brain regions (Li et al., 2002; Vinay and Jean-Xavier, 2008). GABAA receptor-mediated depolarization appears as a critical determinant of early network activities (Cherubini et al., 2011), circuit formation (Ben-Ari, 2002; Akerman and Cline, 2006), neuronal migration (Bortone and Polleux, 2009), and synapse maturation (Aguado et al., 2003). Recent studies also indicate a critical role of KCC2 in both the formation (Li et al., 2007) and functional maintenance (Gauvain et al., 2011) of glutamatergic synapses. These mechanisms appear independent of KCC2 function but instead involve KCC2 interaction with submembrane cytoskeleton.

KCC2 expression and function are tightly regulated by neuronal activity (Fiumelli and Woodin, 2007). This regulation involves trophic factors such as brain-derived neurotrophic factor (BDNF), neuronal intrinsic activity or the activity of excitatory synapses (Kaila et al., 1997; Rivera et al., 2002, 2004; Woodin et al., 2003; Fiumelli et al., 2005; Wang et al., 2006a–c; Kitamura et al., 2008; Lee et al., 2011). In some conditions, neuronal activity may either upregulate KCC2 to strengthen synaptic inhibition in a homeostatic manner or, on the contrary, downregulate KCC2 possibly to enhance the gain of recently active excitatory synapses. In several pathological conditions associated with enhanced excitation, suppression of KCC2 is often observed and may contribute to further alter the balance of excitation and inhibition, leading to excitotoxicity, or anomalous activities such as seizures (Reid et al., 2001; Rivera et al., 2002, 2004; Huberfeld et al., 2007; Pathak et al., 2007; Wake et al., 2007; Li et al., 2008b; Shimizu-Okabe et al., 2011). Therefore, KCC2 appears to mediate a crosstalk between excitatory and inhibitory transmission. It is, therefore, crucial to understand the mechanisms that control its expression and activity. Neuronal activity modulates KCC2 activity through both transcriptional and post-translational modifications. Whereas transcriptional mechanisms may be involved

in regional, developmental, and regulation of KCC2 activity in pathological conditions, post-translational mechanisms of KCC2 regulation occurs in a time scale compatible with a control by fast synaptic transmission (Woodin et al., 2003; Wang et al., 2006b; Fiumelli and Woodin, 2007; Wake et al., 2007; Kitamura et al., 2008; Chorin et al., 2011; Lee et al., 2011). Several recent and comprehensive reviews have addressed the biology of cation-chloride co-transporters (CCCs) both in neuronal (Blaesse et al., 2009) and non-neuronal cells (Gamba, 2005). Here, we will review the recent literature more specifically on (1) the subcellular distribution of KCC2 in relation with excitatory and inhibitory synapses, (2) its basic properties as well as its function at inhibitory and excitatory synapses, (3) its regulation during development and by neuronal activity. We will discuss the cellular and molecular mechanisms underlying its regulation.

STRUCTURE AND DIVERSITY OF KCC2 CO-TRANSPORTERS

The K-Cl co-transporter KCC2 is one of the nine cation chloride co-transporters encoded by the Slc12a one to nine genes [for review see (Gamba, 2005)]. Four KCC co-transporters have been identified so far (KCC1–4). KCC2 is the only KCC isoform exclusively expressed in central neurons (Payne et al., 1996; Williams et al., 1999; Karadsheh and Delpire, 2001; Uvarov et al., 2005). The lack of KCC2 mRNA expression in non-neuronal cells relies on binding of the neuron-restrictive silencer factor (NRSF) to the neuron-restrictive silencer element (NRSE) in KCC2 gene promoter (Uvarov et al., 2005). Two transcription factors up- or down-regulate KCC2 mRNA levels (Uvarov et al., 2006). The transcription factor early growth response 4 (Egr4 or NGFI-C) is a neuron-specific immediate early gene that enhances KCC2 expression (Uvarov et al., 2006). In contrast, the REST transcriptional repressor complex inhibits KCC2 mRNA level by binding to two repressor elements (RE-1) in the KCC2 gene (Yeo et al., 2009). The KCC2 mRNA is expressed with a gradual temporal increase from spinal cord and brainstem to higher brain structures (Li et al., 2002; Stein et al., 2004). Alternative splicing of the KCC2 gene (Slc12a5) gives rise to two KCC2 isoforms, KCC2a and KCC2b (Uvarov et al., 2007). The KCC2a transcript is expressed at low-level between E14-P60 (Uvarov et al., 2007). In contrast, the KCC2b mRNA is up-regulated by 10- and 35-fold in hippocampus and neocortex between E17 and P14 (Uvarov et al., 2007). Similarly to the transcripts, KCC2a protein shows only moderate changes during postnatal development, whereas KCC2b expression is strongly up-regulated (Uvarov et al., 2009). Therefore, KCC2a prevails in neonatal brain whereas KCC2b predominates in adult (Uvarov et al., 2009). KCC2b differs from KCC2a by an extra 40 amino acid residue in its *N*-terminal part that carries a putative binding site for the Ste20-related proline alanine-rich (SPAK) and oxidative stress response-1 (OSR1) kinases (Uvarov et al., 2007).

The KCC2 protein is a glycoprotein with a predicted topology of 12 membrane spanning segments flanked by two cytoplasmic carboxy- and amino-terminal domains of long and short size, respectively (Payne et al., 1996). The intracellular regions represent half of the total size of the molecule and are known so far to be the targets of several kinases and one phosphatase that regulate the function of the co-transporter (**Figure 1**). KCC2 carries putative phosphorylation sites for the Src-family tyrosine kinase [residues Tyr903, Tyr1087, (Lee et al., 2010)], the serine/threonine With no lysine kinase (Wnk) family (Wnk3, (Kahle et al., 2005); Wnk1 at residues Thr906, Thr1006, (Rinehart et al., 2009); Wnk2, (Rinehart et al., 2011), and the Ca^{2+}/phospholipid-dependent protein kinase C (PKC) (residues Thr34, Ser728, Thr787, Ser940, Ser1034, (Payne et al., 1996; Lee et al., 2007). Among the five putative PKC-dependent phosphorylation sites, the Ser940 residue is the major site of PKC phosphorylation in neurons (Lee et al., 2007). This site is specific of the KCC2 transporter (Payne et al., 1996) and can be dephosphorylated by the protein phosphatase 1 (PP1) (Lee et al., 2011).

KCC2 can exist as monomers (∼140 kDa), dimers (∼270 kDa), trimers (∼400 kDa), or tetramers (∼500 kDa) (Blaesse et al., 2006; Uvarov et al., 2009). Immunoblots and co-immunoprecipitation studies from brain extracts reported various oligomeric states of KCC2: KCC2a-KCC2a and KCC2b-KCC2b homo-dimers, KCC2a-KCC2b, and KCC2-KCC4 hetero-dimers (Blaesse et al., 2006; Simard et al., 2007; Uvarov et al., 2009). Although KCC oligomerization domains have not been identified so far, the carboxy-terminal domain appears to be required for oligomerization (Casula et al., 2001; Simard et al., 2004). Thus, truncation of KCC1 in its carboxy-terminus (up to residue 805) fails to oligomerize (Casula et al., 2001). KCC2 oligomers also associate through di-sulfide bounds (Uvarov et al., 2009), as described for other CCCs (Moore-Hoon and Turner, 2000; Blaesse et al., 2006).

CELLULAR AND SUBCELLULAR DISTRIBUTION IN NEURONS

REGIONAL EXPRESSION

KCC2a and KCC2b transcripts are expressed in mature neurons throughout the CNS including all layers of the cortex, neurons of the brainstem, thalamus, olfactory bulb, and spinal cord, CA1–CA4 pyramidal neurons of the hippocampus, granular layer, and cerebellar Purkinje neurons (Payne et al., 1996; Uvarov et al., 2007). Most published immunochemical data on KCC2 have been obtained with antibodies against a carboxy-terminal region of KCC2 common to both KCC2a and KCC2b isoforms (residues 932–1043, commercialized by Sigma, Millipore, AbCam, Life Span. . .). KCC2a and KCC2b isotype specific antibodies were recently generated: KCC2a, amino-terminal residues DPESRRHSVADPRRLPREDVK, (Uvarov et al., 2009); KCC2b, amino-terminal residues CEDGDGGANPGDGN, (Hubner et al., 2001). Finally, an antibody against the second extracellular loop of KCC2 (IFKAEDASGEAAAML residues) was obtained and characterized in Gagnon et al. (2007) but failed to yield specific staining in our hands. Since KCC2b prevails in adults, immunofluorescence (IF) data from adult tissue predominantly reveals the distribution of this isoform. KCC2 is detected in neurons of the cerebellum (Williams et al., 1999; Takayama and Inoue, 2006), all relay nuclei of the thalamus (Bartho et al., 2004), retina (Vardi et al., 2000; Vu et al., 2000; Gavrikov et al., 2006; Zhang et al., 2006; Li et al., 2008a), lateral superior olive (LSO) of the brainstem (Blaesse et al., 2006), neocortex (DeFazio et al., 2000; Szabadics et al., 2006), somatosensory cortex (Takayama and Inoue, 2006, 2010), hippocampus (Rivera et al., 1999; Gulyas et al., 2001), spinal cord (Hubner et al., 2001; Stil et al., 2009),

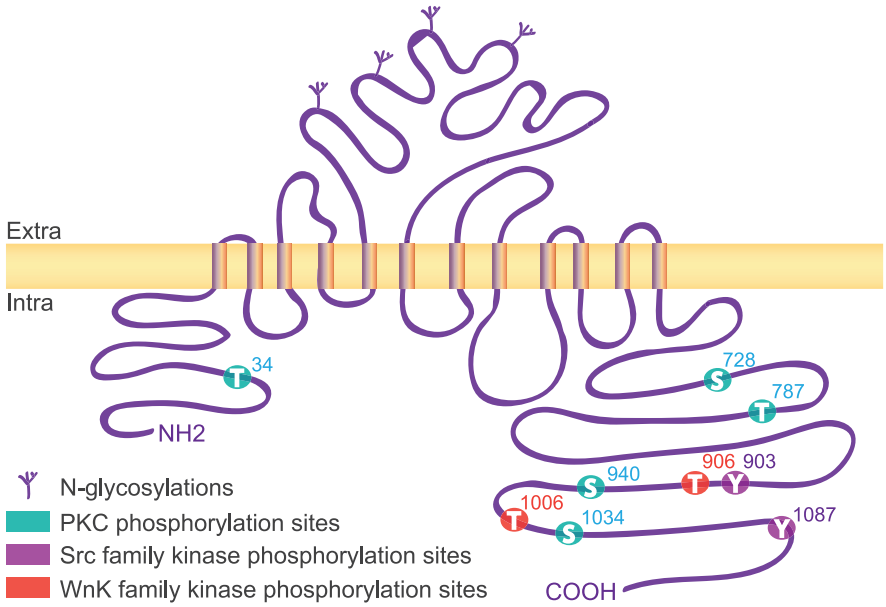

FIGURE 1 | Structure of KCC2. The rat KCC2 co-transporter is a large size (~140 kDa) protein with a predicted topology of 12 membrane spanning segments, a N-linked glycosylated extracellular domain between transmembrane domains 5 and 6, and is flanked by two cytoplasmic carboxy- and amino-terminal domains of 104 and 481 amino acids, respectively. As indicated, the intracellular regions are the targets of several kinases that regulate the function of the co-transporter.

cochlear nucleus (Vale et al., 2005; Yang et al., 2008), and hypothalamic suprachiasmatic nucleus (SCN) (Belenky et al., 2008).

CELLULAR COMPARTMENTALIZATION

KCC2 is restricted to the somato-dendritic compartment and excluded from the axon (Hubner et al., 2001; Szabadics et al., 2006; Bartho et al., 2009) including the initial segment (AIS) (Gulyas et al., 2001; Baldi et al., 2010). KCC2 immunoreactivity is usually evenly distributed along the somato-dendritic axis of neurons of the thalamus (Bartho et al., 2009), the granular layer of the cerebellum (Takayama and Inoue, 2006) and dentate gyrus granule cells (Baldi et al., 2010). However, in OFF bipolar cells and starburst cells of the retina, KCC2 is confined in distal dendrites (Vardi et al., 2000; Gavrikov et al., 2006). Similarly, in hippocampal CA1 pyramidal neurons, KCC2 may preferentially accumulate at GABAergic synapses formed onto distal rather than proximal dendrites (Baldi et al., 2010).

INTRACELLULAR VS. MEMBRANE DISTRIBUTION

Intracellular KCC2 labeling is found at a higher level in immature than in mature neurons. KCC2 decorates the membrane of transport vesicles in dendrites of P2 hippocampal neurons (Gulyas et al., 2001). In contrast, only little cytoplasmic KCC2 is detected in mature hippocampal neurons (Gulyas et al., 2001) as well as neurons of the cochlear nucleus (Vale et al., 2005) and isocortex (Szabadics et al., 2006). In the adult brain, organelles including Golgi apparatus and endoplasmic reticulum do not show KCC2 labeling (Takayama and Inoue, 2006) except in neurons of the SCN (Belenky et al., 2008). Instead, most KCC2 protein is found associated with the plasma membrane both in somatic and dendritic compartments (Williams et al., 1999; Gulyas et al., 2001; Hubner et al., 2001; Vale et al., 2005; Blaesse et al., 2006; Szabadics et al., 2006; Takayama and Inoue, 2006; Belenky et al., 2008; Bartho et al., 2009; Baldi et al., 2010). KCC2 exhibits a high turnover rate. It was shown that the membrane pool of KCC2 is partially (Rivera et al., 2004) or totally (Lee et al., 2007) replaced within 10 min under basal conditions.

SYNAPTIC LOCALIZATION

At the synaptic level, KCC2 is not found within presynaptic boutons except in terminals of the retinal ON bipolar cells (Vardi et al., 2000) and developing photoreceptor cells (Zhang et al., 2006). KCC2-coupled immunogold particles decorate the perisynaptic area of postsynaptic neuronal cell (Gulyas et al., 2001; Bartho et al., 2004; Blaesse et al., 2006; Takayama and Inoue, 2006). Consistent with a major role of the transporter in chloride homeostasis and GABA signaling (see below), KCC2 is found in the postsynaptic membrane near symmetrical inhibitory synapses in brainstem (Blaesse et al., 2006), SCN (Belenky et al., 2008), and spinal cord (Hubner et al., 2001). However, KCC2 also accumulates at or near excitatory synapses formed by cerebellar mossy fiber terminals onto granule cells (Takayama and Inoue, 2006), at terminals formed between the cortico-geniculate fibers and thalamic relay nuclei neurons (Bartho et al., 2004), at synapses in the brainstem (Blaesse et al., 2006) and at glutamatergic synapses in spine heads of principal cells of the hippocampus (Gulyas et al., 2001; Gauvain et al., 2011).

We have studied the subcellular distribution of KCC2 in cultured hippocampal neurons. In line with previous observations (Lee et al., 2007; Watanabe et al., 2009; Gauvain et al., 2011), KCC2 membrane distribution was usually punctate. Using confocal fluorescence microscopy, we found that KCC2

clusters decorate the cell body (**Figures 2A,B**), dendritic shafts (**Figure 2C**) and spines (**Figures 2A–D2**) of mature neurons (29 days *in vitro*). Optical sectioning through a dendrite highlighted the preferential membrane localization of the transporter (**Figures 2D2,D3**). On average, KCC2 IF was about 50% higher near cell surface compared with cytoplasm, reflecting a preferential membrane or sub-membrane localization of the transporter. The fluorescence intensity of KCC2 aggregates in spines was about threefold higher than in cytoplasm and twofold higher than on dendritic shaft, demonstrating KCC2 enrichment in spines. KCC2 clustering was also dependent on spine maturation and was maximal in mushroom-type spines, intermediate in stubby spines and absent in filopodia-like structures. KCC2 expression in relation with excitatory and inhibitory synapses was evaluated in hippocampal neurons co-immunolabeled with the glutamate and GABA receptor anchoring proteins PostSynaptic Density protein 95 kD (PSD95) and gephyrin (**Figures 2E1–G**). KCC2 clusters were found both near inhibitory (**Figures 2E1–E3,G**) and

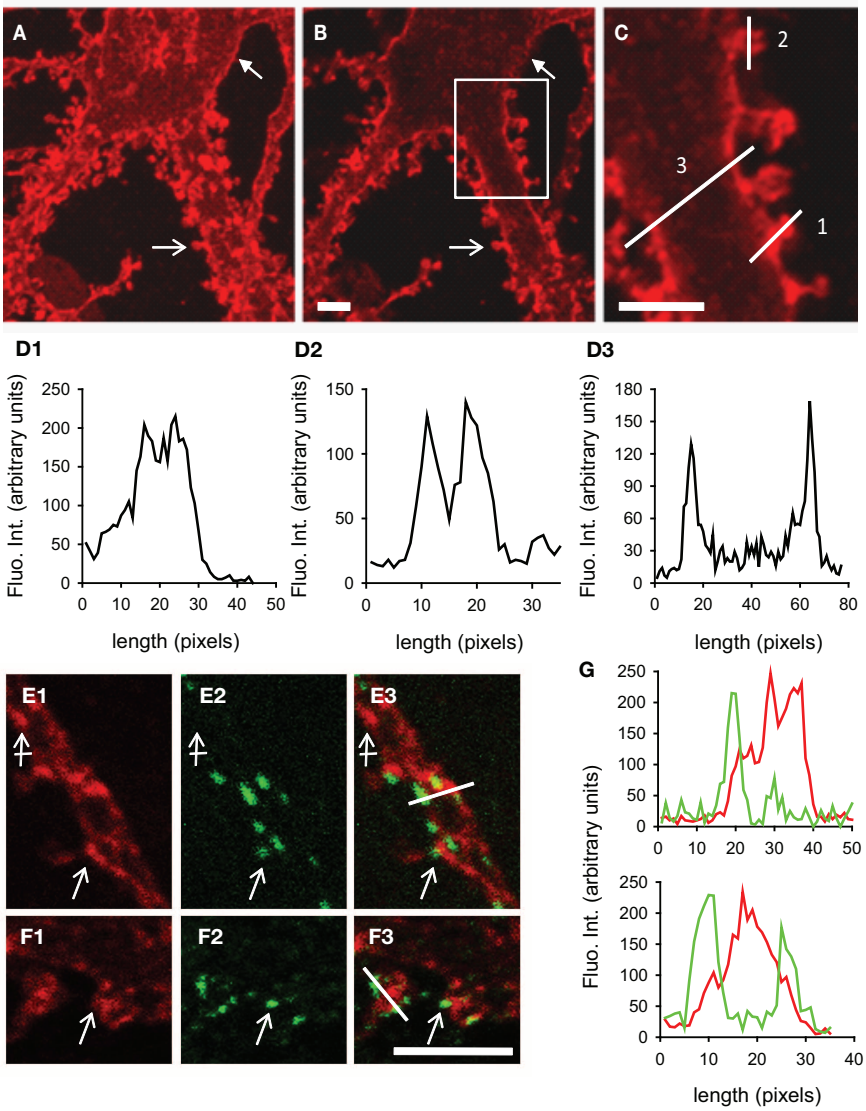

FIGURE 2 | KCC2 clustering in rat hippocampal neurons at 29DIV. (A) Maximum intensity projection of confocal optical sections showing KCC2 at the periphery of somata (filled arrow) and in spines (empty arrow). **(B,C)** optical sectioning from the same neuron as in **(A)**. Boxed region in **(B)** is shown enlarged in **(C)**. Adapted from Gauvain et al. (2011) with permission. Scale bars, 5 μm. **(D)** Fluorescence intensity in arbitrary units per pixel along the lines drawn in **(C)** showing enrichment of KCC2 in dendritic spines **(D1)**, preferential plasma membrane localization of KCC2 in dendritic spines **(D2)** and shafts **(D3)** as compared with the cytoplasm. **(E1–F3)** Dual labeling of KCC2 (red in **E1, E3, F1, F3**) and the GABA and glutamate receptor anchoring proteins gephyrin (green in **E2, E3**) or PSD-95 (green in **F2, F3**). Scale bars, 5 μm. KCC2 forms many clusters on dendritic shafts and spines (empty arrows). Clusters are found at distance from synapses (crossed arrow) as well as near gephyrin-labeled inhibitory synapses (empty arrow in **E1–3**) and PSD95-stained excitatory PSD (empty arrow in **F1–3**). **(G)** Fluorescence intensity in arbitrary units per pixel along the lines drawn in **(E3)** and **(F3)** showing juxtaposition but no colocalization of KCC2 (red) with gephyrin (green, top) or PSD95 (green, bottom).

excitatory (**Figure 2F1–F3,G**) synapses formed on dendrites as well as on the extrasynaptic membrane. However, although concentrated in spines, the co-transporter did not preferentially accumulate within the postsynaptic differentiation (PSD). Instead, it was often localized at the periphery of the PSD (**Figure 2G**). Quantification also revealed the absence of specific KCC2 accumulation at inhibitory synapses (**Figure 2G**). Thus, KCC2 appears to aggregate in close vicinity rather than within synapses, suggesting the existence of specific molecular anchoring mechanisms acting to influence KCC2 clustering.

BASIC PROPERTIES OF THE KCC2 CO-TRANSPORTER

ION CO-TRANSPORT

KCC2 is a secondary active transporter, i.e., it uses the energy of the electrochemical K^+gradient to transport Cl^-. The electrochemical plasma membrane K^+ gradient is generated by the primary active transporter Na-K ATPase (**Figure 3**). KCC2 colocalizes and structurally interacts with the α2 subunit of the Na-K ATPase (Ikeda et al., 2004). Therefore, an alteration of KCC2 membrane expression may impact the Na-K ATPase distribution and/or activity and vice versa. KCC2 functions near thermodynamic equilibrium since the equilibrium potential of K^+ (E_K) is close to the resting membrane potential (RMP) (V_m) (Payne, 1997; Payne et al., 2003). KCC2 can function in either direction depending on the external concentration of K^+ $[K^+]_o$. Even a moderate increase in $[K^+]_o$ may thus reverse Cl^- transport through KCC2. This action may allow K^+ buffering when its extracellular concentration is increased upon sustained neuronal activity (Thompson and Gahwiler, 1989b; Payne, 1997).

WATER TRANSPORT AND THE CONTROL OF CELL VOLUME

Glutamate and GABA/glycine synaptic signaling usually lead to a net influx of Na^+ and/or Ca^{2+} cations and Cl^- anions, respectively. These movements of ions are accompanied by water inflow acting to maintain intracellular osmolarity. Massive water influx may consequently lead to neuronal swelling (Collingridge and Lester, 1989) and, if not compensated, to cell death. Since neurons lack aquaporins (Amiry-Moghaddam and Ottersen, 2003; Andrew et al., 2007) they may use an alternative mechanism to face osmotic challenges. CCCs have been extensively studied in this context of osmotic and volume regulation first in erythrocytes and epithelial cells, two non-neuronal cell types facing large osmotic challenges (Zeuthen and MacAulay, 2002; Hoffmann et al., 2009). It is well established that CCCs function is directly or indirectly coupled to water transport (Zeuthen and MacAulay, 2002; Zeuthen, 2010). Water flows in the same direction as ions to counteract osmotic changes due to CCC activity. The electroneutral Na^+–K^+–$2Cl^-$ NKCC1 co-transporter accumulates intracellular chloride using the electrochemical gradient for Na^+ and K^+ produced by the Na-K ATPase (Gamba, 2005; Blaesse et al., 2009). Both NKCC1 and KCC2 co-transport ions and water and cooperate to maintain ionic and water homeostasis in neurons. It has been estimated that NKCC1 activity triggers a net inflow of 590 water molecules for $1K^+$, $1Na^+$, $2Cl^-$ transported ions. Conversely, KCC2 activity results in the extrusion of 500 water molecules per of $1K^+$ and $1Cl^-$ transported ions [reviewed in (MacAulay and Zeuthen, 2010)]. Consistent with the role of KCC2 in water transport in neurons, experiments using digital holographic microscopy recently revealed that *N*-methyl-D-aspartate (NMDA) receptor activation in neurons triggers water influx through both NMDAR and NKCC1 while delayed activation of KCC2 compensates this influx by mediating a net outflow of water (Jourdain et al., 2011).

The functional properties of NKCC1 and KCC2 co-transporters are reciprocally regulated by serine/threonine phosphorylation. The Wnk kinase family acts as an osmosensor which can lead to reciprocal regulation of NKCC1 and KCC2 in the central nervous system (Kahle et al., 2010). Upon hyperosmotic challenge or in conditions of low $[Cl^-]_i$, activation of Wnk1/3 leads to phosphorylation and activation of NKCC1 through OSR1/SPAK Ste20-type kinases and direct inhibition of KCC2 through phosphorylation of KCC2 Thr906 and Thr1006

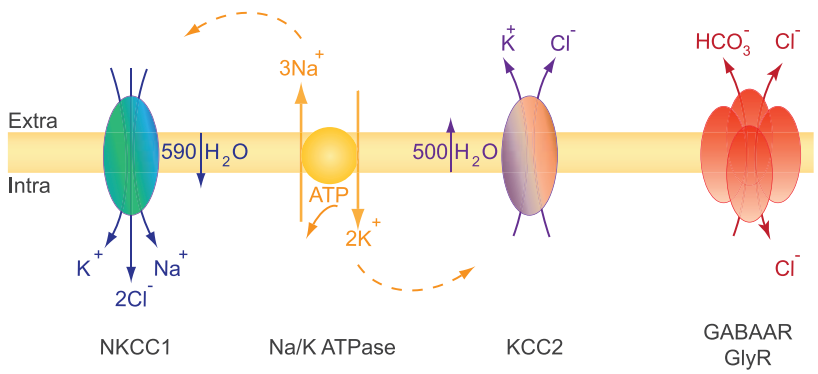

FIGURE 3 | Ions and water transport properties of the co-transporters NKCC1 and KCC2. The secondary active transporters NKCC1 and KCC2 use the energy of the electrochemical Na^+ and K^+ gradients generated by the primary active transporter Na-K ATPase to trigger a net inflow of $1K^+$, $1Na^+$, $2Cl^-$, and a net outflow of $1K^+$, $1Cl^-$ ions, respectively. The activity of each transporter is associated with water influx (NKCC1) or efflux (KCC2). NKCC1 and KCC2 are reciprocally expressed and regulated during development. NKCC1 predominates in early development while KCC2 is the main Cl^- extruder in mature neurons. This expression profile impacts both cell volume regulation and the efficacy and polarity of GlyR and $GABA_AR$ mediated synaptic transmission (see text).

(Gagnon et al., 2006; Rinehart et al., 2009). This results in a net water influx which compensates for cell shrinking due to the hyperosmotic challenge. This process is known as a Regulatory Volume Increase (RVI). Conversely, under hypo-osmotic conditions, activation of KCC2 and inhibition of NKCC1 leads to a Regulatory Volume Decrease (RVD) acting to compensate cell swelling.

INTERACTIONS WITH THE ACTIN CYTOSKELETON

Apart from their ion transport function, several ion channels, pumps, transporters, and exchangers appear to act as cytoskeletal anchors of the plasma membrane [reviewed in (Denker and Barber, 2002)]. Together with other integral membrane proteins, several ion transport proteins anchor actin filaments to the plasma membrane via interactions with the linker proteins of the ankyrin and 4.1 families. A particularly well studied example of such interaction has been described in erythrocytes, where the anion exchanger 1 (AE1) plays a critical role in docking actin cytoskeleton to plasma membrane via ankyrin and 4.1R, thereby influencing cell shape (Jons and Drenckhahn, 1992). KCC2 immunoprecipitation assays recently revealed that the carboxy-terminal domain of KCC2 (KCC2 CTD) directly interacts with 4.1N, a neuronal member of the 4.1 protein family (Li et al., 2007). 4.1N, like other 4.1 family members, possesses a 4.1 Ezrin Radixin Moesin (FERM) domain that binds a variety of transmembrane proteins and a spectrin/actin interaction domain (Baines et al., 2009). Therefore, KCC2 is expected to interact with the submembrane actin cytoskeleton. Such interaction may have multiple implications including (1) to regulate KCC2 clustering and thereby its membrane stability and/or activity, (2) to constrain KCC2 localization to specific membrane microdomains, (3) to control the local organization of actin filaments with downstream effects on subcellular morphology (Mohandas et al., 1992; Cancedda et al., 2007; Li et al., 2007; Gauvain et al., 2011), (4) to regulate cell migration (Wei et al., 2011) and (5) to affect the osmoregulatory response since actin is part of the osmotic sensor of the cell (Mills et al., 1994). These major functions of the KCC2 co-transporter are likely at play in all neuronal compartments where it is expressed. Nevertheless, they may be of particular significance at synapses where they appear to influence synaptic structure and maturation as well as efficacy.

FUNCTIONAL IMPACT OF KCC2 ON SYNAPTIC SIGNALING

FUNCTION IN SYNAPTOGENESIS

Numerous studies have revealed a critical role of KCC2 in synaptogenesis. Early overexpression of recombinant KCC2 in immature hippocampal neurons increases the density of GABAergic terminals, the number of postsynaptic GABAAR clusters as well as the frequency and amplitude of GABAAR-mediated mIPSCs (Chudotvorova et al., 2005). Conversely, the frequency of mIPSCs is reduced in hypomorphic KCC2 mice (Tornberg et al., 2005), suggesting KCC2 is required for the functional maturation of GABAergic synapses. However, the role of KCC2 in the formation of glutamatergic synapses is more controversial. The controversy may result from differences in preparations and experimental methodologies. For instance, early overexpression of KCC2 in *Xenopus laevis* embryos reduced the amplitude and frequency of mEPSCs in tectal neurons (Akerman and Cline, 2006) suggesting elevated $[Cl^-]_i$ may be required for functional maturation of excitatory synapses. Instead, overexpression of KCC2 had no effect on the density of vesicular glutamate transporter isoform 1 (VGlut1)-immunopositive terminals or mEPSC amplitude or frequency in cultured hippocampal neurons (Chudotvorova et al., 2005). This observation, however, contrasts with the effects of the genetic ablation of KCC2 which leads to a reduced number of functional excitatory synapses in immature hippocampal neurons (Li et al., 2007). Finally, in striking contrast with these data, Khalilov et al. (2011) reported a sixfold increase in the density of synaptophysin immunoreactive terminals and increased frequency of spontaneous IPSCs and EPSCs as well as enhanced network activity in CA3 hippocampal neurons from $KCC2^{-/-}$ E18.5 mouse embryos. These discrepancies may result from the timing of both KCC2 manipulations and functional observations and suggest KCC2 differentially modulates synaptogenesis in a very specific time window.

KCC2 may influence synaptogenesis through an ion-transport-independent mechanism (Li et al., 2007; Khalilov et al., 2011). However, the effects of KCC2 on the development of retinotectal circuits rely on a modulation of GABA signaling through shifting transmembrane chloride gradients (Akerman and Cline, 2006). Thus, depolarizing GABA signals may cooperate with NMDAR-mediated transmission to promote the maturation of glutamatergic synapses and the establishment of the balance of excitation and inhibition in developing circuits [for review see (Ben-Ari et al., 2007)].

FUNCTIONNAL IMPACT ON GABA AND GLYCINE SIGNALING

Here, we will present a synthetic view of the well-known impact of KCC2 on inhibitory synaptic transmission and will refer to recent and complete reviews (Ben-Ari, 2002; Ben-Ari et al., 2007; Blaesse et al., 2009). The KCC2-mediated K-Cl co-transport critically determines the electrochemical gradient of chloride ions in neurons. Therefore, a major impact of KCC2 function is on the efficacy or even the polarity of synaptic GABAergic and glycinergic transmissions which both rely on chloride fluxes.

Both GABAARs and GlyRs are primarily permeable to chloride and, to a lesser extent, bicarbonate ions (Bormann et al., 1987). Although these signals are classically considered as 'inhibitory', their polarity and functional impact are dependent on (1) the transmembrane gradients in chloride and bicarbonate ions and (2) the local RMP. Thus, GABAAR-mediated currents are hyperpolarizing only when E_{GABA} (the reversal potential of GABAAR currents, which depends on both E_{Cl} and E_{HCO3}) is hyperpolarized to RMP. Since, under physiological conditions, E_{HCO3} is depolarized as compared to RMP [around −12 mV, (Staley et al., 1995)], a rise in $[Cl]_i$ may be sufficient to depolarize E_{GABA} above RMP, leading to depolarizing actions of GABAAR-mediated currents. This may occur, for instance, during sustained GABAergic activity leading to intraneuronal chloride accumulation (Thompson and Gahwiler, 1989a). It should be noted however, that depolarizing glycine or GABAAR-mediated currents may still be functionally inhibitory due to the electrical shunt of the membrane input resistance generated by the opening of these receptors (Staley and Mody, 1992).

Although measuring $[Cl^-]_i$ in neurons remains a technical challenge potentially subject to many pitfalls (Bregestovski et al., 2009), several studies converge to suggest it may range relatively high values during early postnatal development [25–40 mM; refs in (Blaesse et al., 2009)]. This likely reflects the expression and activity of the NKCC1 transporter which acts to accumulate chloride in neurons and the expression of which precedes that of KCC2 (Plotkin et al., 1997; Hubner et al., 2001). Elevated internal chloride concentration depolarize E_{GABA} above RMP and predicts depolarizing actions of GABA acting on GABAARs in immature neurons. Although this view has been taken for granted for many years (Ben-Ari, 2002), it was recently challenged by experiments suggesting both RMP and E_{GABA} may be strongly dependent on the energy supply to immature neurons, which may be inappropriate in experimental conditions used to measure these variables (Rheims et al., 2009; Ruusuvuori et al., 2010; Tyzio et al., 2011). Nevertheless, several actions of GABA have been associated with membrane depolarization due to low KCC2 function and high intracellular chloride in immature neurons. Those include the generation of population activities such as giant depolarizing potentials (GDPs) *in vitro* (see Ben-Ari, 2002), the maturation of retinotectal circuits (Akerman and Cline, 2006) and cortical interneuron migration (Bortone and Polleux, 2009) *in vivo*.

It is generally agreed that an increase in KCC2 function correlates with a progressive hyperpolarization of E_{GABA} reflecting a reduction of $[Cl^-]_i$ in mature neurons to about 5 mM (Khirug et al., 2008; Tyzio et al., 2008). This developmental switch in chloride electrochemical gradient occurs in almost all brain structures and organisms analyzed, but with variation in the spatio-temporal developmental course (Ben-Ari, 2002). It should be noted however that steady-state $[Cl^-]_i$ is an average and shows intercellular and intracellular variability. GABAAR-mediated currents impinging onto neighboring neurons or even onto different subcellular compartments onto a given neurons may, therefore, be very different. In particular, $[Cl^-]_i$ may differ up to two to threefold between the somatic and dendritic compartments (Duebel et al., 2006) or even from one dendritic branch to the other (Waseem et al., 2010). Similarly, an axo-somatic gradient of E_{GABA} has been reported which may range up to 15–20 mV (Szabadics et al., 2006; Khirug et al., 2008). Thus, GABAAR-mediated currents are usually depolarizing on axon terminals [e.g., (Ruiz et al., 2003); see (Kullmann et al., 2005)] or AISs [(Szabadics et al., 2006; Khirug et al., 2008); but see (Glickfeld et al., 2009)] and hyperpolarizing on somato-dendritic compartments [(Banke and McBain, 2006; Tyzio et al., 2006; Khirug et al., 2008); but see (Tyzio et al., 2008)]. This axo-somatic gradient likely reflects the differential subcellular distribution of KCC2 and NKCC1 (Khirug et al., 2008). In addition, local, activity-dependent alteration of $[Cl^-]_i$ along a single dendrite is correlated with a transient and local shift in E_{GABA} (Dallwig et al., 1999; Staley and Proctor, 1999; Kuner and Augustine, 2000; Isomura et al., 2003; Jedlicka et al., 2011), the dynamics of which likely depends on KCC2 function [see (Doyon et al., 2011)]. Thus, KCC2 dynamically regulates the efficacy of GABA signaling through a local control over $[Cl^-]_i$. Therefore, modulation of KCC2 function may not only impact the steady-state efficacy of GABA signaling through GABAARs but also its dynamic operation. This may occur for instance upon repetitive neuronal activity (Fiumelli and Woodin, 2007) as well as in several pathological conditions which have been shown to both suppress KCC2 expression and affect the efficacy of GABA signaling. These include epilepsy (Cohen et al., 2002; Huberfeld et al., 2007), spinal cord injury (Boulenguez et al., 2010), neuropathic pain (Coull et al., 2003) and others (see Kahle et al., 2008).

FUNCTION AT EXCITATORY SYNAPSES

Although KCC2 primarily influences GABAergic transmission in cortical neurons, its accumulation in dendritic spines near excitatory synapses (Gulyas et al., 2001) suggests a possible interaction with glutamatergic signaling. Most excitatory synapses onto cortical neurons are formed onto dendritic spines which appear concomitantly with the up-regulation of KCC2 during early postnatal development (Rivera et al., 1999; Yuste and Bonhoeffer, 2004). In the absence of KCC2 expression in knock-out animals, spine morphogenesis is largely compromised in immature neurons, leading to anomalously long, filopodia-like protrusions (Li et al., 2007). This defect in spine maturation is associated with a reduced number of functional excitatory synapses as detected by a decreased density of synaptic clusters of the glutamate receptor anchoring proteins PSD95 and Homer and reduced mEPSC frequency. Importantly, this effect can be rescued by expression of a non-functional, *N*-terminal deficient KCC2 and mimicked by expression of the KCC2 CTD (Li et al., 2007). This observation suggests that KCC2 influences spine morphogenesis and functional maturation through a mechanism independent of its ion transport function.

KCC2 CTD directly interacts with the neuronal FERM-domain actin-binding protein 4.1N (Li et al., 2007). F-actin constitutes the main cytoskeleton of dendritic spines and its dynamic organization is known to play a critical role in spine morphogenesis (Tada and Sheng, 2006). In particular, submembrane actin dynamics have been shown to be essential during spine head formation and the transition from filopodium to dendritic spine (Hotulainen et al., 2009). Thus, KCC2–4.1N interaction may contribute to organize submembrane actin cytoskeleton in developing dendritic spines. Consistent with this hypothesis, overexpression of 4.1N FERM domain to prevent KCC2–4.1N interaction is sufficient to prevent spine morphogenesis in hippocampal neurons (Li et al., 2007).

Remodeling of the actin cytoskeleton not only impacts spinogenesis but also affects excitatory synaptic function (Hotulainen and Hoogenraad, 2010). Actin-depolymerizing factors, in particular, induce changes in synaptic function that may reflect changes in glutamate receptor membrane diffusion (Rust et al., 2010). We recently reported that chronic suppression of KCC2 after spine formation does not compromise spine maintenance in mature (>24 DIV) hippocampal neurons (Gauvain et al., 2011). Instead, it is associated with a reduction in the efficacy of excitatory synapses and aggregation of the GluA1 subunit in dendritic spines. Again, this effect is independent of a loss of KCC2 function since it is mimicked by overexpressing the KCC2 CTD but not by application of a selective KCC2 antagonist. Using single particle tracking (SPT) techniques, we showed that suppression of KCC2 expression induced an increase in the lateral diffusion

of rapidly moving, likely extrasynaptic AMPA receptors (Tardin et al., 2003; Petrini et al., 2009) in dendritic spines but not on dendritic shafts. Immobile (likely postsynaptic) receptors were also unaffected. Thus, KCC2 interaction with intracellular partners is essential to spine morphogenesis but not maintenance, and contributes to hinder lateral diffusion of AMPA receptors within dendritic spines, thereby influencing synaptic efficacy (**Figure 4**). A loss of KCC2 clusters in dendritic spines induced by sustained excitatory synaptic activity or under pathological conditions may then induce a rapid homeostatic adjustment of synaptic receptor content and efficacy by acting on the lateral diffusion of AMPA receptors.

Finally, although KCC2 is not necessary for dendritic spine maintenance in mature neurons, its suppression leads to increased spine head volume, an effect that is mimicked by chronic application of a KCC2 antagonist (Gauvain et al., 2011). As described above, KCC function is essential to volume regulation in many cell types (Gamba, 2005). Since KCC2 is the only CCC leading to solute and water extrusion under isotonic conditions (MacAulay et al., 2004; Jourdain et al., 2011), it likely contributes to osmotic regulation in dendritic spines, in particular to counteract cation influx through postsynaptic receptors. These observations predict that changes in KCC2 aggregation or function by synaptic activity may induce activity-dependent modulation of spine volume through local regulation of transmembrane solute and water efflux.

REGULATION OF KCC2 EXPRESSION AND FUNCTION

The expression level and/or transport activity of KCC2 are up-regulated during development (Kahle et al., 2005; Vale et al., 2005; Rinehart et al., 2009; Ludwig et al., 2011a,b). In mature neurons, the activity-dependent regulation of KCC2 contributes to the plasticity of inhibitory synapses under both physiological and pathological conditions. In some conditions, the activity-dependent up-regulation of KCC2 may counteract synaptic excitation by strengthening GABA signaling (Banke and Gegelashvili, 2008; Chorin et al., 2011) whereas in some pathological conditions, KCC2 down-regulation may promote excitotoxicity (Hershfinkel et al., 2009) or seizure (Reid et al., 2001; Rivera et al., 2002, 2004; Huberfeld et al., 2007; Pathak et al., 2007; Wake et al., 2007; Li et al., 2008b; Shimizu-Okabe et al., 2011). Thus, activity-dependent regulation of KCC2 participates in the adjustment of the excitatory-inhibitory balance in neuronal networks.

DEVELOPMENTAL UP-REGULATION OF KCC2

In developing hippocampal neurons, KCC2 expression is increased by the trophic factors neurturin (Ludwig et al., 2011a,b) and BDNF (Ludwig et al., 2011b). BDNF and Neurturin activate similar intracellular cascades that include the src homology two domain containing transforming protein/FGF receptor substrate 2 (Shc/FRS-2). This in turn triggers the extracellular signal-regulated kinase 1/2 (ERK1/2) and the mitogen-activated protein kinase (MAPK) pathway. This effect induces enhanced Egr4 expression and Egr4-dependent activation of KCC2b promoter (Ludwig et al., 2011b). Functional activation of KCC2 also requires oligomerization and/or phosphorylation/dephosphorylation of the co-transporter. Blaesse et al. (2006) showed that membrane expression and mature glycosylation pattern were not sufficient to ensure KCC2-mediated Cl^- extrusion in immature rat LSO neurons. At P3, KCC2 is mainly expressed as a monomeric form in plasma membrane, whereas in more mature neurons (P30) in which Cl^- extrusion was effective, KCC2 was found as oligomers (dimers, trimers, and tetramers). A direct link between oligomerization and ion transport by KCCs is supported by the observation that truncation of KCC1 carboxy-terminal domain prevents both oligomerization and chloride transport (Casula et al., 2001). Phosphorylation and dephosphorylation also play a critical role in KCC2 activation during development. Khirug et al. (2005) observed that the broad-spectrum kinase inhibitor staurosporine, leads to a rapid (5 min) negative shift in EGABA in immature hippocampal cultured neurons.

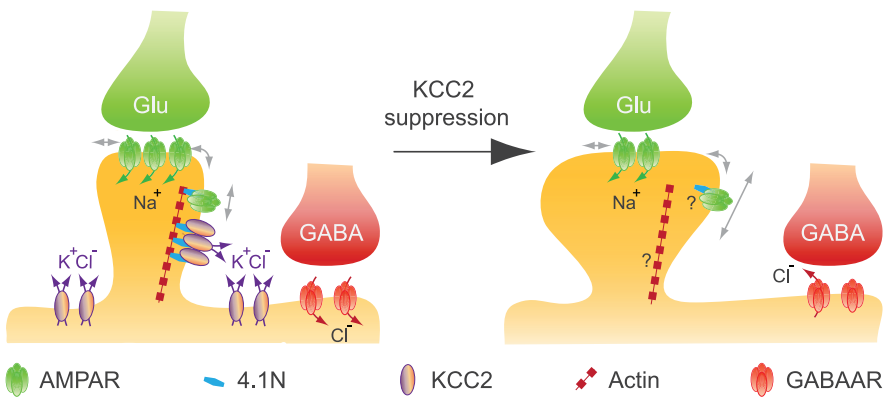

FIGURE 4 | KCC2 controls spine volume and efficacy of glutamatergic AMPAR-mediated synaptic transmission in mature neurons. KCC2 suppression in mature neurons indicates that KCC2 is not required for spine maintenance, while it contributes to both spine head volume regulation and the efficacy of excitatory synapses by physically constraining AMPAR GluA1 subunit in spine head, likely through its interaction with submembrane actin cytoskeleton [adapted from Gauvain et al. (2011)]. A loss of KCC2 clusters induced by sustained excitatory synaptic activity or under pathological conditions may induce a rapid homeostatic adjustment of the efficacy of AMPAR mediated synaptic transmission as well as a reduction in the efficacy of $GABA_AR$-dependent synaptic transmission.

Although this work did not identify the kinase(s) involved or their target(s) (i.e., KCC2 itself and/or molecular partners), KCC2 can be activated by dephosphorylation of Threonine residues. Residues Thr906 and Thr1006 in the CTD of KCC2 are phosphorylated by Wnk1 at birth and dephosphorylated in adult neurons (Rinehart et al., 2009). These phosphorylated residues appear critical for KCC2 function since Wnk1 phosphorylation of KCC2 in mature neurons causes a loss of transport function (Kahle et al., 2005; Rinehart et al., 2009). Although phosphorylation of Ser940 has not been analyzed during development, this residue is phosphorylated in mature hippocampal neurons (Lee et al., 2007), and PKC activation enhances KCC2 function (Banke and Gegelashvili, 2008) through direct phosphorylation of Ser940 (Lee et al., 2007). Therefore, PKC-dependent phosphorylation of Ser940 may also contribute to the developmental or activity-dependent activation of KCC2. Finally, the developmental activation of KCC2 function requires tyrosine kinase activity. Thus, inhibitors of tyrosine phosphorylation such as genistein or lavendustin A impair KCC2 activity in mature hippocampal neurons (Kelsch et al., 2001). KCC2 appears to be a direct target of tyrosine kinases as tyrosine phosphorylation shows a marked increase during postnatal development in cochlear nucleus neurons (Vale et al., 2005). Consistent with this observation, dephosphorylation of Tyr1087 abolishes KCC2 activity in mature neurons (Wake et al., 2007; Watanabe et al., 2009). In conclusion, functional activation of KCC2 during postnatal development appears to rely on dephosphorylation of Thr906 and Thr1006, PKC-dependent phosphorylation of Ser940, and tyrosine kinase phosphorylation of Tyr1087 (**Table 1**).

ACTIVITY-DEPENDENT REGULATION

Neuronal activity dynamically up- or down-regulates KCC2 activity. Rapid up-regulation of KCC2 activity is induced upon activation of group I metabotropic GluRs (mGluRs) and Zn^{2+} extracellular signaling. Tonic activation of both mGluR1 and mGluR5 has been shown to enhance synaptic strength at GABAergic inputs onto CA3 pyramidal neurons through hyperpolarization of E_{GABA} (Banke and Gegelashvili, 2008). This effect may involve synaptic co-release of Zn^{2+} from mossy fibers and subsequent activation of the metabotropic zinc-sensing receptor G-protein coupled receptor 39 (mZnR/GPR39) which in turn causes a hyperpolarizing shift in E_{GABA} by enhancing KCC2 activity and surface expression in CA3 neurons in hippocampal slices (Chorin et al., 2011). mZnR activation triggers Ca^{2+} release from intracellular stores via group I mGluRs, activation of the ERK1/2 pathway and PKC phosphorylation of KCC2 (Banke and Gegelashvili, 2008; Besser et al., 2009; Sindreu et al., 2011). In this context, it is remarkable that synaptic accumulation of Zn^{2+} in mossy fiber terminals and KCC2 activity in pyramidal neurons follow a similar developmental time course (Rivera et al., 1999; Nitzan et al., 2002; Lee et al., 2005; Liguz-Lecznar et al., 2005), suggesting Zn^{2+} signaling may participate in the developmental up-regulation of KCC2 (Chorin et al., 2011). Interestingly however, Zn^{2+} appears to operate a bi-directional control over KCC2 activity. In contrast to extracellular Zn^{2+}, a rise in intracellular Zn^{2+} under pathological conditions such as oxygen-glucose deprivation inhibits KCC2 function resulting in a depolarizing shift in E_{GABA} (Hershfinkel et al., 2009).

KCC2 activity is down-regulated in a variety of physiological and pathological conditions, usually associated with enhanced neuronal activity. These include long term potentiation (Wang et al., 2006b), repetitive pairing of pre- and post-synaptic activities (Woodin et al., 2003), repetitive postsynaptic spiking (Fiumelli et al., 2005), rebound burst activity (Wang et al., 2006a), tetanic stimulation (Kaila et al., 1997), NMDA receptor activation (Kitamura et al., 2008), and epileptic activity (Reid et al., 2001; Rivera et al., 2002, 2004; Huberfeld et al., 2007; Pathak et al., 2007; Wake et al., 2007; Li et al., 2008b; Shimizu-Okabe et al., 2011). All these conditions cause a depolarizing shift in E_{GABA} through reduced KCC2 function and/or expression.

The activity-dependent down-regulation of KCC2 mRNA level involves the BDNF-TrkB signaling and the combination of the Shc/FRS-2 and the phospholipase Cγ (PLCγ) signaling cascades (Rivera et al., 2004). This in turn activates the transcription factor cAMP response element-binding protein (CREB) through phosphorylation (Rivera et al., 2004). At the post-translational level, down-regulation of KCC2 usually relies on Ca^{2+} signaling and involve either Ca^{2+} influx through NMDA receptors (Kitamura et al., 2008; Lee et al., 2011) or L type Ca^{2+} channel (Fiumelli et al., 2005) or Ca^{2+}-induced-Ca^{2-} release from intracellular stores (Fiumelli et al., 2005). Although Ca^{2+} was initially suggested to activate a PKC-dependent phosphorylation of either KCC2 itself or some associated molecules that will in turn inactivate the transporter (Fiumelli et al., 2005), this seems somewhat unlikely since PKC-dependent phosphorylation of KCC2 on Ser940 enhances rather than reduces its activity (Lee et al.,

Table 1 | Phosphorylation/dephosphorylation states of KCC2 at immature or mature stages of development and in conditions of increased activity.

	Immature	Mature	Mature activity-dependent down regulation
Thr906 and Thr1006	Phosphorylated by Wnk kinases (Rinehart et al., 2009)	Dephosphorylated (Rinehart et al., 2009)	?
Tyr1087	Dephosphorylated (Vale et al., 2005)	Phosphorylated (Vale et al., 2005; Wake et al., 2007; Watanabe et al., 2009)	Phosphorylation/Dephosphorylation of Tyr903 Tyr1087 (Wake et al., 2007; Lee et al., 2010)
Ser940	?	Phosphorylated by PKC (Lee et al., 2007)	Dephosphorylated by PP1 (Lee et al., 2011)

2007; Banke and Gegelashvili, 2008). Instead, activity-dependent reduction of KCC2 activity may involve dephosphorylation of Ser940 (Lee et al., 2011) and perhaps phosphorylation of Tyr903/1087 residues (Wake et al., 2007; Lee et al., 2010). NMDAR-dependent Ca^{2+} influx causes PP1 dephosphorylation of KCC2 residue Ser940 (Lee et al., 2011). Thus, PKC-dependent phosphorylation of Ser940 enhances (Lee et al., 2007) while the PP1-mediated dephosphorylation of Ser940 inhibits KCC2 activity (Lee et al., 2011). Lee et al. (2011), therefore, proposed that KCC2 function is controlled by the balance between PKC and PP1 activities. The involvement of residues Tyr903/1087 in the down-regulation of KCC2 by activity is supported by the observation that pilocarpine-induced status epilepticus increases the phosphorylation of KCC2 residues Tyr903/1087 (Lee et al., 2010). However, hyperexcitability induced by BDNF or low external Mg^{2+} as well as oxidative stress all lead to tyrosine dephosphorylation of KCC2 (Wake et al., 2007). Interestingly, in these experiments, tyrosine phosphorylation of KCC2 could only be unmasked once phosphatase activity was blocked. Thus, neuronal activity appears to recruit both kinases and phosphatases, the relative activity of which determines KCC2 phosphorylation state. It is remarkable that developmental up-regulation and activity-dependent down-regulation of KCC2 involve a reciprocal regulation of its phosphorylation (**Table 1**).

REGULATION OF KCC2 BY OLIGOMERIZATION, CLUSTERING AND LATERAL DIFFUSION

Membrane expression and glycosylation of KCC2 precede the onset of KCC2 function and the hyperpolarizing shift in E_{GABA} during postnatal development (Kelsch et al., 2001; Khirug et al., 2005; Vale et al., 2005; Blaesse et al., 2006). In addition, enhanced neuronal activity affects KCC2 at the mRNA and protein expression levels within 1–6 h (Rivera et al., 2002, 2004; Wang et al., 2006c; Wake et al., 2007; Ludwig et al., 2011b) whereas activity-dependent regulation of KCC2 function occurs within minutes (Woodin et al., 2003; Wang et al., 2006b; Fiumelli and Woodin, 2007; Wake et al., 2007; Kitamura et al., 2008; Chorin et al., 2011; Lee et al., 2011). Therefore, regulation of KCC2 function in neurons appears to occur at several, partly interdependent levels and the rapid, activity-dependent modulation of the transporter likely occurs predominantly through post-translational modifications. Activity-induced dephosphorylation of Ser940 and phosphorylation of Tyr903/1087 leads to reduced membrane stability of KCC2 through increased endocytosis and targeting to lysosomal degradation (Lee et al., 2010, 2011). However, the loss of KCC2 activity as detected by increased $[Cl^-]_i$ precedes the removal of KCC2 from cell surface (Wake et al., 2007; Watanabe et al., 2009). Conversely, Zn^{2+}-dependent increase in KCC2 function is observed before the increase in membrane expression (Chorin et al., 2011). These observations suggest that other intermediate mechanisms may be at play to affect KCC2 function independent of its membrane expression level.

As mentioned above, KCC2 oligomerizes and forms clusters at plasma membrane. It is, therefore, tempting to hypothesize that oligomerization and clustering could represent the molecular substrate for such rapid alterations of KCC2 function. So far, only one study has investigated the impact of oligomerization and clustering on KCC2 activity (Watanabe et al., 2009). This study shows that a perturbation of KCC2 oligomerization and clustering by tyrosine kinase inhibitors correlates with a loss of function but no change in membrane expression in hippocampal neurons. Besides, heterologous GT1–7 cells expressing a recombinant KCC2 with either Tyr1087 residue mutated to aspartate (Y1087D) or a deletion of the 28 amino acids distal to the tyrosine phosphorylation site fails to oligomerize and form clusters as wild type KCC2 does (Watanabe et al., 2009). These results support a role for oligomerization and clustering in the regulation of KCC2 function. They also indicate that phosphorylation of Tyr1087 and the carboxyl-terminus of KCC2 play a critical role in oligomerization and clustering.

The mechanisms of KCC2 clustering remain elusive and may involve cholesterol-enriched lipid rafts (Watanabe et al., 2009). Indirect binding of KCC2 to actin through 4.1N interaction (Li et al., 2007) suggests clustering may also involve scaffolding molecules as described for postsynaptic neurotransmitter receptors. Identification of these molecular partners will be of particular importance since the availability of the submembrane scaffold that anchors and stabilizes transmembrane proteins can be rapidly up- or down-regulated by activity (Bruneau et al., 2009) and could be at play to locally control KCC2 aggregation and function.

Lateral diffusion in and outside synapses plays a key role in the activity-dependent regulation of receptor number at synapses (Triller and Choquet, 2008). This raises the question of the impact of lateral diffusion in the control of KCC2 clustering. The mobility of recombinant Flag-tagged KCC2 was analyzed using SPT (Dahan et al., 2003; Bannai et al., 2006). The surface recombinant Flag-tagged KCC2 transporter were labeled with an antibody raised against Flag and subsequently labeled with an intermediate biotinylated Fab fragment and streptavidin-coated quantum dot (QD). QD trajectories were overlaid with fluorescent images of recombinant homer1c-GFP and gephyrin-mRFP clusters in order to identify excitatory and inhibitory synapses, respectively. Trajectories were at/near synapses when overlapping ± 2 pixels (440 nm) with homer1c-GFP and gephyrin-mRFP clusters or extrasynaptic for spots further away (Dahan et al., 2003). As shown in **Figure 5**, KCC2 exploratory behavior (**Figure 5A**) and mobility (**Figure 5B**) is reduced and its confinement (**Figure 5C**) increased in close vicinity of excitatory and inhibitory synapses.

As shown with confocal microscopy (**Figure 2**) and with electron microscopy (Gulyas et al., 2001; Bartho et al., 2004; Blaesse et al., 2006; Takayama and Inoue, 2006), KCC2 is localized at the periphery of the postsynaptic density. Thus, the QDs located in close vicinity of synapses (**Figure 5A**) may correspond to perisynaptic QDs. However, several technical limitations do not permit to strictly separate QDs in the core of the synapse from QDs in the near vicinity of the postsynaptic differentiation (i.e., perisynaptic QDs). Indeed, the optical resolution of the wide field microscope does not permit to precisely segregate the two compartments. Second, due to their large size (20–30 nm), QDs have difficulties to enter the core of the synapse (Groc et al., 2007). Anyway, the extrasynaptic vs. synaptic/perisynaptic diffusion behavior of KCC2 is reminiscent of what was found for neurotransmitter receptors with confined movement of the

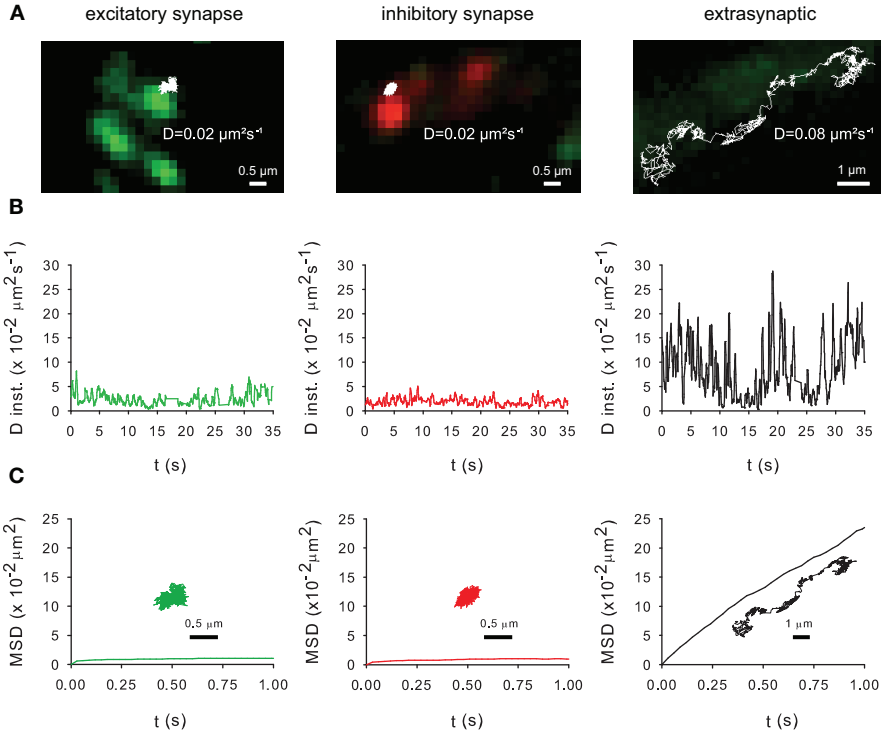

FIGURE 5 | Membrane dynamics of the KCC2 transporter studied with Single Particle Tracking. (A) Representative trajectories of Quantum Dot-bound Flag tagged recombinant KCC2 reconstructed from 35 s recording sequences ($\Delta t = 0.03$ s). QD trajectories were overlaid with fluorescent images of recombinant homer1c-GFP (green) and gephyrin-mRFP (red) clusters in order to identify excitatory and inhibitory synapses, respectively. Note that extrasynaptic QD-bound KCC2 explored larger area of membrane than synaptic/perisynaptic ones. Scale bars for synaptic/perisynaptic and extrasynaptic trajectories, 0.5 µm and 1 µm, respectively. **(B)** Instantaneous diffusion coefficients of the trajectories shown in **(A)**. Note the reduction in diffusion coefficient values and fluctuations for synaptic/perisynaptic QDs as compared with the extrasynaptic QD. **(C)** Time-averaged MSD functions of the trajectories shown in **(A)**. Extrasynaptic and synaptic QDs display linear and negatively bent MSD curves, characteristic of random walk and confined movement, respectively. In all graphs: green and red curves, trajectories at/near excitatory and inhibitory synapses, respectively; black, extrasynaptic trajectory.

protein at/near synapses and free diffusion in extrasynaptic area [ref in (Triller and Choquet, 2008)]. More work is now required to examine whether KCC2 membrane dynamics and clustering are modulated by normal and pathological neuronal activities. A regulation of KCC2 diffusion/clustering might locally affect the net function of KCC2-mediated chloride extrusion. This will be particularly relevant for the regulation of GABA signaling which may be modulated at the subcellular level (Foldy et al., 2010).

CONCLUSIONS AND PERSPECTIVES

The neuronal KCC2 transporter has long been considered only with respect to its function of chloride transport and its subsequent influence on GABA/glycine signaling. It is now becoming clear that KCC2 function in neurons extends beyond the mere transport of ions across the plasma membrane. In particular, KCC2 turns out to play a major role in the maturation and functional maintenance of excitatory synapses where it regulates the density of AMPAR in spines, most likely through actin binding property via 4.1N protein. Interestingly, 4.1N is required for activity-induced exocytosis of GluA1 subunit of AMPAR during long term potentiation (Lin et al., 2009). More work is, therefore, needed to fully characterize the contribution of KCC2/4.1N complexes in AMPAR traffic and long term synaptic plasticity. Another emerging field is that of KCC2 membrane traffic and its regulation by activity. Where does KCC2 exocytosis take place and is it regulated by neuronal activity? If so, this may represent a way to rapidly adjust KCC2 membrane expression and function. We propose that lateral diffusion and clustering of the transporter participate in the rapid regulation of its function. More work is needed to identify the scaffolding molecules that anchor KCC2 to the submembrane cytoskeleton. Whether KCC2 membrane diffusion undergoes a "diffusion trap" mechanism similar to postsynaptic neurotransmitter receptors and whether such mechanism may contribute to the activity-dependent modulation of KCC2 also remain to be established. In this context, it is noteworthy that similar cellular (cell surface stability, clustering, and perhaps lateral diffusion) and molecular (phosphorylation/dephosphorylation) mechanisms are at play to regulate KCC2 and GABAAR at the cell surface [for GABAAR regulation, see (Luscher et al., 2011)]. This raises questions about potential interactions of KCC2 and GABAAR and of their role in the coordinated regulation of GABA/glycine synaptic signaling.

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Co-release of GABA does not occur at glycinergic synapses onto lumbar motoneurons in juvenile mice

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The fast inhibitory neurotransmitters glycine and GABA are co-localized in synaptic terminals of inhibitory interneurons in the spinal cord and co-released onto lumbar motoneurons in neonatal rats. We performed whole-cell voltage-clamp experiments on spinal cord preparations obtained from juvenile (P8–14) mice to determine whether inhibitory currents exhibited GABAergic components in motoneurons of animals of weight-bearing age. Subsequently we established whether or not GABA is co-released at glycinergic synapses onto motoneurons by determining if it conferred modulatory effects on the kinetics of glycinergic currents. Exponential fitting analysis showed that evoked and miniature inhibitory post-synaptic currents (IPSCs) were best-fitted with a single decay time constant. Responses recorded from connected interneuron-motoneuron pairs showed no effect of a benzodiazepine or a $GABA_A$ receptor antagonist. Similarly IPSCs evoked by extracellular stimulation and miniature IPSCs were not affected by either agent, indicating the absence of co-detection. Experimental manipulation of the relative content of pre-synaptic GABA and glycine conferred no effect on post-synaptic responses. It is thus unlikely that GABA is co-released in biologically relevant amounts at glycinergic synapses onto lumbar motoneurons in mice of this age.

Keywords: glycine, GABA, spinal cord, motoneuron, co-release, mouse, patch clamp, paired recordings

INTRODUCTION

Fast inhibition in the nervous system is mediated by γ-aminobutyric acid (GABA) and glycine. Histological studies have shown that both neurotransmitters are present at synaptic terminals in the spinal cord (Bohlhalter et al., 1994; Taal and Holstege, 1994; Todd et al., 1996; Örnung et al., 1996). Recordings of miniature inhibitory post-synaptic currents (mIPSCs) from motoneurons (Jonas et al., 1998) have demonstrated that a high proportion of currents are mediated by both glycine and GABA. Since each mIPSC constitutes the post-synaptic response to an individual quantum of transmitter, the observation suggests that vesicles are loaded with both neurotransmitters which are released together; the notion of co-release was supported by similar observations in recordings from connected interneuron-motoneuron pairs.

Evidence of co-release has been reported in the brainstem of juvenile rats (O'Brien and Berger, 1999) and in the dorsal horn of adult rats (Chéry and De Koninck, 1999). GABA even modulates the glycinergic current and shortens the time constant of the decay phase (Lu et al., 2008) through its action as a partial agonist. Vesicular filling of both neurotransmitters is possible since the vesicular inhibitory amino acid transporter (VIAAT) is shared between GABA and glycine albeit with higher affinity for GABA (Wojcik et al., 2006).

Electrophysiological evidence of co-release on motoneurons (Jonas et al., 1998) however is based on experiments using preparations obtained mainly from young (P6–7) rats. Recordings from neurons in the dorsal horn (Keller et al., 2001) and from interneurons in the ventral horn (González-Forero and Alvarez, 2005) have identified populations of cells in which the extent of co-release decreases with age. In the adult rat, the majority of inhibitory inputs from Ia interneurons and Renshaw cells onto motoneurons are glycinergic (>80%) but a third of glycinergic terminals are also immunoreactive for the glutamic acid decarboxylase (GAD) enzyme (Alvarez et al., 2005). Since GAD is a marker for GABA synthesis, co-release may be a physiologically important mechanism of inhibition by a population of interneurons in the mature spinal cord.

In the present study, we recorded from motoneurons in spinal cord preparations obtained from mice of an age range (P8–14) in which they are almost fully weight-bearing. This is the latest developmental stage at which such recordings can be reliably obtained. The principal aim of the study was to ascertain whether GABA is released from pre-motor glycinergic interneurons and if it has any detectable effect on glycinergic inhibition of motoneurons. We thus performed experiments to detect the contribution of any GABAergic component in glycinergic inhibitory post-synaptic currents (IPSCs). Subsequently we manipulated the relative content of pre-synaptic GABA and glycine to determine if this had any modulatory effect on the kinetics of post-synaptic responses.

MATERIALS AND METHODS

Spinal preparations were extracted from P8–14 mice in which the enhanced green fluorescent protein (EGFP) is expressed under the control of the promoter of the neuronal glycine transporter GlyT2

(Zeilhofer et al., 2005). The transgenic strain was used to assist identification of glycinergic neurons during paired recordings. All experiments were undertaken in accordance with the Animal (Scientific Procedures) Act (UK) 1986.

SPINAL CORD PREPARATION

Animals were anesthetized with urethane 1.8 mg/kg i.p. Intracardiac perfusion was performed with the same extracellular solution as used for the normal artificial cerebrospinal fluid (aCSF) perfused during records of composition (in mM) 113 NaCl, 3 KCl, 25 $NaHCO_3$, 1 NaH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$ and 11 D-glucose. Following decapitation, spinal cords were extracted using standard techniques (Beato, 2008). The procedure comprised of a ventral laminectomy followed by rapid dissection of the cord in oxygenated ice cold aCSF.

Tissue glue (Vetbond, WPI Scientific Instruments) was used to adhere the dorsal side of the cord to an agar block. Transverse slices of thickness 400 μm were cut using a VT1000 vibrating microtome (Leica Microsystems) from the lumbar segments (L2–L5) in oxygenated ice cold solution containing (in mM) 130 K-gluconate, 15 KCl, 0.05 EGTA, 20 HEPES, 25 D-glucose, 3 kynurenic acid, and pH 7.4 (Dugué et al., 2005). For coronal sections, the agar block was glued horizontally to the base of the vibrating microtome and the dorsal horns were sliced away just dorsal to the central canal. Tissue preparations were incubated at 37°C in normal extracellular solution for approximately 45 min prior to experimentation. Experiments were performed at room temperature during which slices were continuously perfused at 5–8 ml/min with the aCSF bubbled with a 95/5% O_2/CO_2 mixture.

PATCH RECORDINGS

Whole-cell voltage-clamp recordings from motoneurons were performed using an Axopatch 200B amplifier (Molecular Devices) and filtered with an eight-pole Bessel filter at 5 kHz. Both voltage and current signals were sampled at 50 kHz using an Axon 1440A interface device (Molecular Devices) and the data were acquired using Clampex 10 software (Molecular Devices). Electrodes were pulled using a P-1000 Flaming/Brown micropipette puller (Sutter Instruments) from thick-walled borosilicate glass GC150F capillaries (Harvard Apparatus) to a resistance of ∼0.5 MΩ. The tips were fire polished to final resistance of ∼1–1.5 MΩ.

Electrodes were filled with an internal solution of composition (in mM) 140 CsCl, 4 NaCl, 0.5 $CaCl_2$, 10 HEPES, 5 EGTA, 2 Mg-ATP, QX-315 Br 3, pH 7.3 with CsOH, and osmolarity of 290–310 mOsM. High intracellular chloride prolongs the decay phase of both glycinergic (Pitt et al., 2008) and GABAergic (Houston et al., 2009) currents through an interaction with amino acids in the pore-lining region of the channels (Moroni et al., 2011). A high chloride internal solution was chosen to improve the signal-to-noise ratio and to allow direct comparison with previously published observations (Jonas et al., 1998).

Cells were visualized using an infrared differential interference contrast (DIC) optics on an Eclipse E600FN (Nikon) with a 40× water-immersion objective. Recordings were performed from motoneurons, identified by their position in the lateral motor column and soma diameter of at least 20 mm (Takahashi, 1992; Thurbon et al., 1998). Motoneurons were voltage-clamped at −60 mV. The series resistance of 4–10 MΩ was compensated by 60–80% and recordings were abandoned if it increased by more than 20%. The typical motoneuron whole-cell capacitance of ∼200 pF gave a corner frequency of 0.2–0.8 kHz.

During all recordings, the aCSF composition included 3 mM kynurenic acid (Sigma) to block excitatory glutamatergic activity. Drugs were bath applied through the perfusion system as detailed below and in the Results section. Applied drugs included 5 μM SR-95531 (Sigma), 0.3–2 μM strychnine (Sigma), 1 μM diazepam (Sigma), 100 nM tetrahydrodeoxycorticosterone (THDOC, Sigma), 20 mM isoniazid (Sigma), 4 mM α-(methylamino) isobutyric acid (MeAIB, Sigma), 1 μM (3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid (TFB-TBOA, Tocris), and 2 mM nipecotic acid (Tocris). Whenever we superfused isoniazid, 1 μM CGP-555845-HCl was applied for the duration of the entire experiment to preclude activation of metabotropic GABA receptors.

In experiments where miniature inhibitory post-synaptic currents (mIPSCs) were recorded, 0.5 μM tetrodotoxin (Tocris) was perfused after a whole-cell patch was established. This concentration of tetrodotoxin was maintained during control recordings but reduced to 0.2 μM during drug application. At least 50 sweeps, of duration 9 s, were recorded for each condition. After the control period, the agents 5 μM SR-95531 or 0.3 μM strychnine were included in the perfusate to isolate glycinergic or GABAergic mIPSCs respectively.

For experiments employing extracellular stimulation, we applied an electrical current through a patch pipette filled with normal aCSF using a constant current DS3 isolated stimulator (Digitimer). After patching a motoneuron in transverse slices, the stimulation electrode was maneuvered within the ventral region of Rexed lamina VIII, in the Renshaw cell area until a response could be elicited. Having established the minimum stimulation intensity required to evoke inhibitory post-synaptic currents (IPSCs) reliably, it was fixed at ∼1.5× threshold. For experiments performed on the coronal preparation, the stimulation electrode was placed in the ipsilateral lateral white matter at least two or three segments rostral or caudal to the motoneuron which was always recorded from the dorsolateral motor nucleus of L5.

PAIRED RECORDINGS

For paired recordings, we used an optical configuration that allowed simultaneous visualization of motoneurons and EGFP positive interneurons in transverse slices. Infrared-DIC transmitted light was collected through the back port of a beam splitter, while a laser scanning confocal D-Eclipse C1 camera (Nikon) mounted on the front port was used to collect light emitted from EGFP positive cells. Having established a stable whole-cell patch on a motoneuron, a second electrode of ∼6 MΩ filled with normal aCSF was introduced into the Renshaw area. Putative pre-synaptic neurons were patched in a loose cell-attached voltage-clamp configuration to stimulate the membrane and record evoked spikes (Barbour and Isope, 2000) using a 1–1.5 V voltage step of 20 μs applied from an ELC-03X (NPI) amplifier.

Figure 1 illustrates how a connection was identified from post-synaptic responses of the motoneuron as IPSCs time-locked to evoked spikes. Typically, 1/100 of tested interneurons were

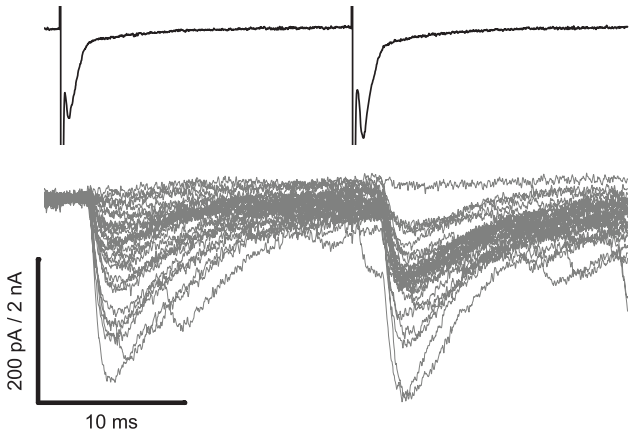

FIGURE 1 | Paired recordings with extracellular loose cell-attached stimulation of an interneuron can identify a connection with a motoneuron. The upper trace illustrates two voltage steps (see Materials and Methods) evoking individual spikes, clearly visible as downward deflections, from an interneuron patched in a loose cell-attached configuration. Post-synaptic responses of the motoneuron, illustrated in the lower trace, demonstrate a connection as IPSCs time-locked to the spikes. Following a 30 ms interval a second spike was evoked to potentiate the synapse and reduce the failure rate.

connected to the recorded motoneuron. Since a number of synaptic connections exhibited a high failure rate, double stimulations were applied to induce paired-pulse facilitation to potentiate responses. Upon finding a connection, the pre-synaptic interneuron was re-patched using a ∼4 MΩ electrode containing an internal solution of composition (in mM) K-gluconate 125, KCl 6, $CaCl_2$ 2, HEPES 10, EGTA 10, Mg-ATP 2, pH 7.3 with KOH, and osmolarity of 290–310 mOsM. In whole-cell current clamp, pre-synaptic cells were stimulated periodically every 9 s using an ELC-03X amplifier (NPI) by application of the minimum positive current required to evoke an action potential reliably.

CONCENTRATION JUMPS

Effects of the GABA-depleting agents on glycine receptors were investigated by performing concentration jump experiments on recombinant rat α1β glycine receptors, the adult isoform. Receptors were expressed in HEK293 cells using standard culture and transfection procedures (Burzomato et al., 2003). Concentration jumps were performed in an extracellular solution of composition (in mM) 102.7 NaCl, 20 Na gluconate, 2 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 14 D-glucose, 15 sucrose, and 20 TEACl, pH adjusted to 7.4 with NaOH (osmolarity ∼320 mOsM). Pipettes were filled with a high chloride solution containing (in mM): 107.1 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 11 EGTA, 20 TEACl, and 2 Mg-ATP.

Outside-out patches were pulled and maneuvered toward a theta tube, of tip diameter of 150 μm, mounted on a piezo-stepper (Burleigh Instruments). While both barrels contained normal extracellular solution, one also included 1 mM glycine. At least 5 fast applications of 1 ms were performed interleaved with 10 s periods for recovery. Before testing each GABA-depleting agent (*i.e.* isoniazid, MeAIB, TFB-TBOA, and nipecotic acid), the patch was first allowed to equilibrate with the new solution. Effects of fast application in control and test conditions on responses were measured using the changes in the peak current.

ANALYSIS OF INHIBITORY CURRENTS

Successfully evoked IPSCs were discriminated offline using Clampfit 10.2 (Molecular Devices) whereas mIPSCs were detected using WinEDR 3.2.4 (Strathclyde Electrophysiology Software). Both evoked IPSCs and mIPSCs were subjected to exponential fitting analysis using MATLAB 7 software (MathWorks). Events were excluded from the analysis if their amplitude was less than 3 standard deviations of the baseline noise, if there were overlapping events within 50 ms, or if the asymptotic decay did not reach 10% of baseline. The Levenberg-Marquardt least-squares iterative algorithm was used to fit one or two exponential components to the decay phase of each post-synaptic current from 95 to 5% of peak amplitude:

$$\hat{I}_1(t) = I_0 + Ae^{-\frac{t}{\tau}}$$

$$\hat{I}_2(t) = I_0 + A_1 e^{-\frac{t}{\tau_1}} + A_2 e^{-\frac{t}{\tau_2}}$$

The fit was selected on the basis of the F-test statistics for the two curves with $F > 4.8$ as a threshold for favoring the second. Since the decay constants were normally distributed, they were amenable to parametric test statistics. Comparisons across treatments were undertaken using one-way ANOVA F statistics, with *post-hoc* tests based on Student's t statistics employing Bonferroni's correction for multiple comparisons. Summary data are presented as mean ± SEM. To confirm successful detection of mixed inhibitory currents, we performed exponential fitting analysis on mIPSCs recorded from motoneurons ($n = 5$) of neonatal (P0–3) mice. Under control conditions, bi-exponential fits consistently represented a proportion of events (14.5 ± 4.5%) that was substantially reduced (to 4.7 ± 1.0%) in the presence of 5 μM SR-95531.

Since mIPSCs were always recorded in the presence of kynurenic acid, inward currents could only have resulted from GABAergic or glycinergic events. We thus quantified the overall inhibitory drive for each sweep by evaluation of the integral of

the entire current trace per unit of time. Using pharmacological isolation of GABAergic or glycinergic events, we used the integral to estimate their relative contributions to overall inhibitory drive.

Prior to integral estimation however we first corrected for slow baseline drifts. We used a low-pass filter at 0.5 Hz that canceled completely the fast-rising events associated with synaptic activity and subtracted the result from the original signal to obtain a drift-free trace. Since the subtraction could impose a non-zero center for the baseline, it was necessary to eliminate any offsetting effects on the integral evaluation by a further subtraction of this bias. We estimated the bias using the mode of the data, which was evaluated by convolution with a Gaussian kernel of a standard deviation $\sigma n^{-0.3}$, where n is the size and σ^2 is the variance of the data (Bhumbra and Dyball, 2010).

RESULTS

EVOKED IPSCs EXHIBITED NO CO-DETECTION

Simultaneous recordings of interneurons and motoneurons were obtained from eight connected pairs. In all cases, strychnine completely abolished evoked IPCSs (data not shown). In a subset of four, responses were tested with bath application of diazepam and gabazine for exponential fitting analysis of the decay phase. Evoked IPSCs were best-fitted with a single exponential in all cases under control conditions ($n = 203$) and in the presence of diazepam ($n = 143$). Across all four pairs, the mean of the averaged time constant τ fitted for all sweeps was 3.3 ± 0.2 ms. The lack of a second component to the exponential fits and the very short mean time constant are consistent with a purely glycinergic component to evoked currents.

Pharmacological modulation of any GABAergic component with diazepam and gabazine confirmed the lack of co-detection of GABA with glycine. A representative example of a paired recording for the different drug treatments is illustrated in **Figure 2A**. Responses of the motoneuron showed no effect by application of 1 μM diazepam or 5 μM SR-95531 of evoked IPSCs. By contrast, responses were abolished after application of 2 μM strychnine.

Group data for the four connected pairs are illustrated in **Figure 2B**. For three of the pairs, one-way ANOVA statistics showed no statistically significant effect of the treatments on the decay constant ($F \leq 0.11$, $P \geq 0.523$). In the fourth pair, the test statistic was significant ($F = 19.3$, $P < 0.001$) as result of a small decrease in the time constant in the presence of diazepam ($\tau = 3.2 \pm 0.1$ ms) compared to control ($\tau = 3.9 \pm 0.1$ ms, $t = 4.64$, $P < 0.001$). A decrease in the time constant however is not consistent with the effect of diazepam enhancing and prolonging the GABA component of evoked IPSCs.

Comparisons of mean amplitudes to those observed in control conditions showed a small decrease in the presence of diazepam ($82 \pm 11\%$) and SR-95531 ($93 \pm 8\%$). Since superfusion of either agent resulted in modest decreases, we attributed the progressive

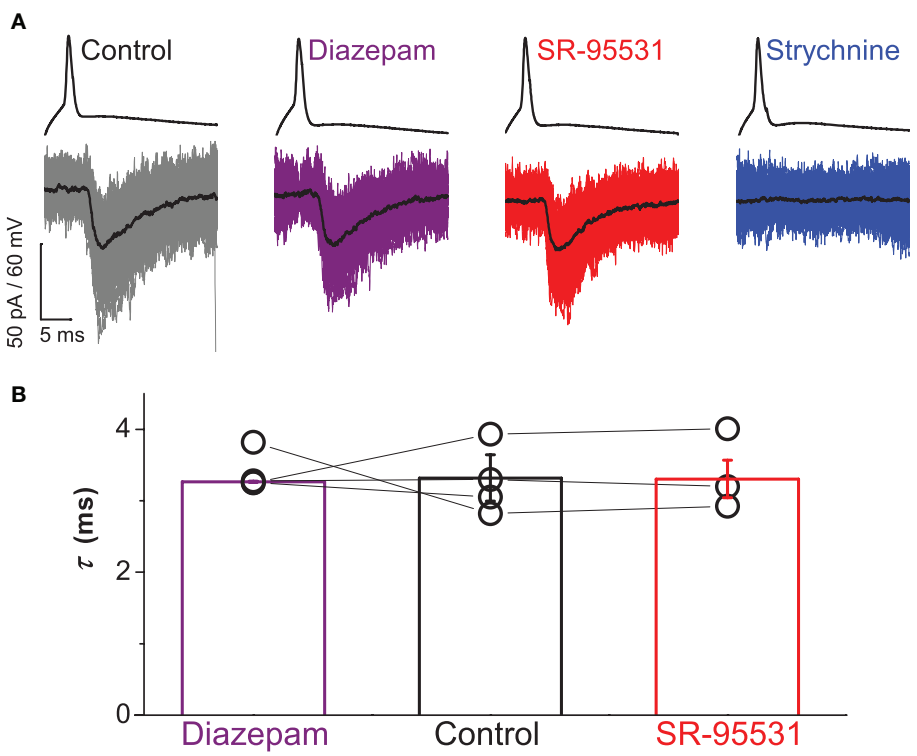

FIGURE 2 | Paired recordings showed no contribution of GABA to evoked IPSCs. Traces in **(A)** illustrate responses from a connected pair representing a single spike elicited in the interneuron above and the evoked IPSCs recorded from the motoneuron below with the mean current overlayed in black. Bath application of neither 1 μM diazepam (purple) nor 5 μM SR-95531 (red) modulated the time constant of evoked IPSCs whereas 2 μM strychnine (blue) abolished all responses. Group data from four connected pairs are represented in graph **(B)**, which illustrates no significant effect of diazepam (purple) or SR-95531 (red) on the time constant (see text). Error bars indicate mean ± SEM.

attenuation of IPSCs to the inevitable run down of neurotransmitter due to dialysis inherent to paired recordings (Diana and Marty, 2003). Analysis of the paired recordings thus indicated that there is no detectable GABAergic component the evoked IPSCs.

Paired recordings were performed on transverse slices and thus were selective for horizontal rather than vertical connections. It is possible that axon terminals from horizontal and vertical projections differ in their neurotransmitter content (Liu et al., 2010) or are apposed to post-synaptic membranes with different compositions of receptors. We thus investigated the effects of extracellular stimulation of ascending or descending connections on evoked IPSCs using the coronal preparation.

Seven motoneuronal recordings were obtained from coronal preparations while stimulating ascending or descending projections. Evoked IPSCs were best-fitted with a single exponential in all cases under control conditions ($n = 223$) and in the presence of diazepam ($n = 223$). Across all cells, the mean of the averaged time constant τ fitted for all sweeps was 5.8 ± 0.9 ms. The lack of a second component to the exponential fits and the very short mean time constant are consistent with a purely glycinergic component to evoked currents.

Superfusion of diazepam or gabazine confirmed the lack of co-detection of GABA with glycine. A representative example of a evoked IPSCs recorded during different drug treatments is illustrated in **Figure 3A**. Responses of the motoneuron showed no effect by application of 1 μM diazepam or 5 μM of evoked IPSCs. By contrast, responses were abolished after application of 1 μM strychnine.

Group data for the seven motoneurons are illustrated in **Figure 3B**. Paired *t*-test statistics evaluated from the average fitted time constant for each cell across all seven motoneurons confirmed no significant effect on the decay kinetics of evoked IPSCs by diazepam ($\tau = 6.1 \pm 0.9$ ms, $t = -1.51$, $P = 0.183$) or SR-95531 ($\tau = 5.7 \pm 1.0$ ms, $t = -1.04$, $P = 0.346$). Analysis of the recordings obtained from coronal preparations thus indicated that there is no detectable GABAergic component in IPSCs evoked by simulation of ascending or descending projections.

To investigate putative GABAergic components of currents mediated by diazepam-insensitive $GABA_A$ receptors, we recorded evoked responses from four motoneurons in transverse slices during bath application of the 200 nM THDOC. Group results are illustrated in **Figure 3C**. In comparison to control conditions ($\tau = 5.5 \pm 0.3$ ms), paired *t*-test statistics showed no significant effect of THDOC on the time constant ($\tau = 5.6 \pm 0.3$ ms, $t = -0.47$, $P = 0.672$).

RECORDINGS OF mIPSCs SHOWED NO CO-DETECTION

Since neither paired recordings nor extracellular stimulation showed demonstrable GABA co-detection in evoked responses, we investigated whether there were any GABAergic components

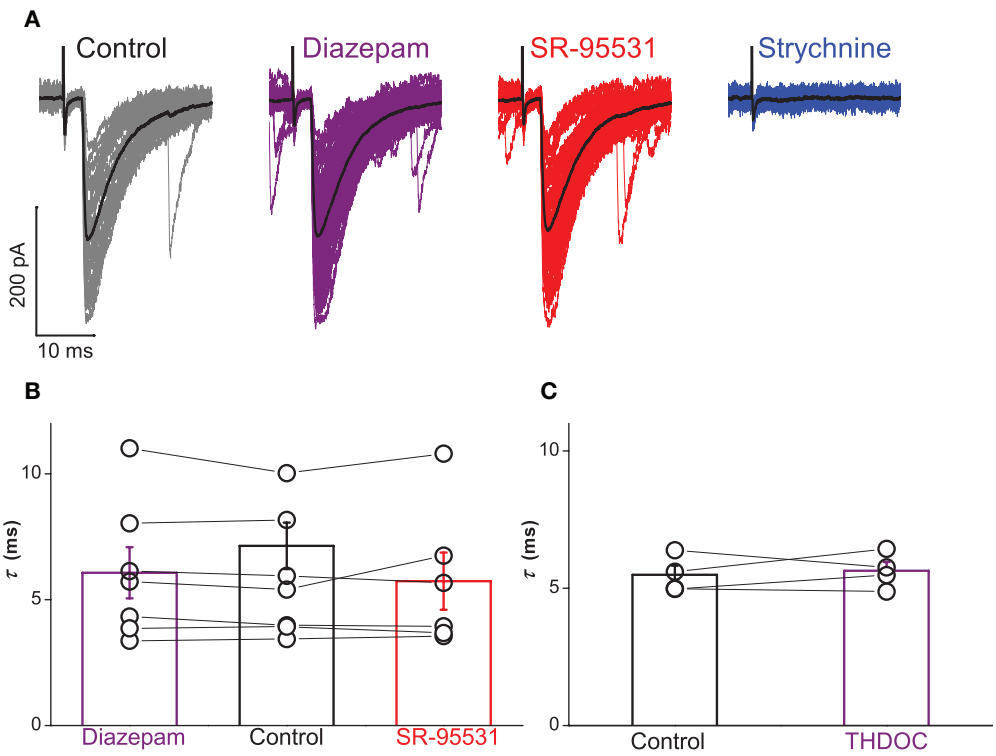

FIGURE 3 | Extracellular stimulation of ascending and descending projections showed no contribution of GABA to evoked IPSCs. Traces in **(A)** illustrate responses of a motoneuron with the mean current overlayed in black. Bath application of neither 1 μM diazepam (purple) nor 5 μM SR-95531 (red) modulated the time constant of evoked IPSCs whereas 2 μM strychnine (blue) abolished all responses. Group data for all motoneurons are represented in graph **(B)**, which illustrates no significant effect of diazepam (purple) or SR-95531 (red) on the time constant. Graph **(C)** shows no significant effect of 200 nM THDOC (purple) on the time constant of responses evoked in transverse slices (see text).

in miniature IPSCs. Miniature currents were recorded from seven motoneurons of which six were tested with 5 μM SR-95531 and four were tested with 1 μM diazepam. Exponential fitting analysis showed that the decay phase of currents were best-fitted with a single exponential in control conditions ($n = 1398$) and in the presence of diazepam ($n = 992$). Across all motoneurons, the mean of the averaged time constant τ fitted for all mIPSCs under control conditions was 6.0 ± 0.9 ms. The lack of a second component to the exponential fits and the short mean time constant are consistent with purely glycinergic miniature currents.

Pharmacological modulation of any GABAergic component with diazepam and gabazine confirmed the lack of co-detection of GABA with glycine. A representative example of mIPSCs recorded from a motoneuron during different drug treatments is illustrated in **Figure 4A**. Exponential fitting analysis showed no effect of diazepam or SR-95531 on the evoked IPSCs.

Group data for the mIPSCs recorded from all the cells are illustrated in **Figure 4B**. The mean relative amplitude of the currents compared to control conditions was not changed in the presence of diazepam ($99.4 \pm 5.0\%$) or SR-95531 ($99.1 \pm 6.9\%$). There was also no significant change on the mean fitted time constant with diazepam ($\tau = 6.2 \pm 1.3$ ms, paired $t = -0.57$, $P = 0.607$) or SR-95531 ($\tau = 5.9 \pm 0.8$ ms, paired $t = 0.50$, $P = 0.618$).

Since the experiments were performed at room temperature, the apparent lack of a second component in mIPSCs could have resulted from a reduction in GABA synthesis from glutamate due to a decrease in the activity of glutamate transporters. We thus recorded mIPSCs from five motoneurons at 32˚C under control conditions and in the presence of diazepam. Exponential fitting analysis showed that the decay phase of currents were best-fitted with a single exponential before ($n = 4111$) and after ($n = 2972$) diazepam administration. As expected for recording at higher temperatures, the mean decay constant was markedly short in control conditions ($\tau = 2.7 \pm 0.2$ ms). Superfusion of diazepam nevertheless had no significant effect on the fitted time constant ($\tau = 3.1 \pm 0.5$ ms, paired $t = -1.06$, $P = 0.348$).

The short time constant and the minimal effects of diazepam and SR-95531 suggested that glycinergic contribution to inhibitory inputs were substantially greater than the GABAergic component notwithstanding the absence of co-detection. Their relative contributions in mIPSC activity at room temperature were evaluated using integral analysis for the recordings in which each component was pharmacologically isolated. A representative example of such a recording during different drug administrations is illustrated in **Figure 4C**. While application of 5 μM SR-95531 had no demonstrable effect on the activity of mIPSCs, they were virtually abolished by 0.3 μM strychnine. The example thus illustrates a substantially greater contribution of a glycinergic inputs compared to inhibition mediated by GABA.

Group data for the mIPSC integral analysis for the six cells recorded are illustrated in **Figure 4D**. In comparison to control conditions, the overall inhibitory drive did not change in presence of SR-95531 in five cells ($99.0 \pm 6.4\%$ of control) whereas it was significantly reduced in only one (to $45.0 \pm 25.7\%$). By

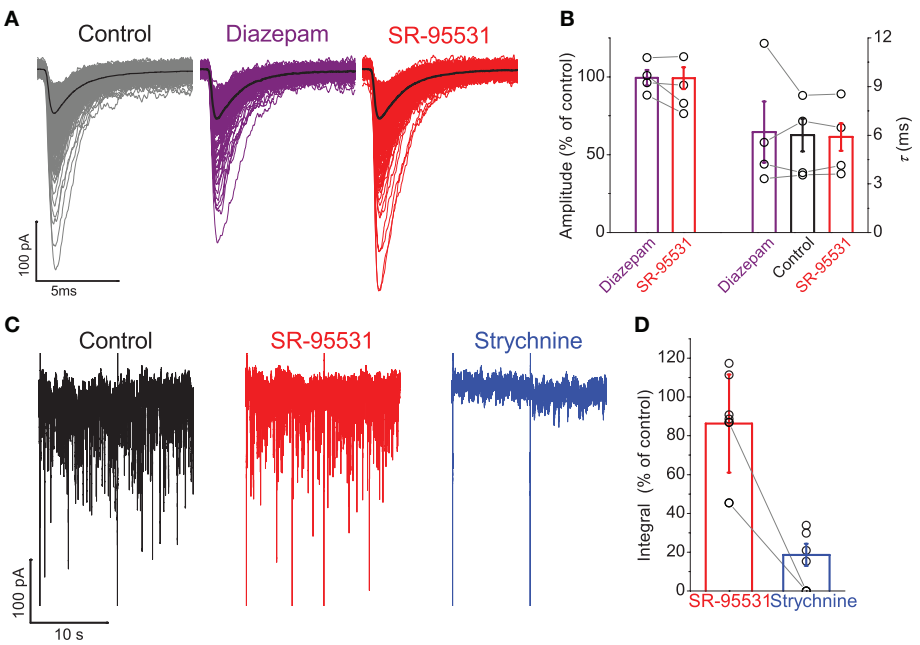

FIGURE 4 | Neither diazepam nor SR-95531 changed the activity of mIPSCs although they were substantially attenuated by strychnine. Traces in **(A)** illustrate mIPSCs recorded from the motoneuron with the mean current overlayed in black, demonstrating no detectable effect of the two agents. Group data from all recorded motoneurons are represented in graph **(B)**, which illustrates no significant effect of diazepam (purple) or SR-95531 (red) on the relative amplitude or time constant of mIPSCs. Traces in **(C)** illustrate recordings from a neuron that were subjected to integral analysis (see text; the biphasic currents resulting from voltage steps are truncated and were excluded from the analysis) to quantify overall inhibitory drive, which was minimally affected by application of SR-95531 (red), but heavily reduced by strychnine (blue). Group data illustrated in graph **(D)** show that overall inhibitory drive is not significantly affected by SR-95531 (red) but substantially attenuated by strychnine (blue) and abolished in some cases.

contrast, out all the seven cells tested with strychnine, activity was completely abolished in three cells and the remaining four exhibited a profound suppression of inhibitory activity following drug administration (to 24.9 ± 4.1%). The integral analysis thus demonstrates a substantial predominance of a glycinergic component to inhibitory inputs in comparison to GABAergic contributions.

GABA DEPLETION DID NOT AFFECT EVOKED IPSCs

While the single exponential profile of IPSCs and pharmacological isolation of a GABAergic component demonstrated no co-detection, it did not preclude possible co-release onto a post-synaptic membrane with a dearth of GABA receptors. Since GABA however is a partial agonist of post-synaptic glycine receptors, its co-release could attenuate glycinergic currents and would shorten their time constant (Lu et al., 2008). Glutamate uptake contributes to GABA synthesis (Mathews and Diamond, 2003) by the action of GAD. Glutamate is either transported into cells or synthesized from glutamine by glutaminase.

In order to deplete GABA, we used 4 mM MeAIB to block glutamine uptake (Varoqui et al., 2000), 1 μM TFB-TBOA to block glutamate uptake (Shimamoto et al., 1998), and 20 mM isoniazid to inhibit GAD (De Koninck and Mody, 1997) thus depleting upstream substrates of GABA and directly inhibiting its synthesis from glutamate. GABA uptake into cells was blocked using 2 mM nipecotic acid. During recordings of glycinergic currents from voltage-clamped motoneurons, responses evoked from extracellular stimulation in the Renshaw cell area were attenuated by ∼70% following administration of isoniazid, MeAIB, TFB-TBOA, and nipecotic acid (data not shown). Since the decrease in peak current was observed within 5 min of drug application, the reduction in response was unlikely to have resulted from perturbations in metabolic processes but as a consequence of direct action of the four drugs on the receptors mediating post-synaptic currents. We thus sought to identify which of the GABA-depleting agents confer the least direct action on glycinergic receptors using concentration jumps.

Figure 5 illustrates responses to fast glycine applications on outside-out patches pulled from HEK293 cells expressing recombinant α1β. Under control conditions (**Figure 5A**), concentration jumps showed uniform responses. In the illustrated example, bath application of 20 mM isoniazid resulted in an attenuation of the current.

Group data illustrated in **Figure 5B** illustrates that all GABA-depleting agents decreased the peak current, which were observed within 5 min of administration. Reductions in mean amplitude were substantial following application of 4 mM MeAIB (25.5 ± 3.9%, $n = 7$), 1μM TFB-TBOA (34.4 ± 9.8%, $n = 5$), and 2 mM nipecotic acid (29.0 ± 4.2%, $n = 7$). The comparatively modest attenuation associated with isoniazid (14.0 ± 3.0%, $n = 5$) thus identified this agent as our choice of drug for depleting GABA.

We confirmed the efficacy of GABA depletion by isoniazid using extracellular stimulation to evoke IPSCs recorded from whole-cell voltage-clamped motoneurons during administration of 20 mM isoniazid in the presence of 1 μM strychnine to isolate GABAergic currents. A representative example of the progressive attenuation in currents is illustrated in **Figures 6A–C**. A plot of the corresponding changes in amplitude (**Figure 6D**) shows that the attenuation was substantial and occurred over a period of 30–45 min, demonstrating an effect more compatible with influences on metabolic processes rather than a direct action on post-synaptic receptors.

Group data represented in **Figure 6E** illustrates the relative change in evoked IPSC amplitude when comparing the peak currents observed 30–45 min after isoniazid administration with those recorded during the control period. In all cases ($n = 4$) there was an attenuation in the response, with an average reduction

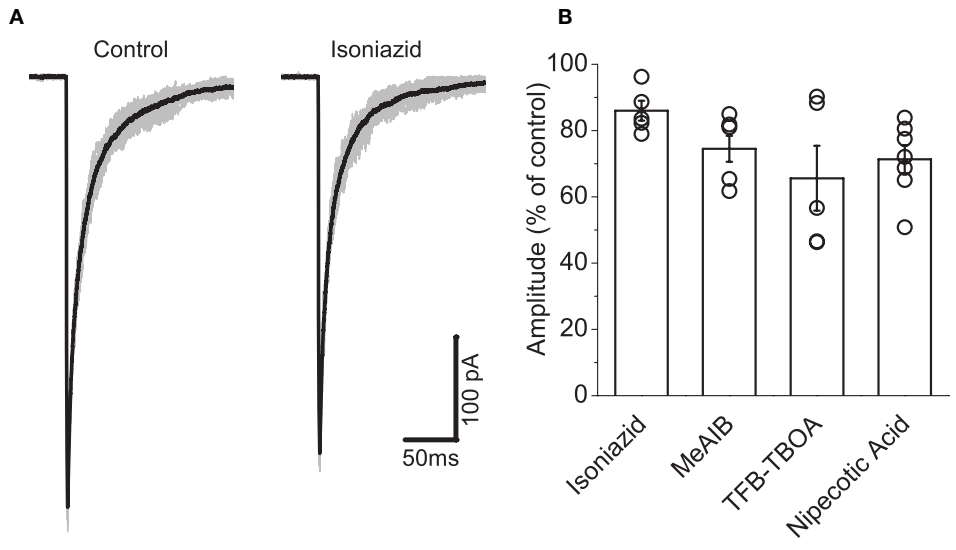

FIGURE 5 | Fast glycine application experiments showed that isoniazid administration produced the least attenuation in current among the GABA-depleting agents. Traces in **(A)** illustrate an example in which a small reduction in the current is observed in the presence of 20 mM isoniazid. Graph **(B)** illustrates isoniazid attenuated the current to the least extent (see text) compared to 4 mM MeAIB, 1 μM TFB-TBOA, and 2 mM nipecotic acid.

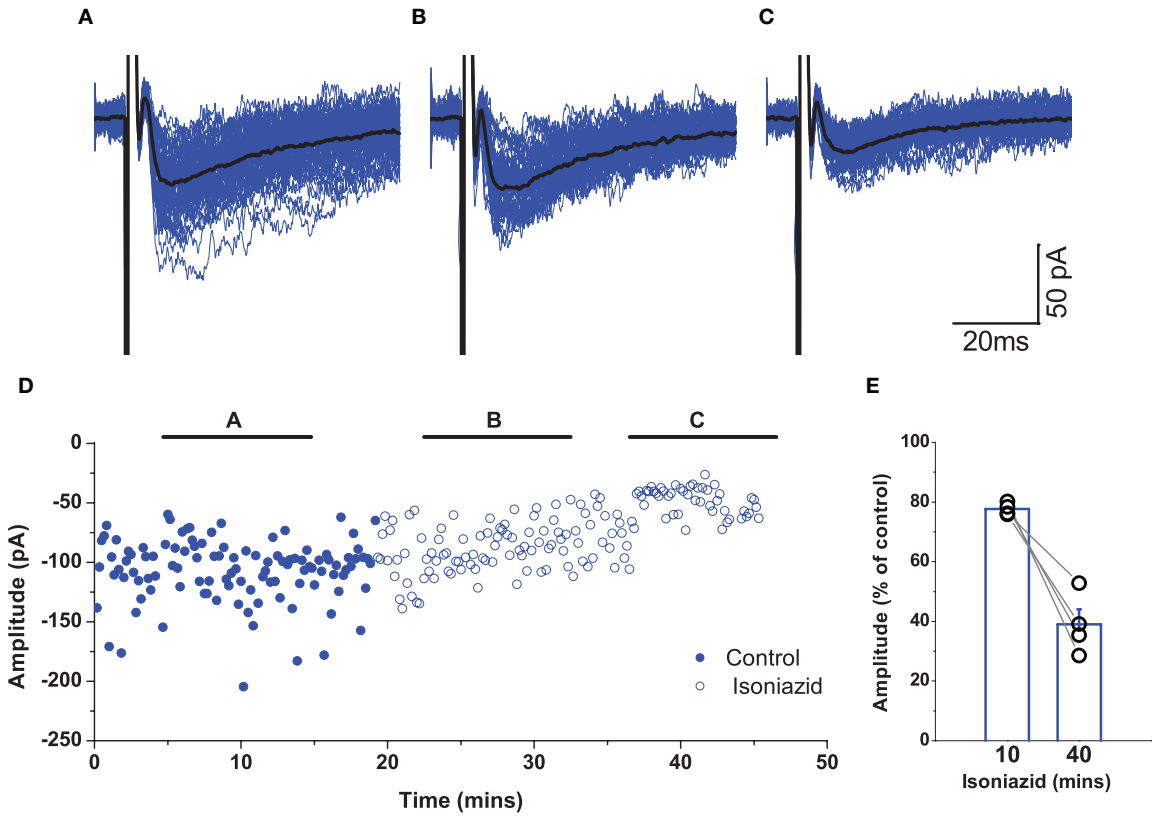

FIGURE 6 | Application of 20 mM isoniazid depleted vesicular GABA over a period of 30–45 min. In the presence of 1 μM strychnine, IPSCs were evoked by extracellular stimulation. The traces illustrate an example of a recording of post-synaptic responses during the control period [trace **(A)**] and two epochs following isoniazid superfusion [traces **(B)** and **(C)**]. Mean responses are overlayed in black. Graph **(D)** plots the changes in amplitude, illustrating the time periods corresponding to **(A–C)**, and shows a gradual but substantial reduction in current. The group data represented in graph **(E)** illustrates that evoked IPSCs in all motoneurons were progressively attenuated during the ∼40 min of isoniazid superfusion.

of 61 ± 5%. The results thus confirm that application of 20 mM isoniazid depletes vesicular GABA over a period of 30–45 min.

Since co-released vesicular GABA would hasten the decay of glycinergic currents (Lu et al., 2008), then any GABA-mediated effect on evoked IPSCs would be perturbed by isoniazid administration over a time scale of 30–45 min during which the decay would become progressively longer. In the absence of strychnine, evoked IPSC recordings were obtained during application of 20 mM isoniazid. A representative example is illustrated in **Figures 7A–C**, which shows only a modest reduction in the size of the current. The plot of corresponding changes in amplitude (**Figure 7D**) however shows the attenuation was modest and occurred over a period of ∼10 min, demonstrating an effect more compatible with direct action on post-synaptic receptors rather than on metabolic processes.

Group data ($n = 6$) are illustrated as graphs showing the effect on the relative amplitude (**Figure 7E**) of isoniazid superfusion after 10 min and after an hour. While a modest attenuation of 13.5 ± 6.6% in the current amplitude was observed after 10 min, the reduction in amplitude after an hour was only 15.8 ± 7.6%. Since there was only a small fast attenuation, the changes in relative amplitude demonstrate an effect more compatible with direct action on post-synaptic receptors rather than on metabolic processes. Comparison of time constants of evoked responses in control conditions with those after an hour of isoniazid superfusion (**Figure 7F**) showed no significant change from $\tau = 5.8 \pm 0.8$ to $\tau = 5.8 \pm 0.8$ ms (paired $t = -0.05$, $P = 0.963$). The results indicate that little or no GABA is co-released in glycinergic synapses investigated in the present study.

GLYCINE LOADING DID NOT AFFECT EVOKED IPSCs

Since GABA depletion did not affect the kinetics of evoked IPSCs, we investigated the effects of loading glycine into pre-synaptic vesicles using paired recordings. Addition of 20 mM glycine to the internal solution of the pipette used to patch the interneuron would favor glycine loading compared to GABA. Any changes to the neurotransmitter content of pre-synaptic vesicles would be detected as a progressive change in post-synaptic responses over time after establishing a whole-cell configuration for the interneuron. After stabilization of the current clamp on the pre-synaptic neuron, trains of 1000 spikes at 50 Hz were delivered to deplete vesicules every 10–15 min.

There were however no progressive changes in evoked currents recorded from connected pairs ($n = 4$). In the representative example illustrated in **Figure 8A**, IPSCs evoked immediately upon establishing a whole-cell configuration were not manifestly

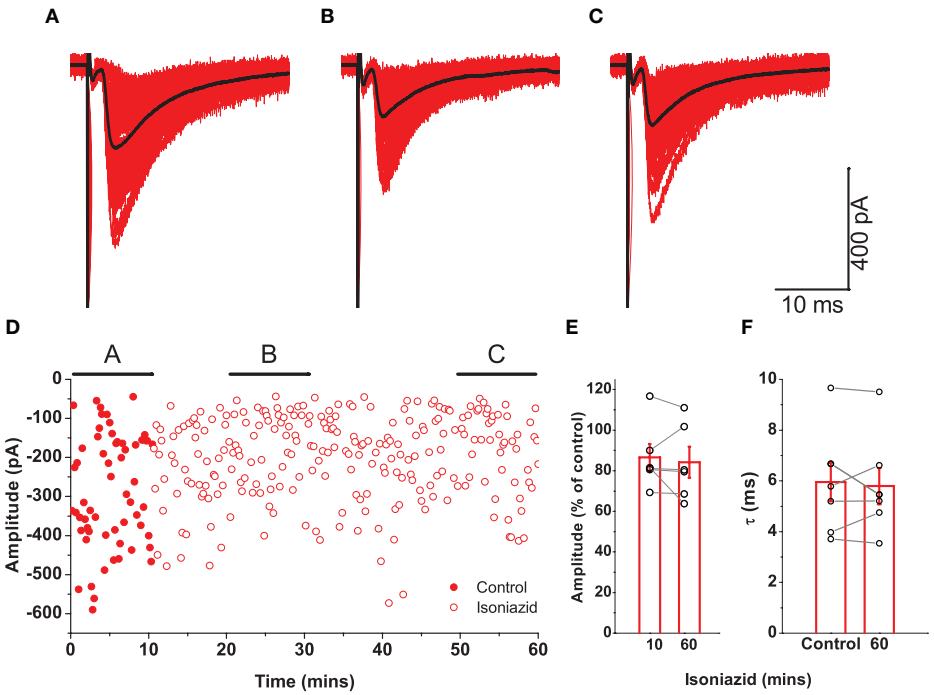

FIGURE 7 | Effects on glycinergic IPSCs by application of 20 mM isoniazid were not compatible with vesicular GABA depletion. The traces illustrate an example of a recording of post-synaptic responses during the control period [trace **(A)**] and two epochs following isoniazid superfusion [traces **(B)** and **(C)**]. Mean responses are overlayed in black. Graph **(D)** plots the changes in amplitude, illustrating the time periods corresponding to **(A–C)**, and shows a modest but rapid reduction in current. The group data for all motoneurons shows that the attenuation of evoked IPSCs [graph **(E)**] after 1 h was no greater than that observed after ∼10 min, and that isoniazid superfusion had no systematic effect on the decay time constant [graph **(F)**].

different to those recorded after ∼45 min. This suggests that the vesicle content was not affected by loading the pre-synaptic terminal with glycine.

Group data for the time constants (**Figure 8B**) and amplitudes (**Figure 8C**) are illustrated as graphs over time. There was no appreciable effect on the decay constant τ, with a mean in control of 4.8 ± 0.4 ms, and of 4.6 ± 0.4 ms after ∼45 min of recording. A Spearman rank test statistic showed in three connected pairs no changes in the decay constant over time ($|r| \leq 0.13$, $P \geq 0.183$), whereas the decrease observed in the fourth ($r = -0.39$, $P < 0.001$) was incompatible with a reduction in vesicular GABA content. Relative mean amplitudes decreased to $98.9 \pm 11.7\%$ thus reflecting no changes on the magnitude of evoked responses beyond a possible counteraction of the effects of run-down associated with dialysis. The group data thus suggests that even if GABA was present in pre-synaptic vesicles, its influence was too small to detect.

DISCUSSION

Analysis of our initial paired recordings identified individual glycinergic synaptic connections in which GABA is not co-detected. Co-detection was also absent in responses to extracellular stimulation of glycinergic projections in the coronal preparation. There was no demonstrable GABAergic contribution to the substantially larger number of inputs associated with the mIPSCs recorded from motoneurons in transverse slices. Our initial results therefore show that glycinergic synapses onto lumbar motoneurons have no ostensible $GABA_A$R-mediated component.

Mixed evoked and miniature IPSCs with pharmacologically isolable GABAergic and glycinergic components have however been observed in recordings from lumbar motoneurons (Jonas et al., 1998). While our results may appear to contrast with those previously reported, the apparent discrepancy is most likely to have arisen as a result of differences in the age and species used for recordings. We used P8–14 mice whereas (Jonas et al., 1998) recorded mainly from P6–7 rats. Mice are already weight-bearing at P8 whereas rats are not until P12. The dissimilar findings may thus reflect the different stages of maturation of the spinal cord for the two animals.

While mIPSC recordings from interneurons in the ventral horn have exhibited co-detection in neonatal rats (González-Forero and Alvarez, 2005), the $GABA_A$R-mediated component is diminished specifically in non-Renshaw cells in juveniles (from P9). In the dorsal horn of the rat (Keller et al., 2001), the extent of co-detection also decreases with age and is absent altogether in the mature state (≥P21). Since our exponential fitting analysis showed neither a second component in evoked or miniature IPSCs nor any pharmacologically isolable GABAergic contribution in evoked responses, co-detection must already be negligible in lumbar motoneurons of P8–14 mice.

Notwithstanding the absence of co-detection, the mIPSC analysis demonstrates that the glycinergic contribution to inhibitory inputs in juvenile mice is substantially greater than the GABAergic

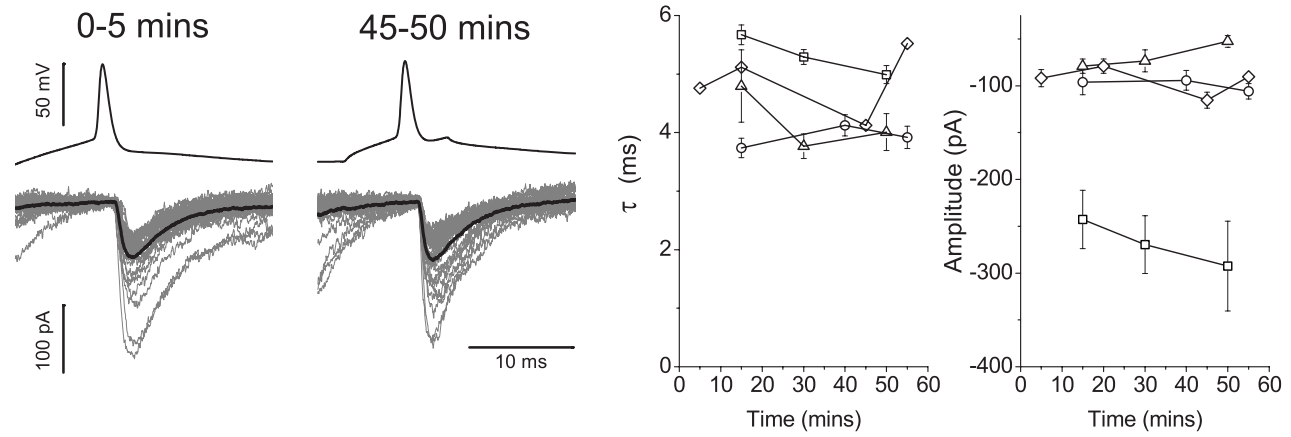

FIGURE 8 | Loading the pre-synaptic cell with 20 mM glycine during paired recordings resulted in no progressive changes in post-synaptic responses. Traces in **(A)** illustrate an example of interneuronal spikes and motoneuronal responses for the first 5 and 45 min after rupturing the membrane of the pre-motor interneuron. Group data ($n = 4$) is illustrated graphically to show changes in the decay constant **(B)** and amplitude **(C)**, neither of which showed systematic changes during the course of the loading glycine into the pre-synaptic vesicles.

component. In the ventral horn there is a postnatal shift in the distribution of mIPSCs from GABAergic to glycinergic inhibition (Gao et al., 2001), a reduction in immunoreactivity of GABA and GAD (Ma et al., 1992), and a decrease in $GABA_AR$ expression (Ma et al., 1993).

We investigated whether the lack of a GABAergic component in the evoked and miniature IPSCs resulted from a paucity of co-released GABA or post-synaptic $GABA_A$ receptors by experimental manipulation of the relative pre-synaptic content of GABA and glycine. Currents evoked by extracellular stimulation were not affected by pre-synaptic depletion of GABA. Paired recordings showed no modulatory effect on post-synaptic responses while glycine was loaded into pre-synaptic vesicles in preference to GABA. We thus infer that GABA is not co-released in physiologically relevant amounts at glycinergic synapses onto lumbar motoneurons in juvenile mice.

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Impact of synaptic neurotransmitter concentration time course on the kinetics and pharmacological modulation of inhibitory synaptic currents

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The time course of synaptic currents is a crucial determinant of rapid signaling between neurons. Traditionally, the mechanisms underlying the shape of synaptic signals are classified as pre- and post-synaptic. Over the last two decades, an extensive body of evidence indicated that synaptic signals are critically shaped by the neurotransmitter time course which encompasses several phenomena including pre- and post-synaptic ones. The agonist transient depends on neurotransmitter release mechanisms, diffusion within the synaptic cleft, spill-over to the extra-synaptic space, uptake, and binding to post-synaptic receptors. Most estimates indicate that the neurotransmitter transient is very brief, lasting between one hundred up to several hundreds of microseconds, implying that post-synaptic activation is characterized by a high degree of non-equilibrium. Moreover, pharmacological studies provide evidence that the kinetics of agonist transient plays a crucial role in setting the susceptibility of synaptic currents to modulation by a variety of compounds of physiological or clinical relevance. More recently, the role of the neurotransmitter time course has been emphasized by studies carried out on brain slice models that revealed a striking, cell-dependent variability of synaptic agonist waveforms ranging from rapid pulses to slow volume transmission. In the present paper we review the advances on studies addressing the impact of synaptic neurotransmitter transient on kinetics and pharmacological modulation of synaptic currents at inhibitory synapses.

Keywords: GABAergic synaptic transmission, GABA concentration time course at the synapse, GABAA receptors, non-equilibrium conditions, pharmacological modulation of GABAA-currents, modifiers of gating, fast-off competitive antagonists, GABAA receptor modeling

INTRODUCTION

The timing, amplitude, and duration of synaptic currents are important determinants for specific neuronal network functions including signal integration, network oscillations, and selection of neuronal assemblies (Freund and Katona, 2007; Klausberger and Somogyi, 2008). Various GABAergic interneurons contact their target cells with synapses showing distinct kinetic features and precise sub-neuronal localization, suggesting that the concerted neuronal network functioning relies on the accurate spatial and temporal properties of synaptic inputs (Spruston, 2008; Klausberger, 2009; Karayannis et al., 2010). The kinetics of post-synaptic currents (PSCs) is commonly ascribed to the gating properties of post-synaptic receptors as exemplified by glutamatergic NMDA receptor- and AMPA receptor-mediated EPSCs showing slow and fast decay, respectively, due to different binding and gating properties of NMDA and AMPA glutamate receptors (Lester and Jahr, 1992). Likewise, GABAergic synapses are characterized by a marked kinetic diversity that is commonly ascribed to the variety of post-synaptic $GABA_A$ receptor ($GABA_AR$) isoforms (Cherubini and Conti, 2001). For instance, receptors containing α1 or α3 subunits (along with β and γ2 subunits) have been demonstrated to underlie fast and slow IPSCs kinetics, respectively (Gingrich et al., 1995; Mozrzymas et al., 2003b; Barberis et al., 2007). In many cases, however, the kinetics of synaptic currents significantly differs from the prediction based solely on the gating properties of post-synaptic receptors. An increasing body of evidence suggests that both at excitatory and inhibitory synapses the profile of neurotransmitter concentration in the synaptic cleft plays a significant role in determining the amplitude and kinetics of synaptic currents. Typically, after release, neurotransmitter is present in the synaptic cleft for only a few hundreds of microseconds implying that activation of post-synaptic receptors occurs in conditions characterized by a high degree of non-equilibrium (Clements, 1996; Mozrzymas et al., 1999, 2003a). Moreover, such a particularly dynamic waveform of synaptic agonist concentration has been demonstrated to play a crucial role in determining the susceptibility of PSCs to various modulators. Importantly, recent studies revealed that for some types of GABAergic interneurons, neurotransmitter transient is particularly slow, lasting tens of milliseconds, raising novel concepts of volume transmission and paracrine modulation of local networks (Szabadics et al., 2007; Balakrishnan et al., 2009; Olah et al., 2009; Karayannis et al., 2010).

In the present review we discuss recent advancement in understanding the role of synaptic GABA concentration profile in

shaping IPSCs kinetics and in pharmacological modulation of GABAergic currents.

ESTIMATING THE NEUROTRANSMITTER TIME COURSE

Having focused this review on the role of agonist transient in synaptic signaling, it is important to bear in mind that the measurement of synaptic agonist time course remains a major challenge. Typically, at the central synapses, cleft width is ∼20 nm, thus precluding placement of a measuring device within the synapse to directly monitor the agonist time course. Moreover, amperometry, a technique widely used for, e.g., monoamines detection, cannot be applied for major inhibitory and excitatory neurotransmitters in the brain (GABA and glutamate) as these aminoacids are not being oxidized on the carbon fibers. It needs to be taken into account that, even in the case of monoamines, amperometry senses these compounds after their spillover from the synapse, as the carbon fiber can only be placed in the release site vicinity. Taking into account these constrains, determination of synaptic neurotransmitter transient has been approached either with computer simulations or with tools enabling to indirectly infer its time course. The latter case exploits compounds that interfere with synaptic transmission in a way that depends on synaptic neurotransmitter waveform. Since the techniques used for estimation of synaptic agonist time course were extensively reviewed elsewhere (Clements, 1996; Mozrzymas, 2004; Scimemi and Beato, 2009), below we provide only a concise description of these methods.

MODEL SIMULATIONS

The spatiotemporal profile of neurotransmitter released from a vesicle can be described using the Fick's equation:

$$\frac{\partial C(x,t)}{\partial t} = D\frac{\partial^2 C(x,t)}{\partial x^2}$$

(1) Where $C(x, t)$ – neurotransmitter concentration at time t and distance x from a reference point, D – diffusion coefficient. Application of this equation requires setting the boundary conditions that basically reflect the geometry of the synapse and diffusion constrains in its surroundings. Although electron microscopy may provide key ultrastructural information, it needs to be considered that this method requires tissue fixation, dehydration, and treatment with chemicals which may induce substantial cell swelling or shrinkage and thereby alter the structure of synapses. Moreover, the diffusion coefficient at the synapse may substantially differ (is typically much smaller) from that determined for bulk aqueous solutions. A number of other phenomena and system features may be considered in the Eq. 1. The simplest model assumes instantaneous point release that is taken into account by setting appropriate initial condition, although more realistic release models (e.g., partial release due to transient releasing pore opening) can be considered. Moreover, neurotransmitter binding and/or buffering binding sites (e.g., by transporters involved in the uptake system and/or post-synaptic receptors) or enzymatic breakdown can be additionally included by adding the respective terms on the right hand side of Eq. 1. This approach provides a macroscopic view of diffusion, as the neurotransmitter concentration is described by a continuous function $C(x, t)$ which reflects a deterministic (zero fluctuation), local value of concentration. Formally, the lack of fluctuations corresponds to the limit behavior of a great number of diffusing particles. A complementary approach, which does account for local fluctuations, is to simulate the agonist diffusion using Monte Carlo method which is based on simulation of a stochastic "random walk" of released neurotransmitter molecules in the "environment" mimicking the 3D structure of the synaptic cleft and its close neighborhood.

LOW AFFINITY COMPETITIVE AGONISTS

A clever and elegant method to estimate the agonist time course in glutamatergic synapses was to "perturbate" synaptic currents with competitive, quickly unbinding antagonists (Clements et al., 1992; Clements, 1996). If the synaptic agonist remained within the synapse for the time comparable to that needed for antagonist dissociation, a non-equilibrium displacement of competitive blocker would be unmasked by a current flowing through unblocked post-synaptic receptors. Such a displacement of the antagonist would be also manifested by a slowdown of synaptic current rising phase. Clearly, the longer the agonist presence in the synapse in comparison to the time needed for unbinding, the larger the displacement of antagonist, and the bigger the current. This approach has been initially used to probe the neurotransmitter concentration profile at glutamatergic synapses (Clements et al., 1992; Clements, 1996), at GABAergic synapses (Overstreet et al., 2003; Barberis et al., 2004, 2005; Karayannis et al., 2010) and, more recently, at glycinergic synapses (Beato et al., 2007; Beato, 2008; Scimemi and Beato, 2009). It is worth emphasizing that, as pointed out by Beato (2008) and Scimemi and Beato (2009), when a dose inhibition curve is fitted using the peak concentration and clearance time as free parameters, these two parameters cannot be unambiguously determined. This limitation can be overcome by either an experimental manipulation that prolongs the clearance time without affecting the peak (Beato, 2008) or by incorporating the time course of the PSCs in the fitting procedure (Overstreet et al., 2003).

MODIFIERS OF GATING

A different option for the use of "perturbating" factors to get an insight into the synaptic agonist time course is represented by the use of compounds that affect post-synaptic receptor properties including binding and gating kinetics (Mozrzymas et al., 1999, 2003a, 2007b; Barberis et al., 2000). The agents influencing agonist binding were particularly useful in estimating the agonist transient. The rationale is that a relatively small up or down regulation of the binding rate exerts a particularly strong impact on the amplitude and time course of currents elicited by a very short exposure to synaptically released agonist. In practice, the pharmacological mechanism of a modulatory action was determined relying on the analysis of current responses to rapid agonist applications and, subsequently, the time course of the agonist transient was optimized to best reproduce the effect of the modulator on the synaptic currents. Thus, determination of agonist transient kinetics was not a unique goal of studies in which specific agents were investigated but rather it was a necessary step to reconcile the observed effects of modulators on synaptic and exogenously evoked currents.

A more detailed comparison of the techniques based on the use of rapidly dissociating antagonists and modifiers of gating is discussed below in the Section "Consideration of Synaptic

Agonist Transient Sheds New Light on Pharmacological Modulation of Post-Synaptic Currents."

NEUROTRANSMITTER CONCENTRATION PROFILE SHAPES SYNAPTIC CURRENTS

Since the duration of the synaptic agonist pulse is much shorter than the mean open time of synaptic channels, the activation of post-synaptic receptors takes place in conditions of substantial non-equilibrium. An important consequence of this particular activation pattern is that, in contrast to what is assumed in classical equilibrium pharmacology, the strength of the synaptic agonist pulse depends on both the peak agonist concentration and pulse duration. This concept is illustrated in (**Figure 1A**), where the peak of simulated IPSCs evoked by the same GABA concentration (1 mM) strongly depends on the duration of GABA transient.

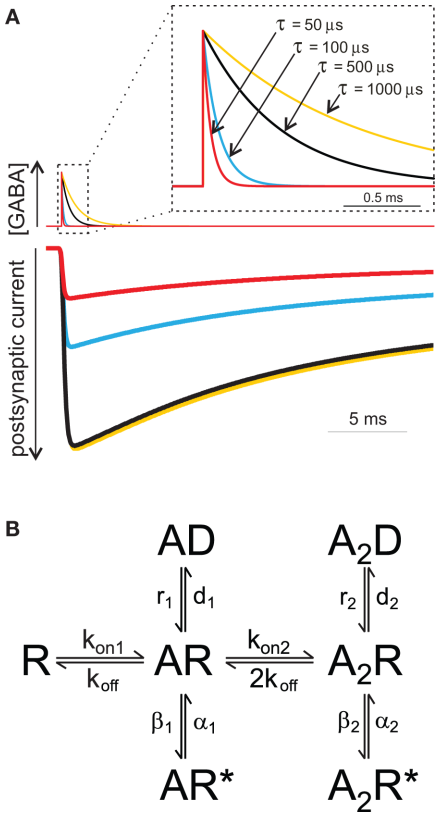

FIGURE 1 | The amplitude of simulated IPSCs depends on the neurotransmitter time exposure. (A) Currents simulated using the Jones and Westbrook model (rate constants from Mozrzymas et al., 1999) elicited by four different GABA pulses. At GABA pulses with τ lower than ~500 μs, the current amplitude strongly depends on GABA pulse length. Please note that current evoked by fast clearance GABA pulses (τ = 50 μs) is markedly smaller that that obtained by longer GABA exposures. **(B)** Jones and Westbrook's (1995) model. It assumes the sequential binding of two GABA molecules and includes open (AR*, A_2R^*) and desensitized (AD, A_2D) states originating from both mono- and doubly liganded closed states (AR, A_2R). This gating scheme has been extensively used in the investigation of the gating properties of $GABA_AR$ and, although oversimplified, this model reproduces the basic features of $GABA_AR$ (Jones and Westbrook, 1995; Mozrzymas et al., 2003b).

During the last decade, in order to demonstrate that brief neurotransmitter synaptic exposures could limit the activation of post-synaptic receptors, different strategies have been adopted. One of them was the use of polymers such as dextran which slows down neurotransmitter diffusion thereby prolonging the neurotransmitter presence in the synaptic cleft. At glutamatergic and GABAergic synapses, synaptic currents recorded in the presence of dextran showed indeed higher amplitude (Min et al., 1998; Perrais and Ropert, 2000; Barberis et al., 2004) confirming that, generally, synaptic exposure may be a limiting factor for post-synaptic receptor activation. This observation has been corroborated by using an alternative approach based on pharmacological modulation of post-synaptic $GABA_AR$ (see also below). For instance, benzodiazepines (BDZ) that are believed to up regulate GABA binding rate (Rogers et al., 1994; Lavoie and Twyman, 1996), enhance mIPSCs amplitude as they effectively increase the occupancy of bound states following a short receptor exposure to synaptically released agonist (Frerking et al., 1995; Perrais and Ropert, 1999; Mozrzymas et al., 2007b). These two approaches, although useful to unveil non-equilibrium synaptic conditions, fail to provide quantitative details about agonist transient, especially when their use is limited to the analysis of synaptic currents. Moreover, it is not precisely known to what extent dextran penetrates the synaptic cleft and interferes with neurotransmitter diffusion within the synapse. In addition, as discussed in detail below, BDZs not only alter $GABA_AR$ affinity but also affect receptor gating thus complicating the assessment of their effect on agonist binding during synaptic transmission. While the influence of non-equilibrium conditions on the PSCs peak amplitude has been broadly acknowledged, the impact of neurotransmitter concentration profile in shaping the decay kinetics of synaptic currents is more elusive, and whether or not synaptic-like neurotransmitter exposures can unmask gating features not observed for longer agonist pulses is still a matter of debate. This is mainly due to the lack of knowledge on the gating mechanisms of $GABA_AR$ activated by synaptic-like GABA pulses (~1–3 mM, ~0.1 ms). The fastest ultra-rapid perfusion systems, indeed, cannot reliably reproduce such neurotransmitter pulse since, at best, they are able to deliver pulses in the range of ~0.4–1 ms (Jonas, 1995), although in most studies, including ours, minimal exposure is >1 ms. In order to overcome this limitation the following strategy has been adopted. If in non-equilibrium conditions the agonist pulse strength depends on both agonist concentration and application duration ($C \times t$), it is possible to deliver pulses with a strength that is "equivalent" to the synaptic one simply by lowering the agonist concentration and keeping the value of $C \times t$ similar to that estimated for PSCs. Using this approach, Jones and Westbrook (1995) showed that ultra-weak GABA pulses (10 μM, 2 ms) evoked currents that deactivated faster than those elicited by saturating GABA pulses (10 mM, 2 ms). Similar results were reported by Banks and Pearce (2000). Which mechanisms underlie the current deactivation speed-up observed at ultra-weak GABA pulses? An increasing body of evidence points to the involvement of monoliganded states of $GABA_AR$. Although $GABA_AR$ fully activates following the binding of two GABA molecules, it has been proposed that a single GABA molecule would be sufficient to induce a low probability open state (Macdonald et al., 1989; Jones and Westbrook, 1995; Baumann et al., 2003). This

hypothesis was suggested by the observation that $GABA_AR$ mean open time critically depends on the agonist concentration being shifted toward short openings at low, submicromolar GABA doses. Based on this theory, Jones and Westbrook (1995) proposed that ultra-short applications of low GABA concentrations (10 μM, 2 ms) would largely limit the binding reaction to the monoliganded state(s), therefore promoting low probability openings leading to fast deactivation kinetics. More recently Petrini et al. (2011) applied a mutagenesis approach directed to impair a single GABA binding site and provided a more direct evidence that currents mediated by singly bound states of $GABA_AR$ show fast deactivation. In the same study, the possibility that monoliganded states of $GABA_AR$ could participate in shaping IPSCs kinetics during synaptic transmission was taken into consideration. In particular, model simulations of GABA release in the synaptic cleft suggested that, compared to disk center, receptors located at the disk periphery sense markedly lower GABA concentrations, thus activating preferentially in the singly bound state(s) (**Figure 2**). Thus, singly bound synaptic receptors characterized by a fast deactivation contributed to the acceleration of IPSCs decay kinetics. The largest contribution of singly bound states was found when the synapse works in conditions far from saturation, e.g., at low number of molecules released and/or large synaptic radius. In contrast, large amount of released neurotransmitter and/or small synapse radius generate saturation of post-synaptic receptors thus favoring receptor activation in the doubly bound state(s) (**Figures 2B–E**). These data, although strongly relying on model simulations, confirm that IPSCs kinetics could be finely tuned by the relative proportion of receptor activating in the mono- and doubly bound state, respectively, that in turn depends on both strength of pre-synaptic release and synapse geometry (**Figure 2E**). It has to be pointed out that the results of Petrini et al. (2011) predict, on average, ∼11% IPSCs decay acceleration due to contribution of $GABA_AR$ activating in the monoliganded state (**Figure 2D**). Although this could appear as a moderate effect, it is expected to be relevant in network tasks strongly relying on the dynamic equilibrium between excitation and inhibition such as signal integration and fast oscillation. Worth of mention, in the case of non-equivalence between the two $GABA_AR$ binding sites (as reported for instance for glutamate kainate receptors, see below), during activation by short synaptic GABA exposure, the agonist would preferentially bind to the site with higher affinity thus determining larger probability of singly bound activation (Benke et al., 2004; Minier and Sigel, 2004). The aforementioned

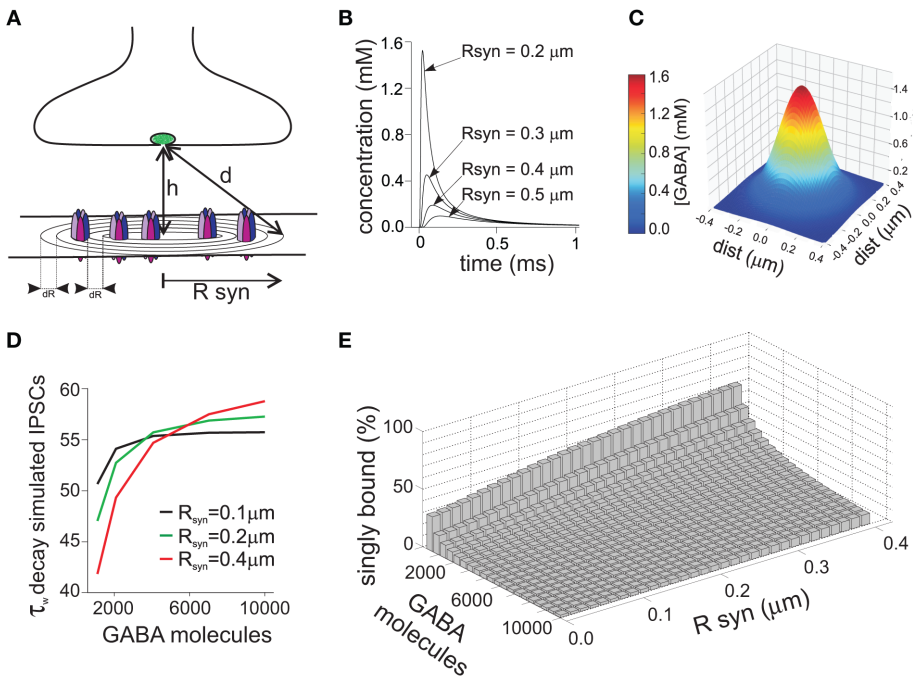

FIGURE 2 | Receptors located at different distances from the disk center sense a different GABA concentration due to non-homogeneity of the neurotransmitter concentration profile. (A) Schematic representation of the GABAergic synapses. The distance from the releasing site to the synaptic site is indicated by *d*, while R_{syn} represents the synaptic disk radius. **(B)** Simulated time course of the GABA concentration following release of 4000 GABA molecules, measured at different distances from the disk center. Please note that in the vicinity of the disk center the GABA concentration peak is markedly higher with respect to the disk periphery. **(C)** Simulated spatial profile of GABA diffusion in the cleft obtained 30 μs after the instantaneous release of 4000 GABA molecules. **(D)** Dependence of the decay time of simulated IPSCs on the number of GABA molecules released. At low number of GABA molecules released, receptors located at the disk periphery sense a low dose of GABA, activating preferentially in the monoliganded configuration, and contributing therefore to IPSCs speed-up. Please note that the IPSCs decay was also influenced by the synapse radius. **(E)** Summary of the relative weight of monoliganded $GABA_AR$ on the simulated IPSCs at different GABA molecules released and synaptic radiuses. Please note that conditions generating low saturation (low number of molecules released and/or large synaptic radiuses) favor the singly bound state activation, while high concentration pulses at small synapses determine almost complete activation in doubly bound state. Modified from Petrini et al. (2011) with permission.

IPSCs speed-up due to $GABA_AR$ singly bound states could provide a partial explanation to the observation that GABAergic currents are systematically faster than currents evoked by brief exogenous GABA pulses. A potential role for monoliganded states in explaining such discrepancy has been previously studied by Banks and Pearce (2000) who compared the amplitude and the deactivation time constant of currents elicited by both exogenous brief saturating GABA pulses (2 ms, 10 mM) and "synaptic equivalent" pulses obtained by lowering the GABA dose (2 ms, 10 μM). In comparison to currents elicited by saturating pulses, those evoked by low GABA concentrations showed deactivation kinetics accelerated by 35% and peak amplitude reduced by 96%. Interestingly, currents evoked by low concentration GABA pulses still showed deactivation kinetics twofold slower than IPSCs. The strong sensitivity of the peak current to weak GABA pulses associated with a moderate dependence of the deactivation kinetics led Banks and Pearce (2000) to conclude that brief synaptic exposure could not account for the fast IPSCs kinetics. They suggested therefore that the reason for the slow response observed in excised patch (as compared to synaptic current) could be due to the presence of high affinity extra-synaptic receptors. Although this hypothesis is reasonable, several observations complicate this scenario. Currents elicited in excised patches containing recombinant $GABA_AR$ subtypes known to mediate synaptic currents (e.g., $\alpha1\beta2\gamma2$) show slower deactivation than IPSCs decay (and similar to that obtained in patches pulled from neurons). It could be objected that receptors expressed in recombinant systems lack some important factors modulating the IPSCs kinetics. This hypothesis is certainly plausible, although manipulations of proteins known to compose or interact with scaffold proteins at GABAergic synapses show only a moderate effect on IPSCs decay time (Petrini et al., 2003; Marchionni et al., 2009). In keeping with this, Pugh and Raman (2005) proposed that the patch excision itself would stabilize GABA desensitization with consequent currents slow down, although the molecular determinants for such gating modification have not been identified yet. Other lines of evidence argue against the possibility that synaptic $GABA_AR$ would show different gating properties with respect to recombinant receptors or native receptors recorded in excised patches. For instance, currents elicited by UV laser photolysis of caged-GABA in diffraction limited spots (∼400 nm) at individual intact GABA synapses still display deactivation kinetics markedly slower than IPSCs (Trigo et al., 2009; Matsuzaki et al., 2010). Similarly, focal micro-iontophoretic GABA applications restricted to ∼600 nm diameter spots (Heine et al., 2008) fail to reproduce the IPSCs kinetics (Barberis et al., 2011). It has to be pointed out, however, that both uncaging and microiontophoresis deliver GABA pulses different from those occurring at the synapse. This is due to the fact that the "GABA spot" is bigger than the size of most of central synapses activating extra-synaptic receptors that typically do not participate in synaptic transmission. In addition, most of the commercially available GABA-caged compounds partially act as competitive blockers, potentially contributing to current slowdown (Trigo et al., 2009). In summary, although several lines of evidence show that neurotransmitter concentration profile plays a major role in shaping the amplitude and the kinetics of IPSCs, the precise assessment of its contribution will require the development of new devices capable to deliver, at intact synapses, neurotransmitter pulses in a temporal and spatial range relevant for synaptic transmission. It is important to mention that other factors are likely to affect the IPSC decay kinetics. These may include modulation of $GABA_AR$ gating by intracellular chloride concentration (Houston et al., 2009), phosphorylation state of various $GABA_AR$ subunits (β or γ), (Kittler and Moss, 2003; Houston et al., 2008) and interaction with yet unidentified accessory subunits or scaffolding proteins such as, e.g., gephyrin (Christie et al., 2002; Fritschy et al., 2003; Arancibia-Carcamo and Moss, 2006).

ROLE OF DESENSITIZATION IN SYNAPTIC CURRENT

Desensitization process has been recognized to profoundly shape the deactivation process of $GABA_AR$-mediated currents (Jones and Westbrook, 1995; Mozrzymas et al., 2003b). Indeed, due to slow unbinding rate, after GABA removal, the receptor may experience several transitions between bound open, closed, and desensitized state(s), implying that desensitization does prolong the deactivation kinetics. One of the most straightforward demonstrations of this phenomenon is that the deactivation time course depends on the degree of receptor desensitization induced by GABA pulses of different time durations. For instance, the kinetics of current relaxation after a 3 s pulse of saturating GABA is markedly slower than that observed at brief (2–3 ms) pulses (Jones and Westbrook, 1995; Mozrzymas et al., 2007b; Petrini et al., 2011). It could be hypothesized that shortening the GABA pulse length from 2 to 3 ms down to synaptic-like exposures (∼0.1 ms) would induce even lower extent of desensitization with consequent faster deactivation kinetics. However, by considering only the doubly bound states, the deactivation time course predicted by the Jones and Westbrook's (1995) model (**Figure 1B**) would not show major differences between currents evoked by ultra-short pulses (0.1 ms) and those elicited by longer (2–3 ms) pulses. The reason for this prediction is that the main mechanism of desensitization onset following brief exposure is receptor trapping into the desensitized state due to slow unbinding and resensitization rates (k_{off}, r_2, **Figure 1B**; Jones and Westbrook, 1995; Mozrzymas et al., 2003b, 2007b). Thus, although it might look at the first glance counterintuitive, most of rapid accumulation in the fast desensitized state takes place following application of brief GABA pulses depending weakly on the exposure time. On the other hand, pulses longer than 2–3 ms would favor accumulation of receptors in the desensitized states, including slower ones, contributing thus to observed slow down of deactivation kinetics. Glutamatergic synapses, in contrast, provide an excellent example of how synaptic exposure can efficiently shape the decay kinetics of synaptic currents by interfering with desensitization. The deactivation of AMPA and kainate receptors, contrary to $GABA_AR$, is accelerated by desensitization that, in these receptors, is very fast and profound (**Figure 3A**). The GluK2/K5 subtype of kainate receptor (composing synaptic receptors) mediate fast deactivating currents ($\tau \sim 3$ ms) when activated by long (1 mM, 100 ms) glutamate pulses, whereas short glutamate exposures (1 mM, ∼1.5 ms) unmask markedly slower deactivation kinetics ($\tau \sim 46$ ms, **Figures 3A,B**; Barberis et al., 2008). This behavior results from the fact that GluK2/K5 receptors possess two different types of binding sites showing distinct affinity and desensitization properties. In particular, glutamate binding to the high affinity sites induces poor desensitization while activation of

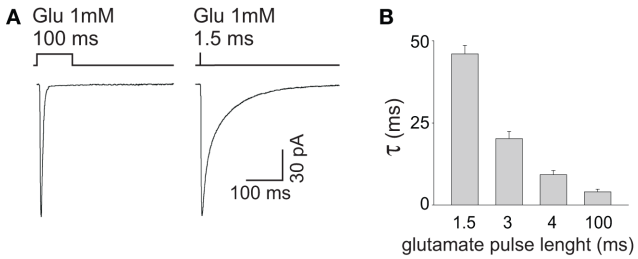

FIGURE 3 | The decay kinetics of currents mediated by GluK2/K5 kainate receptors strictly depends on the length of glutamate pulse. **(A)** Representative traces of GluK2/K5 currents elicited by long (100 ms) and brief (1.5 ms) pulses of glutamate 1 mM. Please note that the current elicited by brief glutamate pulses (right panel) deactivates markedly slower that that obtained at long glutamate exposures (left panel). **(B)** Dependence of GluK2/K5 current deactivation kinetics upon glutamate pulse length. Modified from Barberis et al. (2008) with permission.

low affinity binding sites determines fast and profound GluK2/K5 desensitization (Mott et al., 2010). During fast synaptic activation, therefore, the high affinity/poorly desensitizing binding site is expected to be preferentially activated mediating hence slow decaying responses.

CONSIDERATION OF SYNAPTIC AGONIST TRANSIENT SHEDS NEW LIGHT ON PHARMACOLOGICAL MODULATION OF POST-SYNAPTIC CURRENTS

As discussed above, kinetic studies brought increased awareness that the time course of synaptic neurotransmitter transient plays a crucial role in determining the kinetics of synaptic currents. Moreover, pharmacological modulation of synaptic signals may critically depend on synaptic agonist waveform even in the cases in which drug action is purely post-synaptic. This is particularly evident for modulators affecting agonist binding to post-synaptic receptors. Indeed, for a brief and non-saturating agonist transient, even a slight modulation of the binding rate may result in a marked effect on bound receptor occupancy and therefore on the amplitude and time course of synaptic currents. Thus, the specificity of synaptic conditions may be responsible, at least partially, for kinetic and pharmacological differences observed between synaptic currents and responses evoked by exogenous agonist applications. As shown below, these differences may become a source of information on the time course of synaptic agonist. A critical limitation, when studying the pharmacology of PSCs, is that synaptic neurotransmitter transient is *a priori* unknown and may show substantial variability. Moreover, synaptic agonist cannot be easily manipulated thus precluding the use of classical pharmacological analyses based on dose-response relationships. For this reason, receptor kinetics and mechanisms of its modulation need to be determined relying on the analysis of rapid agonist applications. The weakness of this approach, however, is that the kinetics is determined not for synaptic receptors but for recombinant ones or for receptors in patches excised from neurons (containing a mixture of synaptic and extra-synaptic receptors). Moreover, while IPSCs are expected to be particularly sensitive to agents affecting binding, most modulators affect both binding and gating (opening/closing, desensitization, unbinding) of $GABA_AR$. In general, even in a pharmacological study based on currents evoked by precisely defined agonist concentration "jumps," it is not straightforward to "dissect" the effect on binding from modification of other transition rates. As shown in an elegant study by Jones et al. (1998) this task becomes a considerable challenge even when determining only two rate constants (binding and unbinding rates) that define the receptor affinity. In general, assessment of specific binding and gating rate constants from the analysis of evoked current time course is difficult because any current characteristics (e.g., rise time or decay kinetics) may potentially depend on all the rate constants and on occupancies of all the channel states (Colquhoun, 1998; Mozrzymas et al., 2003b). In the past decade, the idea to combine high resolution studies of receptor gating while considering the specificity of synaptic conditions dictated by rapid agonist transient has been implemented by us and others to explore the modulatory mechanisms of several pharmacological agents. Below we provide examples of such studies to emphasize important practical implications of agonist transient features in the context of PSC modulation. It is worth noting that determination of synaptic neurotransmitter waveform and description of pharmacological mechanisms can be regarded as synergistic components of these studies. Indeed, high resolution determination of receptor gating is crucial to describe the properties of post-synaptic receptors while assessment of synaptic neurotransmitter time course enables to extrapolate this knowledge to synaptic conditions. Conversely, optimization of agonist transient waveform offers the opportunity to verify to what extent the receptor gating determined from current responses (receptors in excised patches) is adequate for the analysis of synaptic currents. Importantly, besides relevant pharmacological information, most of these studies provided consistent indications regarding the time course of synaptic neurotransmitter transient.

EFFECT OF EXTRACELLULAR pH ON mIPSCs AND ON CURRENT RESPONSES

In the context of the role of synaptic GABA time course, it has been particularly insightful to consider the effect of extracellular pH on mIPSCs and on current responses elicited by exogenous GABA. Strikingly, while currents evoked by saturating GABA showed a strong (several fold) and monotonic decrease in amplitude with increasing pH (**Figures 4A,B**), synaptic currents were characterized by a biphasic pH-dependence: an increase at pH = 6.5 (with respect to that at pH = 7.2), steep decrease at pH values below 6.5 and nearly no effect when alkalinizing the extracellular saline (**Figures 4C,D**). An intuitive explanation for this discrepancy could be that synaptic receptors and those in excised patches might show different kinetic and pharmacological properties (Wisden et al., 1996; Banks and Pearce, 2000; Cherubini and Conti, 2001; Fritschy and Brunig, 2003). However, the kinetic features such as rise time and decay kinetics of IPSCs and of current responses to saturating GABA showed a qualitatively similar pH-dependence (Mozrzymas et al., 2003a). This observation suggested that the major differences lay on receptor activation conditions rather than on receptor properties. Indeed, while rapid application was set to assure saturation (10–30 mM GABA applied for >1 ms), synaptic neurotransmitter was likely to show smaller peak concentration

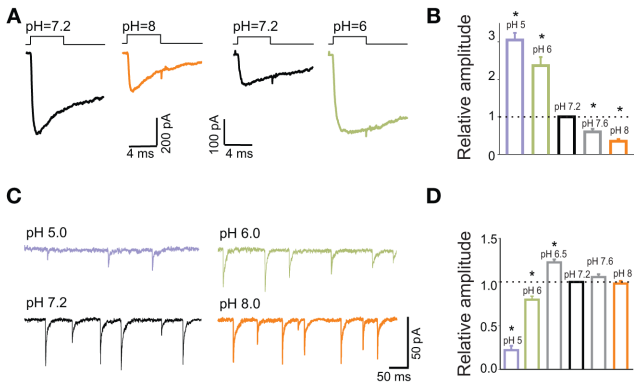

FIGURE 4 | Current responses to applications of saturating GABA concentrations show different pH-sensitivity than mIPSCs. (A) Typical current responses to applications of saturating (GABA) at pH = 7.2 black) and at pH = 8 (orange) and pH = 6 (green). Traces are presented in pairs (pH = 7.2, 8.0 and pH = 7.2, 6.0) that were recorded from the same excised patches. **(B)** Statistics of relative amplitudes (normalized to that measured at pH = 7.2). Note a strong and monotonic pH-dependence. **(C)** Typical traces of mIPSCs recorded at various pH values as indicated. **(D)** pH-dependence of mIPSC amplitudes. Note a qualitatively different proton effect in comparison to that observed for current responses **(B)**. Modified from Mozrzymas et al. (2003b) with permission.

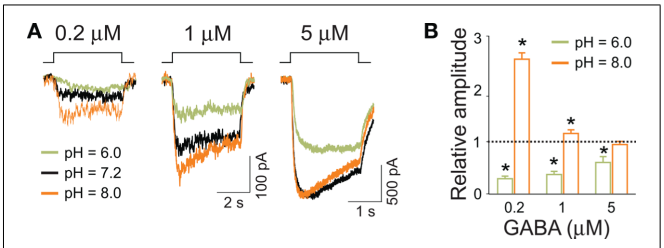

FIGURE 5 | Amplitudes of currents elicited by low GABA concentrations are up regulated by basic and down regulated by acidic pH. (A) Typical current traces elicited by applications of GABA concentrations indicated above current traces at different pH values indicated by colors (green – 6.0, black – 7.2, orange – 8.0). Note that for (GABA) = 1 μM, alkalinization of extracellular solution enhances the current amplitudes. **(B)** Statistics for relative changes in current amplitudes (with respect to that measured at pH = 7.2) for pH = 6.0 (green bars) and pH = 8.0 (orange bars). Note that especially at very low (GABA; 0.2 μM) basic pH strongly enhances the amplitude, the effect that is qualitatively opposite to that observed at saturating (GABA; **Figure 4**). Modified from Mozrzymas et al. (2003b) with permission.

and faster clearance. Moreover, kinetic studies of current responses evoked by rapid GABA applications revealed that decrease in pH strongly down regulated the binding and desensitization rates. In conditions of saturating GABA application, it is thus expected that when decreasing pH, the current amplitude would increase as the open state occupancy would increase at the expense of desensitization and this prediction was consistent with experimental findings (Mozrzymas et al., 2003a; **Figures 4A,B**). In contrast, for synaptic currents which are evoked by non-saturating and very brief GABA transient, a decrease in the binding rate at low pH would reduce the occupancy of synaptic bound receptors. At pH = 6.5, mIPSC amplitude is enhanced because reduction of desensitization rate counter balanced the reduction in the occupancy of bound states. However, a further acidification and ensuing reduction of binding rate, largely suppressed the recruitment into bound states resulting eventually in a decrease of mIPSC amplitude. Thus, a particular pH-dependence of mIPSCs resulted from an interplay between proton-induced changes in the microscopic binding/gating and dynamic conditions of receptors activation by synaptically released neurotransmitter. Model simulations indicated that, as expected, the shorter the GABA transient the larger the discrepancies between pH-dependence of responses to saturating (GABA) and mIPSCs. Fitting the data to a modified Jones and Westbrook's (1995) model indicated the time constant of agonist clearance of approximately 100 μs.

Recently, Dietrich and Morad (2010) provided intriguing evidence that protons might be involved in modulation of synaptic currents in the scale of a single GABAergic synapse. Consistent with our previous reports, they have observed that mild acidification increased the mIPSC amplitude (Mozrzymas et al., 2003a, 2007a). However, when increasing the buffering power, mIPSC amplitude decreased and this effect was ascribed to alkalinization of synaptic milieu. These (and other) findings led Dietrich and Morad (2010) to the proposal that vesicular release is associated with a local acidification that boosts the synaptic currents. While the data obtained for current responses and mIPSCs in hippocampal culture turned out to be consistent, at least at a qualitative level, it needs to be considered that neurons might express several different types of receptors, characterized by potentially different pH-sensitivity. It seemed thus important to verify the observed pH-dependence on a homogeneous $GABA_AR$ population, representative for synaptic receptors. Analysis of protons effects on recombinant α1β2γ2 receptors (Mercik et al., 2006) qualitatively reproduced our major observations obtained on neuronal culture (Mozrzymas et al., 2003a) including a strong pH-dependence of amplitudes evoked by saturating (GABA) and a marked pH effect on the rising phase of current evoked by non-saturating GABA. An intriguing finding observed first in neuronal culture was that acidic pH (6.0) decreased and alkaline pH (8.0) increased current amplitude evoked by low (GABA; 1 μM; **Figures 5A,B**; Mozrzymas et al., 2003b) – i.e., the opposite to what observed for saturating (GABA; **Figures 3A,B**. Importantly, at qualitative level, these inverse effects of acidic and basic pH on currents evoked by low or high (GABA) was reproduced on recombinant α1β2γ2 receptors (Mercik et al., 2006) implying that such a peculiar proton sensitivity may be a feature of a homogeneous population of $GABA_ARs$ (but see Pasternack et al., 1996). These observations are consistent with reduction of the binding and desensitization rates when lowering pH. Thus, at low (GABA) receptor occupancy is minimal and the impact of desensitization is low [contrary to the situation at saturating (GABA)]. Thus, in these conditions, acidification would lead to a decrease in current amplitude as it would further reduce the occupancy of bound receptors while down regulation of a weak desensitization would not be sufficient to counterbalance this reduction (**Figures 5A,B**; Mercik et al., 2006; Mozrzymas et al., 2007b). On the contrary, alkalinization would enhance both binding and desensitization, but in conditions of very low (GABA), gain in the binding rate would not be counterbalanced by increased

desensitization giving rise eventually to enhancement of current amplitude (**Figures 5A,B**; Mercik et al., 2006; Mozrzymas et al., 2007a).

MODULATION OF mIPSCs BY CHLORPROMAZINE

Chlorpromazine (CPZ) is a neuroleptic and its primary target are dopamine receptors (Snyder et al., 1974; Seeman, 1980) but several other receptors and channels are affected by this compound (Peroutka and Synder, 1980; Zorumski and Yang, 1988; Ogata et al., 1989; Muller et al., 1991; Bolotina et al., 1992; Benoit and Changeux, 1993; Lidsky et al., 1997). Since CPZ administration could be accompanied by seizures (Toone and Fenton, 1977; Itil and Soldatos, 1980) we were interested in describing the action of this drug on GABAergic transmission (Mozrzymas et al., 1999). CPZ strongly reduced mIPSC amplitude and accelerated its decay having no significant effect on the onset kinetics. Surprisingly, CPZ exerted a dramatically stronger inhibitory effect on mIPSCs than on the current responses elicited by exogenous GABA [especially when applying high (GABA)]. Moreover, at variance to mIPSCs, the rising phase of current responses to non-saturating (GABA) was clearly slowed down by CPZ. In attempt to clarify this discrepancy we have first determined the modulatory mechanism whereby CPZ affected $GABA_A$Rs. Recordings of current responses to rapid GABA applications together with quantitative analysis based on model simulations indicated that the major CPZ effect was to reduce the binding rate and to increase the unbinding rate with only minor effects on other transition rates (opening/closing, desensitization). It may look surprising that such a simple mechanism (a "mere" weakening of binding) might produce such a variety of kinetic alterations of mIPSCs and of current responses (and that the CPZ effects were substantially different in these two current types). The key to reconcile these diverging findings was again to consider the differences between synaptic transient and exogenous agonist applications. Thus, similar to acidic pH, strong effect of CPZ on mIPSC amplitude was found to result mainly from reduction of binding rate and ensuing lower bound receptor occupancy. The lack of a significant CPZ effect on the mIPSC rising phase was a consequence of a very short receptor exposure to synaptic agonist (ca. 100 μs) for which reduction of binding rate resulted in a decrease in amplitude rather than in a change in the onset rate. On the contrary, longer exogenous applications of GABA (1 mM, 2–5 ms) revealed additionally a slowdown of the current rising phase. Taking altogether, this study revealed not only the mechanism whereby CPZ modulate $GABA_A$Rs but also provided the first to our knowledge experimentally based estimation of GABA synaptic transient using, as a tool, the modifier of post-synaptic receptor gating. It is worth noting that inhibitory CPZ effect on GABAergic currents appears consistent with seizures induction by this drug although its causality remains to be proved.

EFFECT OF BENZODIAZEPINE RECEPTOR AGONISTS ON GABAergic CURRENTS

Benzodiazepine receptor agonists (BRAs) are positive modulators of GABAergic currents that have been recognized for their sedative, anti-convulsant, anxiolytic, and hypnotic effects. At variance to another group of clinically relevant drugs, barbiturates, BRAs do not activate $GABA_A$Rs by themselves (but see Campo-Soria et al., 2006), but they rather up regulate the activity of receptors activated by the agonist. Studies on recombinant receptors provided detailed information regarding sensitivity of different $GABA_A$R subtypes to BRAs (Rudolph and Mohler, 2004, 2006; Wafford, 2005) and the localization of BDZ binding site has been indicated (Pritchett et al., 1989; Sigel et al., 1990; Duncalfe et al., 1996; Sigel and Buhr, 1997; Kloda and Czajkowski, 2007). The most widely accepted mechanism of BRA modulation of $GABA_A$R is an increase in the receptor affinity while the effect on the kinetics of conformational transitions between bound states remains a matter of debate. Interestingly, the mechanism relying on BRA-induced increase in receptor affinity has been challenged by Rusch and Forman (2005) as well as by Downing et al. (2005) who studied spontaneously active $GABA_A$R mutant and proposed that the major BRA effect is due to an up regulation of the receptor efficacy. In a series of experiments carried out in one of our laboratories (Mercik et al., 2007; Mozrzymas et al., 2007b; Wojtowicz et al., 2008) the modulatory mechanisms of GABAergic currents by BRAs with particular focus on the impact of specific synaptic conditions dictated by rapid agonist transient have been investigated. To our surprise, the potentiating effect of BRAs on current responses to exogenous agonist was limited to currents evoked by (GABA) several fold smaller than EC50. Moreover, at (GABA) higher than 30 μM, BRAs induced a reduction of current amplitude. BRA-induced down regulation of responses evoked by saturating (GABA) was confirmed on recombinant $GABA_A$Rs ($\alpha1\beta2\gamma2$) implying that this effect cannot be ascribed to a non-homogeneity of the receptor subtypes in the patches excised from cultured neurons (Mercik et al., 2007). This BRAs effect was attributed to an up modulation of receptor desensitization (Mozrzymas et al., 2007b). Moreover, these compounds prolonged the deactivation phase of the current responses but, again, this effect was limited to (GABA) much lower than EC50. At the same time, BRAs markedly enhanced mIPSC amplitudes and prolonged their decay kinetics. These results led to the conclusion that synaptically released GABA, in spite of reaching relatively high peak concentrations, is far from saturation mainly due to its very short duration. Moreover, as recently pointed out by Petrini et al. (2011), the spatial profile of synaptic agonist is likely to show marked non-homogeneities within the synaptic cleft raising the possibility that receptors located at the periphery of the synaptic disk could experience lower agonist concentration than the central ones. Thus, these peripheral receptors would be more susceptible to an up regulation by BRAs and thereby could boost mIPSC sensitivity to these compounds. Most importantly, experiments with BRAs (Mozrzymas et al., 2007b) confirmed a critical role of synaptic conditions dictated by a rapid agonist transient. Moreover, these studies indicated that in addition to the binding reaction (binding and unbinding rate) BRAs might up regulate the occupancy of the desensitized state (Mercik et al., 2007; Mozrzymas et al., 2007b). The latter finding is compatible with the observation of increased current fading in the presence of BDZ reported by Lavoie and Twyman (1996) and a similar observation by Mellor and Randall (1997). Moreover, in a recent report, Karayannis et al. (2010) showed that the decaying phase of slow IPSC recorded from the neurogliaform cells in the hippocampus is accelerated by zolpidem and diazepam providing an indirect evidence for BDA-induced up regulation of the

desensitization process. In a recent report by Bianchi et al. (2009), it has been proposed that BDZs modulated $GABA_A$Rs (α1β3γ2 subtype was examined) by reducing only the unbinding rate (k_{off}). However, while data indicating BDZ-induced reduction of k_{off} confirm previous reports, no evidence was presented for the lack of BDZ effect on the binding rate (k_{on}). An up regulation of k_{on} has been consistently reported in several studies addressing this problem (Macdonald and Olsen, 1994; Rogers et al., 1994; Lavoie and Twyman, 1996; Mozrzymas et al., 2007b).

CONCOMITANT MODULATION OF GABAergic CURRENTS BY PROTONS AND BDZ

As pointed out above, the major mechanism whereby BDZs modulate $GABA_A$Rs is to increase binding and desensitization while protons exert an opposite action (Mozrzymas et al., 2003a, 2007a). It is thus interesting to check for the combined action of these two modulators on synaptic currents. While acidification strongly reduces desensitization, BDZ impact on this process is relatively weak. On the other hand, BDZ and protons exert the opposite action on binding and both effects are strong. It is thus expected that in conditions of a brief synaptic transient, combined effect of BDZ and acidic pH on agonist binding would be mutually compensatory but the current would be enhanced due to a net suppression of desensitization and ensuing higher occupancy of the open states. This prediction was confirmed in our recent study (Wojtowicz et al., 2008) in which BDZ impact on mIPSCs was tested in neurons in conditions of acidic and physiological pH (**Figures 6A,B**; Wojtowicz et al., 2008). In addition, analysis of the effect of BDZ and protons on current responses to rapid agonist application, provided evidence that the modulatory effects of these compounds are additive. It is worth noting that these results are likely to have some clinical implications as some brain disorders, such as hypoxia, ischemia, and hypoglycemia, are associated with acidosis of the extracellular fluid in the brain tissue (Kraig et al., 1986; Bengtsson et al., 1990; Katsura et al., 1993).

ZINC EFFECT ON GABAergic CURRENTS

Zinc ions are abundant in the CNS and have been found to be co-released with glutamate from mossy fiber boutons in the hippocampus (Slomianka, 1992). Several functions of zinc have been implicated in physiology and pathology and it has been proposed to perceive zinc as biological signal ion (Frederickson et al., 2005; Sensi et al., 2009). Zinc has been demonstrated to reduce the amplitude of GABAergic IPSCs (Defazio and Hablitz, 1998; Barberis et al., 2000; Mellor et al., 2000) and this effect was accompanied by substantial alterations in their time course (decrease in the onset rate and faster decay). In our study (Barberis et al., 2000), the mechanisms of zinc-mediated modulation were studied by analyzing the effect of this compound on synaptic currents and on current responses elicited by rapid GABA applications. The analysis of these currents revealed that the effect of zinc is complex and involves: decrease in binding (k_{on}) and increase in unbinding rate (k_{off}), slower entrance into the fully bound open state (β2) and modified desensitization (decrease in d_2 and r_2). Importantly, similar to what observed for other modulators reducing agonist binding, zinc ions caused a considerably larger inhibition of mIPSCs than of the current responses to exogenous GABA. Model simulations confirmed that a stronger inhibitory effect of these divalent cations on mIPSCs was a consequence of reduced binding rate in combination with short and non-saturating synaptic GABA transient.

Since it is known that the inhibitory effect of zinc is strongly attenuated by the presence of γ2 subunit, the impact of this ion on α1β2γ2 and on α1β2 receptors was considered (Barberis et al., 2002). Besides confirming that γ2 subunit-containing receptors are at least two order of magnitude more resistant to the blockade by zinc, profound differences were found in the effect of these ions on the onset and desensitization kinetics of these receptors. Importantly, the effect of zinc ions on the α1β2γ2 receptors closely resembled that observed for neuronal ones (Barberis et al., 2000) confirming thus that the pattern of current modulation of α1β2γ2 receptors may be representative, at least qualitatively, for neuronal population of $GABA_A$R.

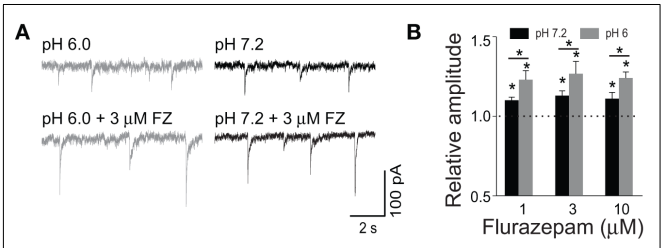

FIGURE 6 | Low pH increases mIPSC susceptibility to modulation by flurazepam. (A) Examples of mIPSCs recorded at −70 mV at pH = 6.0 (gray) and pH = 7.2 (black) in the absence (upper traces) and presence of 3 μM flurazepam (lower traces). **(B)** Statistics of flurazepam effect on mIPSC amplitude at pH 7.2 (black bars), and at pH = 6.0 (gray bars). Note that the acidic pH increased the relative enhancement of mIPSC amplitude by flurazepam. Modified from Wojtowicz et al. (2008) with permission.

VOLTAGE-DEPENDENCE OF GABAergic CURRENTS

Several studies provided evidence that the time course of GABAergic currents shows a substantial voltage-dependence (Barker and Harrison, 1988; Weiss et al., 1988; Jones and Harrison, 1993; Pearce, 1993; Lukatch and MacIver, 1997; Mellor and Randall, 1998; Krishek and Smart, 2001). Since the mechanisms underlying the modulation of GABAergic currents by voltage have not been fully elucidated, we have performed a kinetic analysis of current responses evoked by rapid applications of exogenous GABA (Pytel et al., 2006). I–V relationship for currents evoked by a saturating GABA concentration showed an inward rectification and this effect was accompanied by a faster onset as well as a larger rate and extent of desensitization at positive voltages. Moreover, at low GABA concentration (1 μM) currents were outwardly rectifying and at a non-saturating GABA concentrations (10–300 μM) current onset was significantly faster at positive voltages. Model simulations indicated that the major effect of membrane depolarization was to increase the rates of binding, desensitization, and of opening. The major factor implicated in the inward rectification was the increase in the occupancy of the desensitized state at positive voltages. Interestingly, this interpretation appears supported by the observation that at acidic pH (6.0), i.e., in conditions in which desensitization is markedly weakened, the inward rectification is nearly abolished (Pytel et al., 2005). However, when confronting the voltage-dependence of mIPSCs and of current

responses to high (GABA), striking differences were found (Pytel and Mozrzymas, 2006). In contrast to the current responses, mIPSCs showed a strong outward rectification and decay kinetics of mIPSCs was substantially prolonged when changing the membrane voltage from negative to positive. A likely explanation for this observation is that positive voltage, by enhancing the binding rate, might increase the recruitment of synaptic receptors into the bound states after synaptic agonist release. Thus, the switch from a negative to positive polarization would exert a qualitatively similar effect as BRAs.

OENANTHOTOXIN

Oenanthotoxin (OETX) is a polyacetylenic alcohol found in plants of genus *Oenanthe*, known to be among the most poisonous in the European flora. It is known since pre-Roman times that it induced contraction of facial muscles giving rise to sinister smile (*risus sardonicus*). Since OETX action was found to induce also epileptic-like activity (Chauvel et al., 1978; Louvel and Heinemann, 1980, 1983) we have addressed the impact of this toxin on GABAergic currents (Appendino et al., 2009; Wyrembek et al., 2010). Interestingly, this compound exerted a complex action on $GABA_A$Rs including strong reduction of amplitude, profound change in the current time course and use-dependent blocking action suggesting the open channel block. Most strikingly, however, the effect of this toxin was much more potent on mIPSCs than on current responses. While IC50 for mIPSCs was approximately 0.1 μM, for currents evoked by exogenous saturating or subsaturating GABA concentrations, IC50 was more than one order of magnitude higher (Wyrembek et al., 2010). Again, this discrepancy was attributed to a strong reduction of the binding rate by this toxin that resulted in a dramatic decrease in receptor occupancy during short-lasting synaptic application. Interestingly, OETX also down regulated receptor desensitization, an effect that would be expected to increase the occupancy of the open conformation. However, at submicromolar concentrations, OETX so strongly down regulated the agonist binding during synaptic exposure that the overall occupancy of bound receptors was greatly reduced thus obscuring the effect of a weakened desensitization, similarly to what observed for mIPSCs at strongly acidic pH (Mozrzymas et al., 2003a). Altogether, OETX affected GABAergic currents by a complex mechanism including allosteric modulation of binding and gating receptor properties and possibly by a direct block of the channel pore. Most importantly, a particularly large sensitivity of mIPSCs to OETX with respect to current responses to exogenous GABA was attributed to concomitant reduction of the receptor binding rate and short duration of GABA transient in the synapse.

INTERMINGLED IPSC MODULATION AND NEUROTRANSMITTER TRANSIENT DYNAMISM

Although the nature of described above modulating factors was different (chemical compounds and electric field associated with membrane polarization) and the structures of pharmacological agents ranged from ions (protons or zinc) to complex molecules, it is worth noting some common features of their action and to propose some general observations emerging from these studies. The first and perhaps the most apparent is that the agonist transient time course needs to be perceived as a key regulator of synaptic current susceptibility to pharmacological modulation. It is noteworthy that for all modulators considered above, mIPSCs showed markedly larger sensitivity than the current responses, elicited by exogenous GABA applications, even if the latter ones were evoked using a technique designed to best reproduce synaptic conditions. Kinetic analysis carried out for all the modulators confirmed the general rule that the larger the impact on the agonist binding rate the larger the sensitivity of the synaptic currents to the modulator. Importantly, each time when confronting the effect of a given modulator on current responses evoked by rapid agonist applications and on the synaptic currents one gets a possibility to optimize the parameters describing the synaptic GABA transient time course. When performing such a procedure for data obtained on hippocampal neuronal culture for different modulators of binding and gating (Mozrzymas et al., 1999, 2003a; Barberis et al., 2000) the clearance time course was consistently estimated close to 100 μs. However, when applying the technique based on the use of rapid competitive blockers, predominant time constant for agonist clearance in similar models was considerably longer, ranging between a few hundreds of microseconds up to 1 ms (Clements et al., 1992; Scimemi and Beato, 2009). This discrepancy may result from several drawbacks of both approaches. In the case of available rapidly dissociating competitive antagonists, their unbinding time constant is at least several hundreds of microseconds, i.e., comparable with the agonist clearance time constants determined when using these compounds. Thus, one possibility is that determination of agonist transient waveform might be at the limit of the resolution of this method. A very fast clearance component of approximately 100 μs has been postulated by several Monte Carlo and model simulations (Holmes, 1995; Clements, 1996; Kleinle et al., 1996; Wahl et al., 1996; Glavinovic, 1999; Franks et al., 2002; Ventriglia and Maio, 2003; Petrini et al., 2011). It needs to be born in mind, however, that modeling of agonist transient as a pure diffusion might lead to overestimation of the clearance rate as in the synaptic and subsynaptic environment, the diffusion coefficient might be much lower than in the free solution due to, e.g., larger viscosity of extracellular fluid, interactions with extracellular matrix components or limited space (Nicholson and Sykova, 1998; Vorisek and Sykova, 2009). On the other hand, as already mentioned, the method of transient determination based on the use of binding and gating modifiers (Mozrzymas et al., 1999, 2003a; Barberis et al., 2000) might be biased by the fact that the modulators, besides modifying binding affect also gating and their effect might differ between synaptic and extra-synaptic receptors (and also between excised patch and whole-cell configurations). Synaptic currents can be modulated by cell adhesion molecules (Dalva et al., 2007; Huang and Scheiffele, 2008) and to some extent by a number of scaffolding proteins (Tyagarajan and Fritschy, 2010; Tyagarajan et al., 2011), the effects that are likely to be lost or severely impaired upon patch excision or intense cell dialysis. Thus, it seems reasonable to assume that basing on studies using both types of techniques, the predominant component of agonist clearance at the GABAergic synapse in the considered models (neuronal cell cultures) lies within the range between one hundred up to a few hundreds of microseconds.

VARIETY OF NEURONAL TYPES AND A VARIETY OF SYNAPTIC CONDITIONS

Pharmacokinetic studies outlined above were carried out either on cultured neurons or on cell lines expressing recombinant receptors. Several factors may alter the synaptic transmission in cultured neurons with respect to their counterparts in more physiological models (slices or *in vivo*). Cultured neurons, after a harmful enzymatic and mechanical treatment, are grown in artificial environment and, although some developmental mechanisms are maintained (Ortinski et al., 2004; Swanwick et al., 2006; Pytel et al., 2007), neuronal differentiation is altered, and synaptic connections are formed largely randomly due to dispersion of naturally occurring cues. Importantly, large volume of bulk aqueous solutions around neurons may greatly affect the diffusion conditions favoring faster neurotransmitter clearance from the synapses. Moreover, in neuronal cultures, glial cells are typically suppressed to avoid their overgrowth. Taking into consideration the extensive and rapidly growing body of evidence that astrocytes are strongly involved in the functioning of synapses, giving rise to the concept of a tri-partite synapse (Araque et al., 1999; Perea et al., 2009; Pereira and Furlan, 2010), it may be expected that the lack or deficit of astrocytes could profoundly affect the synaptic transmission. Astrocytes are known to set not only the physical barriers for free diffusion within and in the immediate surroundings of the synapse but also to play a pivotal role in the neurotransmitter uptake (Conti et al., 2004; Bergeles and Edwards, 2008). Especially in the case of glutamate, excitatory amino acid transporters (EAAT1 and EAAT2) are very efficient in removing free extracellular glutamate and are highly concentrated in astrocytes assuring an efficient control of the spatiotemporal profile of synaptically released glutamate (Diamond, 2005; Tzingounis and Wadiche, 2007; Bergeles and Edwards, 2008; Scimemi et al., 2009; Thomas et al., 2011). Electrogenic nature of glutamate transporters (2 Na^+ ions, 1 H^+ ion, 1 glutamate in, 1 K^+ ion out) made it possible to track their kinetics by analyzing transporter-mediated currents evoked by exogenous applications of glutamate to excised patches (Bergles et al., 1997, 2002). These studies provided evidence that glutamate binding and its translocation to the inner side are rapid, although their full cycle (including glutamate release in the cytoplasm) requires approximately 10–20 ms (for the transporter EAAT2, Bergles et al., 2002). This implies that transporters would contribute to the fast clearance of synaptic neurotransmitter by rapidly binding the released molecules, acting therefore as neurotransmitter buffer (Scimemi et al., 2009).

Importantly, GABAergic interneurons are greatly diversified with respect to their morphology and function (Klausberger and Somogyi, 2008). The time duration (assessed as mean decay time constant) of GABAergic currents may range between little more than 1 ms for synapses formed by parvalbumin positive basket interneurons (Bartos et al., 2002) up to nearly 100 ms for currents in thalamic reticular nucleus (Huntsman and Huguenard, 2000, 2006; Mozrzymas et al., 2007a) or in neurogliaform cells in hippocampus and cortex (Szabadics et al., 2007; Olah et al., 2009; Zarnowska et al., 2009; Karayannis et al., 2010; Capogna and Pearce, 2011). This heterogeneity of synaptic currents resulting from interneuron type and target cell identity is often obscured when studying IPSCs in neuronal cultures and therefore the estimation of synaptic transient may be biased by data averaging for different types of neurons (both pre- and post-synaptic) while conditions of synaptic transmission are likely to be profoundly affected by artificial conditions in culture. A variety of synaptic conditions in a slice model has been observed by Hajos et al. (2000) who examined the impact of zolpidem on mIPSCs in different types of neurons in eight brain regions and provided evidence for different extent of occupancy of post-synaptic receptors (incomplete vs. complete) depending on cell type and localization in the brain.

Glutamatergic synapses are known for their particularly high pre- and post-synaptic morphological heterogeneity. Indeed, the shape of synaptic terminals may range between tiny "en passant" boutons and giant depolarizing terminals. Interestingly, these two extreme forms of pre-synaptic terminals may occur within the same axon (e.g., in hippocampal mossy fibers) which may additionally show a fascinating feature of target cell specific properties (Maccaferri et al., 1998; Pelkey and McBain, 2008). Similarly, the morphology of the post-synaptic structures may vary between small varicosities and filopodia to extensive thorny excrescences (Fiala et al., 2008). Taking additionally into account that synaptic structures can be tightly ensheathed by astrocytes that, as mentioned, are strongly involved in controlling the spatiotemporal profile of synaptic agonist, it is not surprising that the time course of synaptic currents may critically depend on the synaptic structure. Indeed, Barbour et al. (1994) have reported that AMPA/kainate receptor-mediated EPSCs recorded in parallel fibers are characterized by a slower decay than those measured from interneurons while synaptic receptor properties in these cell types were very similar. This difference was attributed to different spine and synapse geometry that favors prolonged presence of glutamate around dendritic spines in the case interneurons. More recently, Cathala et al. (2005) applied an elegant approach based on functional, pharmacological, morphological, and analytical techniques to address the mechanisms of developmental speeding of AMPA receptor-mediated mEPSCs in mouse cerebellar granule cells. Surprisingly, they found that developmental acceleration of mEPSCs is unrelated to any clear switch in the subunit stoichiometry of the post-synaptic receptors. However, 3D reconstruction of mossy fiber terminals and granule cell dendrites revealed that the post-synaptic density areas in young (P8) animals were more than four times larger than those in adult ones (P40). In contrast, P8 mossy fiber terminals were much smaller and less convoluted than those in adults and these differences in dendritic shape resulted in an increased number of diffusional sinks in P40. Model simulations confirmed that these morphological changes of synapses are sufficient to affect the synaptic glutamate concentration waveform and thereby to underlie the developmental acceleration of the glutamatergic mEPSCs.

Although most studies based on combined functional and morphological approaches concerned glutamatergic synaptic transmission, in the last years important reports appeared concerning also GABAergic synapses. Biro et al. (2006) have estimated quantal parameters for IPSCs measured in pairs of CCK-expressing interneurons and CA3 pyramidal cells and for the same pairs

performed a *post hoc* EM determination of the number of boutons mediating IPSCs and the number of active zones per CCK-positive bouton. Surprisingly, quantal analysis indicated fivefold more functional releasing sites than those identified structurally. Biro et al. (2006) have proposed that this apparent discrepancy resulted from variation of synaptic GABA transient generated by multivesicular release in conditions of particularly low post-synaptic receptor occupancy. This mechanism may be regarded as a pre-synaptic, release probability-dependent scaling of PSCs at a single GABAergic synapse. Barberis et al. (2005) provided another example of pre-synaptic changes of GABA transient. By using the rapidly dissociating $GABA_AR$ antagonist, TPMPA they showed in an *in vitro* model, that in "young" neurons, displacement of TPMPA was more efficient than in "older" ones indicating that GABA uniquantal release is developmentally regulated.

As already mentioned, time duration of GABAergic synaptic currents measured in different projections may vary for nearly two orders of magnitudes. Initially, the phenomenon of IPSCs characterized by a particularly slow kinetics contrasting with predominant population of fast IPSCs has been observed in the hippocampus (Pearce, 1993; Banks et al., 1998). Later, IPSCs with slow kinetics were found also in other brain regions including the neocortex and thalamus as well as other brain regions (Kapur et al., 1997; Huntsman and Huguenard, 2000, 2006; Capogna and Pearce, 2011). Subsequent studies addressed the mechanisms underlying slow IPSCs and, not surprisingly, in different cell types distinct pre- or post-synaptic factors were implicated. Slow IPSCs in the thalamic RT interneurons are among the slowest GABAergic synaptic currents in the CNS (Huntsman and Huguenard, 2000, 2006; Browne et al., 2001). Extensive body of evidence based on slice recordings and recombinant receptors analysis consistently indicated that the slow IPSCs in these cells reflected a particular kinetics of post-synaptic α3 subunit-containing $GABA_AR$ (Verdoorn, 1994; Gingrich et al., 1995; Barberis et al., 2007; Mozrzymas et al., 2007a; Schofield and Huguenard, 2007). In contrast, in the cortical neurogliaform cells, slow IPSC kinetics was reported for synapses in which low affinity α1, β2/3, and γ2 subunit-containing receptors (typically implicated in fast transmission) were found to be predominant. In the case of these neurons, slow IPSC time course was attributed to a particularly slow GABA transient (Szabadics et al., 2007). This mechanism was recently further supported by an elegant functional and utrastructural study in which a remarkable observation was made that pre-synaptic boutons on axons of these cells were extremely dense but most of them did not form the classical synapse including a precisely juxtaposed post-synaptic density (Olah et al., 2009). Thus, wide synaptic clefts or GABA released from boutons distant from recipient $GABA_ARs$ provide a consistent explanation for slow GABA transient described by Szabadics et al. (2007). Such a peculiar synaptic arrangement of neurogliaform cells was proposed to underlie the volume release of GABA, raising thus a novel concept that GABA released from these neurons might exert a paracrine effect on the local neuronal networks (Olah et al., 2009). In parallel, Karayannis et al. (2010) explored the mechanisms of slow IPSCs in the hippocampal neurogliaform cells. Importantly, using pharmacological tools, they have found that α1 and γ2 subunit-containing receptors were involved in mediating slow currents evoked by neurogliaform cell activation, similar to what reported by Szabadics et al. (2007) in the cortex. Elegant quantitative analysis and modeling led Karayannis et al. (2010) to the conclusion that slow kinetics IPSCs generated by neurogliaform cells in the hippocampus results from unusually slow GABA transient in the synapses formed by these cells. Moreover, their ultrastructural studies revealed that neurogliaform cells formed membrane-to-membrane appositions that were lacking a clear synaptic structure. This result corroborates the structural findings by Olah et al. (2009) in the cortical neurogliaform cells.

The above examples of studies carried out on the GABAergic transmission in models believed to closely reproduce physiological conditions, indicate an amazing variety of kinetic patterns that have not been previously observed in cultured neurons. It is worth to bear in mind that some GABAergic interneurons (e.g., parvalbumin positive basket cells) are responsible for the fastest point-to-point synaptic transmission (Bartos et al., 2002) while neurogliaform cells, which release the same neurotransmitter often in a close vicinity of basket neurons, give rise to the slowest GABAergic synaptic signals in the hippocampus, implicated in a paracrine action (Olah et al., 2009). Taken altogether, both post-synaptic receptor properties and conditions of synaptic activation, dictated by the spatiotemporal profile of synaptically released agonist, were found to play a crucial role in shaping the GABAergic synaptic currents including the fastest and the slowest signals.

CONCLUDING REMARKS

Although tools allowing to directly monitoring the concentration of synaptic agonist in GABAergic and glutamatergic synapses are still lacking, our knowledge about its time course has been considerably enriched in the last two decades. Experimental approaches used to explore the dynamics of synaptic neurotransmitter are based on indirect information such as displacement of quickly unbinding competitive antagonists or modifiers of binding/gating receptor properties. While the former one was used intentionally to determine the agonist transient, the latter approach turned to be a necessary step to explore the mechanisms of pharmacological modulation. Indeed, for several agents, differences in pharmacological modulation of synaptic signals and of responses elicited by agonist applications could have been reconciled by considering the specific synaptic conditions set by the agonist transient. Investigations of the time course of synaptic neurotransmitters shed new light on our understanding of not only modulatory mechanisms but also extended our knowledge on the physiology of synaptic transmission in specific neurons. In particular, brief and spatially non-homogeneous GABA transient may favor openings of singly bound, peripherally localized synaptic receptors which would accelerate the IPSC decay kinetics. While early studies on cultured neurons implicated very rapid kinetics of synaptic agonist clearance, recent investigations carried out on brain slices revealed that in various types of interneurons, agonist transient kinetics may vary by nearly two orders of magnitude. It is worth emphasizing that synaptically released neurotransmitter, especially in the synapses showing slow transient, is spilling over, and affects the synapse neighborhood that may include other synapses, perisynaptic neuronal receptors mediating tonic transmission and astrocytes that emerged as powerful modulators of

synaptic functions. Thus, synaptic agonist transient is not only setting the transmission conditions at a given synapse but is also largely determining the interaction of the synapse with its local environment.

Taking altogether, the precise description of the synaptic conditions resulting from the spatiotemporal neurotransmitter profile is a crucial step in exploring the synaptic transmission and its modulation. It is expected that development of new (e.g., optical) tools enabling high resolution tracking of agonist profiles at different synapses would mark an important step forward in deciphering the function of neuronal networks.

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A role for GAT-1 in presynaptic GABA homeostasis?

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In monoamine-releasing terminals, neurotransmitter transporters – in addition to terminating synaptic transmission by clearing released transmitters from the extracellular space – are the primary mechanism for replenishing transmitter stores and thus regulate presynaptic homeostasis. Here, we analyze whether GAT-1, the main plasma membrane GABA transporter, plays a similar role in GABAergic terminals. Re-examination of existing literature and recent data gathered in our laboratory show that GABA homeostasis in GABAergic terminals is dominated by the activity of the GABA synthesizing enzyme and that GAT-1-mediated GABA transport contributes to cytosolic GABA levels. However, analysis of GAT-1 KO, besides demonstrating the effects of reduced clearance, reveals the existence of changes compatible with an impaired presynaptic function, as miniature IPSCs frequency is reduced by one-third and glutamic acid decarboxylases and phosphate-activated glutaminase levels are significantly up-regulated. Although the changes observed are less robust than those reported in mice with impaired dopamine, noradrenaline, and serotonin plasma membrane transporters, they suggest that in GABAergic terminals GAT-1 impacts on presynaptic GABA homeostasis, and may contribute to the activity-dependent regulation of inhibitory efficacy.

Keywords: GABA, GABA transporters, GAT-1, mIPSCs, knock-out mice

INTRODUCTION

In his Nobel Lecture, given on December 12, 1970, Julius Axelrod summarizes his seminal work on noradrenaline (NET) metabolism and uptake. In the paragraph entitled *Effect of drugs on neuronal uptake* he describes how blocking NET uptake using cocaine or other drugs resulted in a dramatic decrease in tissue [^{3}H] NET and addresses the question of whether, in addition to blocking uptake, "these drugs could prevent storage or release of [^{3}H] NET" (Axelrod, 1971). Ever since, the possible role of transport mechanisms on transmitter storage and release of neurotransmitters has been essentially neglected. Advent of molecular cloning and knock-out technologies made it possible to unveil the nature of proteins mediating neurotransmitter transport (transporters) and to investigate their functional role *in vivo*. Mice lacking dopamine (DAT), NET, or serotonin (SERT) transporters exhibit an increase of extracellular lifetime and levels of monoamines and a decrease of both their intracellular storage and release (Torres et al., 2003). These findings indicate that, besides terminating synaptic transmission by clearing released transmitters, monoamine transporters mediate replenishment of transmitter stores, thus regulating presynaptic homeostasis. Here, we analyze whether the same principle applies to GABAergic synapses[1].

GABA SYNTHESIS AND TRANSPORT: A COMPLEX *SCENARIO*

GABA SYNTHESIS

Most GABA is synthesized from glutamate (Glu) by glutamic acid decarboxylase (GAD; Roberts and Frankel, 1950, 1951). In mammalian brain, GAD occurs in two molecular forms, GAD_{65} and GAD_{67}; the former preferentially synthesizes GABA for vesicular release, the latter for cytoplasmic stores (Soghomonian and Martin, 1998). Indeed, ratio of GAD_{65} to GAD_{67} is higher in synaptic vesicle fractions than in cytosol (Solimena et al., 1993). GAD_{65} may be anchored to synaptic vesicles by forming a complex that includes the vesicular GABA transporter VGAT, an integral membrane protein of synaptic vesicles responsible for their filling (McIntire et al., 1997). This may provide a structural and functional coupling between synthesis and vesicular packaging of GABA (Hsu et al., 2000; Jin et al., 2003). Interestingly, [^{3}H]GABA newly synthesized from [^{3}H]Glu by synaptic vesicle-associated GAD is taken up preferentially into vesicles over cytosolic GABA (Jin et al., 2003). Minor sources of GABA, such as putrescine, spermine, spermidine, and ornithine, offer a negligible contribution to releasable GABA.

Glu used for GABA synthesis may originate from diverse sources.

Glu–GABA/glutamine cycle

Glutamate derived from glutamine (Gln) is an important GABA precursor (Bradford et al., 1983; Sonnewald et al., 1993). Released GABA is taken up by astrocytic transporters (i.e., GAT-3; Minelli et al., 1996), and catabolized to the tricarboxylic acid (TCA) cycle intermediate succinate by GABA transaminase and succinate semialdehyde dehydrogenase; the resulting α-ketoglutarate is then transformed to Glu which is converted to Gln by Gln synthetase (Martinez-Hernandez et al., 1977). Gln is then extruded from astrocytes by SNAT3, a system N transporter (Chaudry et al., 1999, 2002; Boulland et al., 2002), and taken up by axon terminals. Gln influx into neurons is thought to be mediated by SNAT1 and/or SNAT2, two system A transporters (Fricke et al., 2007); this view is compatible with expression of SNAT1 and SNAT2 in most GABAergic cells (Melone et al., 2004, 2006; Conti and Melone, 2006). In neurons, Gln is converted to Glu by phosphate-activated glutaminase (PAG; Kvamme et al., 2001).

[1]Space constraints prevent a detailed analysis of all brain regions; accordingly, most of the studies reported and the unpublished material presented here concern the cerebral cortex.

PAG immunoreactivity (ir) is detected in ~18% of all puncta expressing VGAT, which is expressed in all GABAergic terminals (Chaudhry et al., 1998; Minelli et al., 2003), and electron microscope studies show that ~20% of all PAG+ axon terminals making synaptic contacts form symmetric synapses (**Figures 1A,B**).

TCA cycle

Glutamate is synthesized in all cells from intermediates in the TCA cycle, and neurons are capable of *de novo* synthesis of Glu from TCA cycle intermediates, indicating that neuronal TCA cycle contribute significantly to Glu synthesis (Peng et al., 1993).

Glu transporters

Glutamate used for GABA synthesis could derive from the action of Glu transporters (GluT). EAAC1 is a neuronal GluT expressed also by GABAergic neurons (Rothstein et al., 1994; Conti et al., 1998a): in hippocampal slices from EAAC1 antisense-treated animals incubated in the presence of DON and gabaculine, newly synthesized [^{14}C]GABA from [U–^{14}C]Glu is lower in the presence of the Glu transport inhibitor DL-threo-β-hydroxy-aspartic acid; moreover, patch-clamp recordings of miniature IPSCs (mIPSCs) conducted in CA1 pyramidal neurons demonstrated a significant decrease in mIPSC amplitude, indicating decreased tonic inhibition (Sepkuty et al., 2002; see also Mathews and Diamond, 2003; Hartmann et al., 2008). The possible contribution of other GluTs (e.g., EAAT4 and GLT-1) to GABA synthesis remains to be verified.

GABA TRANSPORT

Four GABA transporters have been identified: GAT-1, GAT-2, GAT-3, and BGT-1 (Borden, 1996). GAT-1 is localized almost exclusively to axon terminals forming symmetric synaptic contacts and, in neocortex, to some astrocytic processes (Minelli et al., 1995; Conti et al., 1998b); GAT-2 is mainly expressed in the leptomeninges and in ependymal and choroid plexus cells, and to a lesser extent in neurons and astrocytes (Conti et al., 1999); GAT-3 is exclusively localized to distal astrocytic processes (Minelli et al., 1996); and BGT-1 is not localized to the CNS (Borden, 1996; Cherubini and Conti, 2001; Conti et al., 2004). Thus, the only GABA transporter that can contribute directly to GABA replenishment in terminals is GAT-1.

ARE MECHANISMS SUBSERVING GABA SYNTHESIS AND TRANSPORT EXPRESSED IN ALL GABAERGIC TERMINALS?

Crucial to the theme raised in this Perspective is whether the two mechanisms for GABA replenishment (synthesis and transport) are segregated in different terminals. Recent co-localization studies show that both GADs and GAT-1 are expressed in 90% of VGAT+ terminals (**Figure 1B**). Considering technical limitations, these values indicate that virtually all GABAergic terminals express both GADs and GAT-1 – i.e., that virtually all GABAergic neurons can synthesize GABA and take it up from extracellular *milieu* – and that GABAergic terminals cannot be differentiated on the basis of their mechanism of GABA replenishment.

INSIGHTS FROM KO MICE

GAT-1 deficient mice exhibit normal reproduction and life span, but have reduced body weight and higher body temperature fluctuations; they display motor disorders, including gait abnormality, 25–32 Hz tremor, reduced rotarod performance, and locomotor activity; they also display mild anxiety or nervousness (Jensen et al., 2003; Chiu et al., 2005).

In CA1 pyramidal cells, cerebellar granule and Purkinje cells, and neocortical layer II/III pyramids of KO mice, $GABA_A$ receptors (R)-mediated tonic conductance is significantly increased. Frequency, amplitude, and kinetics of spontaneous IPSCs are unchanged, whereas the decay time of evoked IPSCs is prolonged in KO mice (Jensen et al., 2003; Chiu et al., 2005; Bragina et al., 2008). In neocortex, high-frequency stimulation of GABAergic terminals induces large $GABA_A$R-mediated inward currents associated with a reduction in amplitude and decay time of IPSCs; the recovery process is slower in KO than in WT mice (Bragina et al., 2008). Thus, GAT-1 has a prominent role in both tonic and phasic $GABA_A$R-mediated inhibition, in particular during sustained neuronal activity (Bragina et al., 2008). The effects reported in KO mice are ascribable to impaired GABA uptake and subsequent reduction of GABA clearance from the synaptic cleft, a conclusion that would rule out any significant role of GAT-1 on presynaptic homeostasis.

However, analysis of mIPSCs in hippocampal pyramidal cells recorded in the presence of tetrodotoxin show that whereas mIPSCs have similar rise times, decay time constants, and

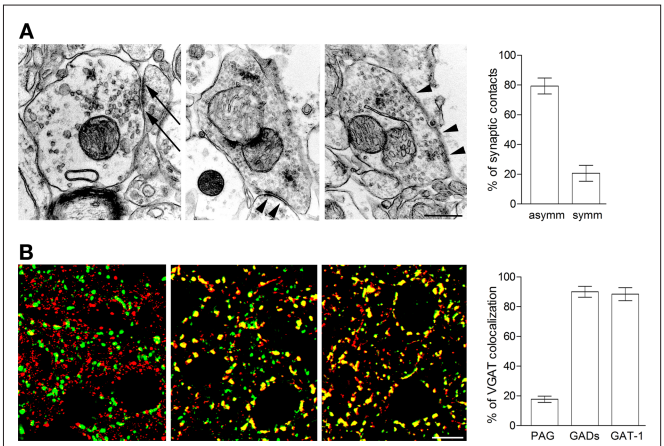

FIGURE 1 | Anatomical insight into GABAergic presynaptic terminals. (A) Electron microscope studies of PAG immunoreactivity. Both axon terminals forming asymmetric (*arrows*) and symmetric synapses (*arrowheads*) contain PAG-ir. Layer II–III; parietal cortex. Rat brains perfused and post-fixed for 48 h with 1% PFA and antiproteolytic cocktail (Melone et al., 2006); vibratome sections processed with mouse anti-PAG primary antibodies (1:250; Kaneko et al., 1987) and avidin–biotin. Quantitative data from two animals. **(B)** Co-localization studies of VGAT/PAG (*left*), VGAT/GADs (*middle*), and VGAT/GAT-1 (*right*). In all series, green codes for VGAT. VGAT/PAG series: tissue preparation as in A; anti-VGAT antibodies (1:500; Synaptic System; No. 131003); anti-PAG antibodies (1:250; Kaneko et al., 1987). VGAT/GADs series: tissue perfused and post-fixed for 24 h with 4% PFA; anti-VGAT antibodies (1:50; Synaptic System; No. 131011); anti-$GAD_{65/67}$ antibodies (1:800; Millipore; AB1511). VGAT/GAT-1 series: tissue perfused and post-fixed for 2 h with 4% PFA; anti-VGAT antibodies (1:50; Synaptic System; No. 131011); anti-GAT-1 antibodies (1:500; Minelli et al., 1995). For studies employing PAG antibodies, fixative conditions were selected on the basis of the results of preliminary studies performed to ascertain optimal conditions. Layer II–III; parietal cortex. Percentage of VGAT puncta co-localized with PAG, GADs, and GAT-1 defined by analysis of 60–80 microscopic cortical fields (from layers I–VI) from two animals for each antigen. Scale bars: 0.25 μm (*upper row*) and 5 μm (*lower row*).

amplitudes in WT and GAT-1 KO, their frequency in GAT-1 KO animals is reduced to about one-third of that recorded in control animals (Jensen et al., 2003; **Figure 2A**). Since reduced frequency of miniature events reflects presynaptic changes of quantal transmission (del Castillo and Katz, 1954; Clements, 1993), these findings indicate that in GAT-1 KO mice a presynaptic deficit does exist. Interestingly, simultaneous application of the GAT-1 inhibitor NNC-711 and of TBOA reduces mIPSC amplitudes, whereas high-frequency stimulation of CA1 afferents determines an activity-dependent increase in mIPSCs quantal size, that is produced by GAT-1-mediated GABA uptake and EAAC1-mediated Glu uptake in terminals of inhibitory interneurons (Hartmann et al., 2008).

Moreover, western blotting studies from neocortex of WT and GAT-1 KO mice show that whereas VGAT levels are unchanged, GAD65/67 and PAG levels are increased by 35% and 18%, respectively, in GAT-1 KOs compared to WTs (Bragina et al., 2008; **Figure 2B**). These studies show that in conditions in which GAT-1-mediated GABA uptake is lacking, the biosynthetic machinery is up-regulated. Interestingly, although expressed in a limited number of GABAergic neurons, PAG expression increases significantly, indicating that the Glu–GABA/Gln cycle may play a not negligible role in GABA synthesis. Most importantly, by showing that in GAT-1 KO mice the levels of GADs, the fundamental enzyme for GABA biosynthesis, and PAG, an enzyme responsible for the generation of some of the Glu used for GABA synthesis, are up-regulated, these results suggest that the lack of GAT-1 reduces intraterminal GABA, which in turn triggers the increased expression of PAG and GAD. These observations strengthen the hypothesis that in physiological conditions GAT-1 exerts an influence on GABA homeostasis in GABAergic terminals.

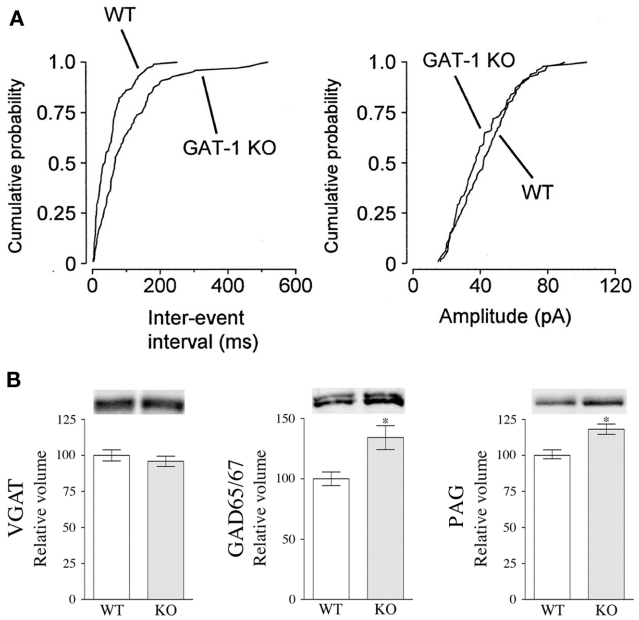

FIGURE 2 | GAT-1 may contribute to presynaptic GABA storage and release. (A) mIPSC inter-event intervals (*left*) and amplitudes (*right*) in WT and GAT-1 KO hippocampal neurons. Note the longer inter-event intervals for GAT-1 KO mIPSCs compared to WT ($p < 0.01$; From Jensen et al., 2003). **(B)** Western blotting studies on cortical cellular extracts show increased expression of $GAD_{65/67}$ and PAG, but not VGAT, in GAT-1 KO mice. Age of all animals was comparable. VGAT and $GAD_{65/67}$ data from Bragina et al. (2008). For PAG analysis, homogenates (10 μg/μl) were exposed to anti-PAG antibodies (1:1000; Akiyama et al., 1990).

CONCLUSION

In monoamine-releasing terminals, neurotransmitter transporters are crucial for presynaptic homeostasis, and this notion prompted us to verify whether GAT-1 plays a similar role in GABAergic terminals. Re-examination of existing literature from this perspective and some recent data gathered in our laboratory permits some initial conclusions: (1) in physiological conditions, GABA homeostasis in GABAergic terminals is dominated by the activity of GADs; conceivably, GABA transported by GAT-1 contributes to the cytosolic stores of GABA; (2) analysis of GAT-1 KO, besides demonstrating the effects of reduced clearance, reveals the existence of changes (altered mIPSCs frequency; GADs and PAG up-regulation) compatible with an impaired presynaptic function; (3) notwithstanding the paucity of data, it appears safe to conclude that in GABAergic terminals GAT-1 impacts on presynaptic homeostasis, though less so than DAT, NET, and SERT in monoamine-releasing terminals; (4) these conclusions open new and interesting problems; among these, the following appear of some interest: Where and how is GAT-1-derived GABA compartmentalized in GABAergic terminals? What is the relationship between GAT-1 transported GABA and vesicle filling? How dynamic relationships between GABA taken up by GAT-1 and GABA derived from other sources are regulated in diverse physiological conditions, including activity-dependent plasticity? What is, at both the mechanistic and functional level, the link between GAT-1 and mechanisms regulating GABA release? How does the presynaptic role of GAT-1 contribute to the pathophysiology of neuropsychiatric diseases such as epilepsy and schizophrenia?

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GABA potency at $GABA_A$ receptors found in synaptic and extrasynaptic zones

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The potency of GABA is vitally important for its primary role in activating $GABA_A$ receptors and acting as an inhibitory neurotransmitter. Although numerous laboratories have presented information, directly or indirectly, on GABA potency, it is often difficult to compare across such studies given the inevitable variations in the methods used, the cell types studied, whether native or recombinant receptors are examined, and their relevance to native synaptic and extrasynaptic $GABA_A$ receptors. In this review, we list the most relevant isoforms of synaptic and extrasynaptic $GABA_A$ receptors that are thought to assemble in surface membranes of neurons in the central nervous system. Using consistent methodology in one cell type, the potencies of the endogenous neurotransmitter GABA are compared across a spectrum of $GABA_A$ receptors. The highest potency for GABA is measured when activating extrasynaptic-type α6 subunit-containing receptors, whereas synaptic-type α2β3γ2 and α3β3γ2 receptors exhibited the lowest potency, and other $GABA_A$ receptor subtypes that are found both in synaptic and extrasynaptic compartments, showed intermediate sensitivities to GABA. The relatively simple potency relationship between GABA and its target receptors is important as it serves as one of the major determinants of $GABA_A$ receptor activation, with consequences for the development of inhibition, either by tonic or phasic mechanisms.

Keywords: $GABA_A$ receptor, synaptic and extrasynaptic receptors, GABA, neurons

INTRODUCTION

The neurotransmitter γ-aminobutyric acid (GABA) targets $GABA_A$ and $GABA_B$ receptors which, in the mature central nervous system (CNS), provide the main basis for inhibitory neurotransmission and the subsequent integration of neuronal excitation (Luscher and Keller, 2004). When considering how effective an inhibitory GABAergic synapse will be in terms of reducing cell excitation, a number of factors are important. At the presynaptic terminal, this includes the GABA concentration and time profile in the synaptic cleft following GABA release. In addition, GABA overspill from inhibitory synapses to perisynaptic $GABA_A$ receptors will also be important. Postsynaptically, other factors come into consideration such as the subunit composition of $GABA_A$ receptors; the number and density of these receptors and their targeting to precise inhibitory synaptic compartments; and their residence time at synapses before endocytosis or lateral mobility causes them to exit the synaptic environment – these are all of equal significance (Moss and Smart, 2001; Jacob et al., 2008; Luscher et al., 2011). Many of the above factors will also be relevant to the effectiveness of extrasynaptic $GABA_A$ receptors in underpinning tonic inhibition.

One further factor that remains paramount to the effectiveness of GABA in activating specific isoforms of synaptic and extrasynaptic $GABA_A$ receptors is the potency of GABA. The activation of the receptor by GABA will depend on several factors, including the speed of the initial binding reaction, the potential for a shut but pre-activated receptor state, the final gating reaction that causes the channel to open, and the potential for the receptor to rapidly enter into one or more desensitized states. These factors will all impact on the potency of GABA to varying extents. Despite knowing that GABA can appear more potent on some receptor isoforms compared to others, what is currently lacking is a controlled and consolidated comparison, under identical conditions, of a series of receptor isoforms that are regarded as physiologically relevant to inhibitory synaptic and extrasynaptic $GABA_A$ receptors.

$GABA_A$ receptors are pentamers formed from a selection of 19 subunits: α(1–6), β(1–3), γ(1–3), δ, ε, θ, π, and ρ(1–3; Sieghart, 1995; Korpi et al., 2002). Although the potential for receptor diversity in neurons is considerable this is naturally contained by two principle factors: differential gene expression, whereby specific neuronal subtypes usually express a subset of $GABA_A$ receptor subunit genes (Wisden et al., 1992; Pirker et al., 2000); and the imposition of receptor subunit assembly rules that cause particular subunits to preferentially co-assemble (Taylor et al., 1999, 2000; Klausberger et al., 2000, 2001), e.g., α6 and δ subunits in cerebellar granule neurons (Jones et al., 1997). Despite the potential for receptor heterogeneity, the majority of $GABA_A$ receptors will contain two α subunits, two β subunits, and a γ subunit (Farrar et al., 1999). This is particularly relevant for synaptic αβγ $GABA_A$ receptors since a number of intracellular proteins have been shown to interact with these subunits regulating $GABA_A$ receptor transport to synaptic sites, their anchoring at synapses, turnover and degradation, and possibly assembly (e.g., α1–3 subunits and gephyrin, Tretter et al., 2008, 2011; Mukherjee et al., 2011; α, β subunits and Plic1, Bedford et al., 2001; β subunits and Hap1, Kittler et al., 2004; γ2 subunits and gephyrin and GABARAP, Wang et al., 1999;

Kneussel et al., 2000; and GODZ, Keller et al., 2004; see Luscher et al., 2011 for review). In addition, it should be noted that αβγ $GABA_A$ receptors also diffuse laterally in the surface membrane (Thomas et al., 2005; Triller and Choquet, 2005; Bogdanov et al., 2006), where they can also be found in significant numbers in extrasynaptic compartments of neurons (Kasugai et al., 2010).

$GABA_A$ receptors that are specifically considered to populate the extrasynaptic domain principally contain the δ subunit and often a specific α subunit, such as α4 (e.g., thalamic relay cells or dentate granule cells) or α6 (cerebellar granule cells; Sieghart and Sperk, 2002). However, extrasynaptic $GABA_A$ receptors are not restricted to those containing δ subunits as further evidence suggests that α5βγ and other αβγ receptors will be present in this domain (Thomas et al., 2005; Glykys et al., 2008). Moreover, αβ $GABA_A$ receptors can also form a constituent part of the extrasynaptic $GABA_A$ receptor population (Sieghart and Sperk, 2002; Mortensen and Smart, 2006). It is presently unclear whether homomeric $GABA_A$ receptors (e.g., β), apart from those containing ρ subunits, are expressed in significant numbers compared to more frequent αβγ and αβδ isoforms.

Ascertaining the most physiologically relevant $GABA_A$ receptors that are expressed in the CNS is not straightforward. Extensive *in situ* hybridization, immunocytochemical, and immunoprecipitation studies, using complementary DNA or RNA probes and subunit-selective antisera, together with transgenic mice, have been used to deduce the distribution profiles for the majority of individual $GABA_A$ receptor subunits (Wisden et al., 1992; Whiting et al., 1995; Pirker et al., 2000; Korpi et al., 2002). From such studies, $GABA_A$ receptor subunit compositions have been deduced with the aid of corroborating functional and pharmacological data. As a result, it is now possible, to tentatively list the likeliest native $GABA_A$ receptor subtypes that are expressed in the CNS (Sperk et al., 1997; Hutcheon et al., 2004; Olsen and Sieghart, 2008).

For native $GABA_A$ receptors *in situ*, one of the most important factors determining their functional response to released GABA is the potency of the neurotransmitter at specific receptor isoforms. Although over previous decades, some studies have examined the action of GABA in detail on a variety of $GABA_A$ receptor isoforms, some of which (e.g., α1β2γ2) are clearly relevant neuronal isoforms (Sigel et al., 1990; Verdoorn et al., 1990), these predate the wealth of immunocytochemical and immunoprecipitation data that is now available. These studies have modified our perception of physiologically relevant neuronal $GABA_A$ receptor isoforms. Here, we reappraise the potency of GABA at recombinant $GABA_A$ receptor isoforms designed to emulate the most prevalent $GABA_A$ receptors that are expressed in neuronal tissues, and also discuss the relative importance of the various subunits.

METHODS FOR ASSESSING GABA POTENCY

In providing an assessment of GABA potency, the relevant $GABA_A$ receptor isoforms can be conveniently expressed in heterologous expression systems such as *Xenopus* oocytes or human embryonic kidney cells (HEK293). Normally, HEK293, CHO, Ltk, and other such immortalized cell lines are preferred, not only because they efficiently accommodate protein assembly and cell-surface insertion, but also because of their smaller cell size compared with oocytes, where the speed of drug application can be compromised often leading to an underestimation of ligand potency. By using HEK cells, the $GABA_A$ receptors are not exposed to endogenous regulators such as neurosteroids or Zn^{2+} that may affect GABA potency and pH is closely controlled. It is possible that phosphorylation may alter GABA potency, but under basal conditions, where kinases are not specifically and directly activated, this is unlikely to be a confounding factor. Moreover, phosphorylation often involves a change in GABA current amplitude rather than an alteration to GABA sensitivity (e.g., Krishek et al., 1994).

HEK CELL CULTURE AND EXPRESSION OF RECOMBINANT $GABA_A$ RECEPTORS

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin-G, and 100 mg/ml streptomycin, and maintained at 37°C in a humidified 95% air/5% CO_2 atmosphere (Krishek et al., 1994; Wooltorton et al., 1997). Cells were transfected with equimolar ratios of cDNAs encoding for α1–6, β1–3, γ2S, δ, ε, and θ $GABA_A$ receptor subunits, representing the predominant $GABA_A$ receptor subunits expressed in the CNS.

WHOLE-CELL VOLTAGE-CLAMP ELECTROPHYSIOLOGY

Whole-cell GABA-activated and spontaneous currents were recorded from transfected HEK cells using patch clamp recording with electrodes filled with a solution containing (mM): 120 KCl, 1 $MgCl_2$, 11 EGTA, 30 KOH, 10 HEPES, 1 $CaCl_2$, and 2 K_2ATP; pH 7.2 with 1 M NaOH. The HEK cells were constantly superfused with a Krebs solution containing (mM): 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.52 $CaCl_2$, 11 Glucose, and 5 HEPES; pH 7.4. Membrane currents were recorded from voltage clamped cells at −60 mV, and routinely compensated for series resistance (Rs) of >70%, and filtered at 5 kHz. For assessing GABA potency on physiologically relevant $GABA_A$ receptor isoforms we used a U-tube fast drug application system (Mortensen and Smart, 2007). The recording parameters were designed to ensure near identical experimental conditions and thus valid comparative determinations of GABA potency.

MEASURING GABA POTENCY

This requires GABA concentration response relationships to be determined by normalizing GABA currents to the response induced by a maximal, saturating concentration of GABA (I_{max}) and subsequently curve fitting the data using the Hill equation:

$$I/I_{max} = \left(1/(1 + (EC_{50}/[A])^n\right),$$

where the GABA potency, EC_{50}, represents the concentration of the agonist ($[A]$) inducing 50% of the maximal current evoked by a saturating concentration of the agonist and n is the Hill coefficient. The potency of GABA can then be simply deduced from the relative EC_{50} values for each curve and potency ratios can also be derived from these data.

Given that dose response data are distributed on a logarithmic scale, EC_{50} values are converted to pEC_{50} values using: $pEC_{50} = -\log(EC_{50})$. Unlike EC_{50}s, the pEC_{50} values are distributed on a linear scale from which mean ± SEM values can be obtained. To facilitate data interpretation, mean pEC_{50} values can

be transformed into EC_{50} values. The potency histograms shown in this review depict left ordinate axes corresponding to mean pEC_{50} values ± SEM, and right ordinate logarithmic axes for EC_{50} values (note that the error bars only relate to pEC_{50}).

Finally, some receptor subunit combinations can exhibit spontaneous channel activity in the absence of GABA. To determine the level of spontaneous activity of, for example, ε subunit-containing receptors, the maximal inhibition of spontaneous channel activity was observed as a decrease in the membrane holding current in the presence of a saturating concentration of the allosteric $GABA_A$ receptor blocker, picrotoxin (1 mM; $I_{PTX,\,Max}$). This was quantified by dividing $I_{PTX,\,Max}$ by the total range of GABA channel activity ($I_{PTX,\,Max} + I_{GABA,\,Max}$), according to the following ratio:

$$\%\ \text{spontaneous activity} = I_{PTX,Max} / \left(I_{PTX,Max} + I_{GABA,Max}\right),$$

where $I_{GABA,\,Max}$, is the maximal current activated by a saturating concentration of GABA at the same spontaneously opening receptors (Mortensen et al., 2003).

RESULTS

COMPARISON OF GABA POTENCY ON $GABA_A$ RECEPTOR α-SUBUNITS

All six α subunits were sequentially expressed with β3 and γ2 subunits to compare GABA potency by determining the GABA EC_{50}s. GABA concentration response curves have been established by measuring whole-cell currents (**Figure 1Aa**) for a range of GABA concentrations on α1–6 subunit-containing receptors

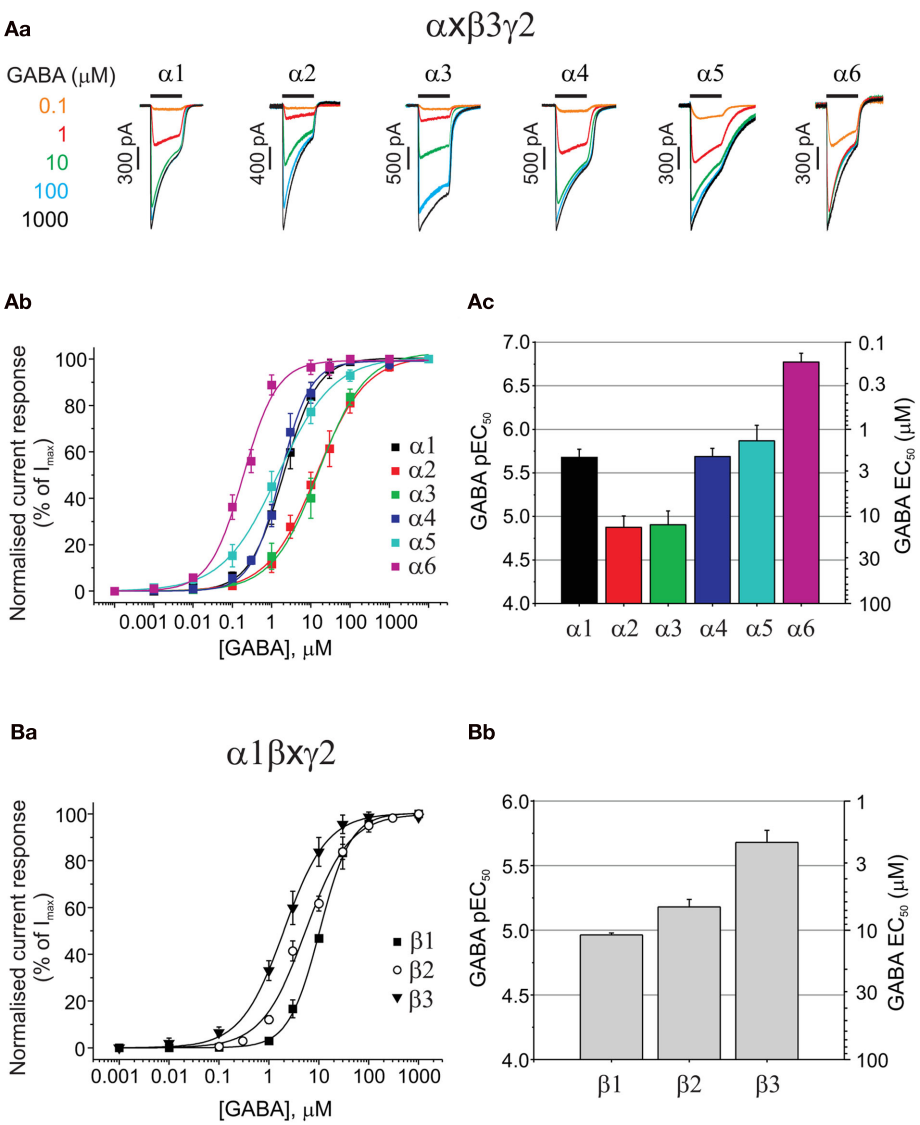

FIGURE 1 | Assessing the impact of α, and β $GABA_A$ receptor subunits on GABA potency. (Aa) Superimposed membrane currents activated by GABA concentrations ranging from 0.1 μM to 1 mM for α1–6β3γ2 $GABA_A$ receptors expressed in HEK293 cells. Note the slow desensitization for α3β3γ2 and slow deactivation for α5β3γ2. **(Ab)** GABA concentration response curves for α1–6β3γ2. Points are mean ± SEM (5–7 cells). **(Ac)** Bar chart of mean GABA pEC_{50} values ± SEM (left ordinate) and equivalent EC_{50} values (right ordinate) for α1–6β3γ2 receptors. **(Ba)** Displays GABA concentration response curves for α1β1–3γ2 receptors ($n = 5$–34 cells), and **(Bb)** depicts the pEC_{50} values (mean ± SEM) and the equivalent EC_{50} values (mean).

(**Figure 1Ab**). It is apparent from the EC_{50} values for each isoform that the α-subunit-containing receptors form three distinct groups, with GABA exhibiting its lowest potency at α2 and α3 containing receptors (EC_{50}: 13.4 and 12.5 μM, respectively; **Figure 1Ac**; **Table 1**), increasing to an intermediate potency for activating α1, α4, and α5-containing receptors (2.1, 2.1, and 1.4 μM, respectively; **Figure 1Ac**; **Table 1**), with the highest potency measured for α6 subunit-containing receptors (0.17 μM; **Figure 1Ac**; **Table 1**). The difference in potency between α2/3- and α6-containing receptors is ∼80-fold.

Although in such experiments GABA is usually delivered to single $GABA_A$ receptor expressing cells with a latency of 20–30 ms, it is clear from the current profiles that the rate of desensitization was not simply related to GABA potency as both α2 (low GABA potency) and α6 (high potency) receptor isoforms showed relatively fast current desensitization, whilst α3 (low potency) exhibited the slowest desensitization kinetics (**Figure 1Aa**). By comparing between α subunit isoforms, it is clear that only α5 showed a dramatically slow current deactivation (**Figure 1Aa**). All these recombinant $GABA_A$ receptor isoforms (and others reviewed below) that incorporate the γ2 subunit, display robust expression in HEK293 cells with maximal GABA currents in the range of 2–4 nA (**Table 1**) without exhibiting any spontaneous activity.

GABA BINDS MOST TIGHTLY TO SYNAPTIC-TYPE β3 SUBUNIT-CONTAINING RECEPTORS

The importance of the β subunit (1–3) for GABA potency has been examined in receptors co-expressing α1 and γ2 (**Figures 1Ba,b**). GABA EC_{50} values for α1β1γ2 (10.9 μM), α1β2γ2 (6.6 μM), and α1β3γ2 (2.1 μM) were significantly different (ANOVA, $P = 0.0022$), with the β3-containing isoform being the most sensitive to GABA. Membrane current profiles were similar and all isoforms showed robust expression in HEK cells after only 14–18 h (GABA I_{max} values (pA) for α1β1γ2: 3575 ± 799, α1β2γ2: 2230 ± 193, and α1β3γ2: 3367 ± 662; **Table 1**). A similar rank order of GABA potency has been observed from comparative expression studies of human $GABA_A$ receptor constructs expressed in *Xenopus* oocytes, although the EC_{50} values were higher overall by ∼2–3-fold (Hadingham et al., 1993).

GABA POTENCY AT α4 AND α6 SUBUNIT-CONTAINING $GABA_A$ RECEPTORS

Some of the most abundant extrasynaptic $GABA_A$ receptors are formed from α4βδ (Jia et al., 2005; Belelli et al., 2009) and α6βδ subtypes (Farrant and Nusser, 2005). There is also evidence in support of extrasynaptic α4β and α6β receptors in the CNS (Bencsits et al., 1999; Sinkkonen et al., 2004) as well as synaptic and/or extrasynaptic α4βγ and α6βγ $GABA_A$ receptors (Quirk et al., 1994; Peng et al., 2004). Whereas α4-containing receptors have a wide distribution throughout the brain, α6 subunits are exclusively expressed in cerebellar granule cells and the cochlear nucleus (Pirker et al., 2000). GABA EC_{50} values for α4β3 (0.97 μM), α4β3γ2 (2.1 μM), and α4β3δ (1.7 μM) $GABA_A$ receptors displayed similar sensitivities to GABA, although GABA is slightly more potent in activating α4β3 compared to α4β3γ2 ($P = 0.0172$; **Figure 2A**; **Table 1**). Similarly, for α6-containing receptors, GABA

Table 1 | GABA potencies and maximum currents.

Isoform	Cellular location	Main brain areas/cell types	GABA pEC_{50} (EC_{50})	GABA max currents (pA)
α1β3γ2S	S/(E)	Widespread in the brain	5.679 ± 0.0932 (5), 2.1 μM	3367 ± 662 (5)
α2β3γ2S	S/(E)	Widespread	4.874 ± 0.1308 (5), 13.4 μM	3056 ± 435 (5)
α3β3γ2S	S/(E)	Reticular thalamic nucleus, hypothalamic nuclei, dentate granule cells, noradrenergic cells in locus coeruleus	4.904 ± 0.1592 (5), 12.5 μM	3776 ± 305 (5)
α4β3γ2S	S/(E)	Thalamic relay cells (weak)	5.689 ± 0.0930 (5), 2.1 μM	2574 ± 292 (8)
α5β3γ2S	E/S	Hippocampal pyramidal cells	5.869 ± 0.1782 (7), 1.4 μM	2642 ± 938 (5)
α6β3γ2S	(S)/E	Cerebellar granule cells, cochlear nucleus granule cells	6.772 ± 0.1034 (5), 0.17 μM	2446 ± 445 (5)
α1β1γ2S	S/(E)	Restricted distribution	4.965 ± 0.0149 (5), 10.9 μM	3575 ± 799 (5)
α1β2γ2S	S/(E)	Widespread and most abundant	5.180 ± 0.0593 (34), 6.6 μM	2230 ± 193 (18)
α4β3	E	Thalamic relay cells	6.014 ± 0.0559 (5), 0.97 μM	328 ± 67 (5)
α4β3δ	E	Thalamic relay cells	5.776 ± 0.1147 (5), 1.7 μM	1224 ± 264 (7)
a6β3	E	Cerebellar granule cells	7.122 ± 0.0954 (5), 0.076 μM	490 ± 125 (5)
α6β3δ	E	Cerebellar granule cells	6.760 ± 0.1174 (5), 0.17 μM	706 ± 148 (5)
α1β2	E	Widespread distribution	5.771 ± 0.0624 (5), 1.7 μM	1863 ± 333 (5)
a3β3	E	Thalamus, hypothalamus, locus coeruleus	5.346 ± 0.0556 (5), 4.5 μM)	3924 ± 288 (6)
α1β2δ	E	Hippocampal interneurons	5.430 ± 0.0738 (5), 3.7 μM	398 ± 147 (5)
α4β2δ	E	Hippocampal dentate granule cells	6.040 ± 0.1227 (5), 0.91 μM	1544 ± 263 (5)
a3β3θ	E	Hypothalamic nuclei, locus coeruleus	5.473 ± 0.0886 (5), 3.4 μM	1680 ± 508 (5)
a3β3ε	E	Hypothalamic nuclei, locus coeruleus	6.064 ± 0.0738 (5), 0.86 μM	811 ± 300 (5)

GABA potency data for isoforms of the $GABA_A$ receptor representing the most likely subtypes found in the CNS. Their putative cellular locations are indicated (S, synaptic, or E, extrasynaptic; and when this is noted in parentheses, the location and/or distribution is assumed based on the available literature). GABA potency is represented as a pEC_{50} (mean ± SEM) and the number of experiments (n) is shown in parentheses. For easier interpretation, mean pEC_{50} values are also transformed into EC_{50} values. Mean maximum currents are provided as mean ± SEM.

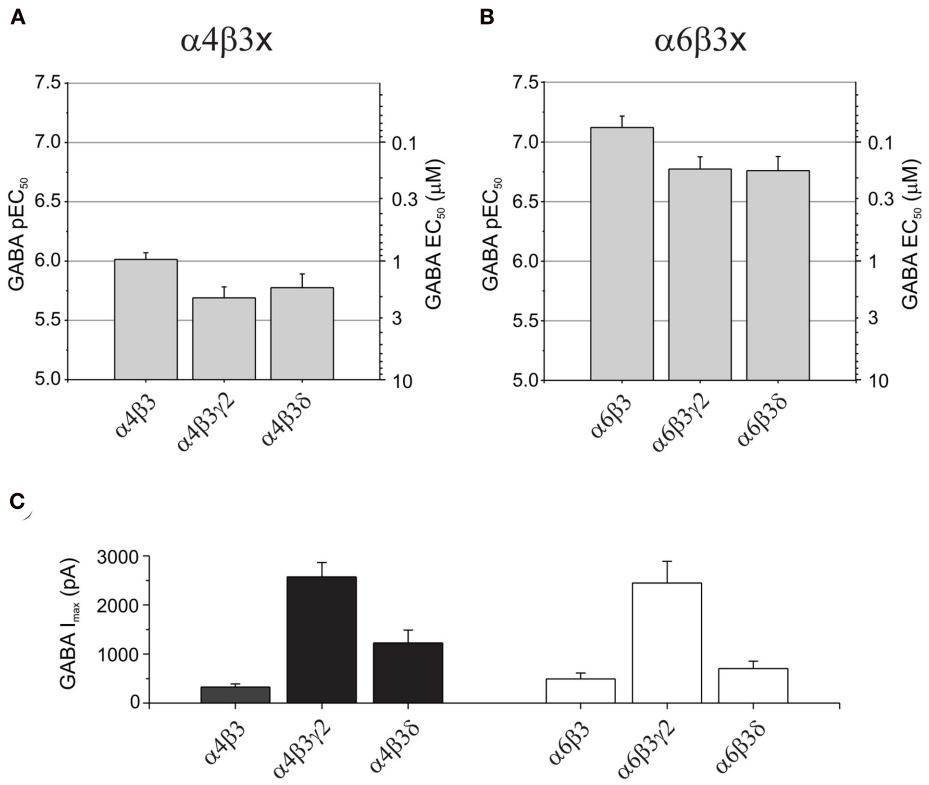

FIGURE 2 | GABA potencies and maximum currents at α4- and α6-containing $GABA_A$ receptors. Bar charts showing pEC_{50} (mean ± SEM) and EC_{50} (mean) values for: **(A)** α4β3, α4β3γ2, and α4β3δ, and **(B)** α6β3, α6β3γ2, and α6β3δ $GABA_A$ receptors. Note that α6-containing receptors consistently display a higher potency for GABA than α4-containing receptors. **(C)** Bar charts of the maximum GABA-induced currents (mean ± SEM) for α4- and α6-containing $GABA_A$ receptors expressed in HEK293 cells.

is again more potent at α6β3 (EC_{50}: 0.076 μM) than either α6β3γ2 (0.17 μM; $P = 0.0377$) or α6β3δ (0.17 μM; $P = 0.0437$; **Figure 2B**; **Table 1**).

Interestingly, by comparing α4- with α6-containing receptors, GABA consistently exhibited a higher potency at α6-containing receptors ($P = 0 < 0.001$; **Figures 2A,B**; **Table 1**). Furthermore, α4β3γ2 or α6β3γ2 receptors also showed significantly higher expression levels compared appropriately with either α4β3 and α6β3 or α4β3δ and α6β3δ receptors ($P < 0.01$; **Figure 2C**; **Table 1**).

GABA POTENCY AT EXTRASYNAPTIC-TYPE αβ RECEPTORS

In addition to α4 and α6 subunit-containing receptors, there is now evidence supporting the existence of αβ $GABA_A$ receptors at extrasynaptic locations in cerebellar granule cells and hippocampal pyramidal cells (Brickley et al., 1999; Mortensen and Smart, 2006). The genes encoding for α1, β2, and γ2 subunits are clustered on human chromosome 5q34 (mouse: 11, rat:10; Simon et al., 2004) and following their co-expression, could be one reason why α1β2γ2 receptors are one of the most abundant $GABA_A$ receptor isoforms in CNS neurons (Sieghart, 1995; McKernan and Whiting, 1996; Mehta and Ticku, 1999), and potentially why α1β2 receptors can be assembled and inserted in extrasynaptic membrane compartments in the same neurons and brain regions as α1β2γ2 receptors. However, gene clusters are not absolute predictors of receptor subunit combinations since the α6 subunit gene, *GABRA6*, also clusters with those for α1, β2, and γ2 (Simon et al., 2004) yet has a much more restricted expression profile.

In noradrenergic locus coeruleus cells and hypothalamic and thalamic nuclei, α3, θ, and ε subunits are expressed (Sinkkonen et al., 2000; Pape et al., 2009) and it is possible that extrasynaptic α3β2/3 receptors form in these brain areas. In addition, there is indeed evidence for α4β3 (Bencsits et al., 1999) and α6β3 isoforms (Sinkkonen et al., 2004) being present extrasynaptically in thalamic and cerebellar neurons respectively, where α4β3δ and α6β3δ are also expressed.

When comparing the potency of GABA in activating these αβ receptors, GABA was least potent at α3β3 (4.5 μM, $P < 0.01$), exhibited intermediate potency at α1β2 (1.7 μM) and α4β3 (0.97 μM), and displayed the highest potency at α6β3 receptors (0.076 μM, $P < 0.001$; **Figures 3Aa,b**; **Table 1**).

Curiously, the maximum GABA currents obtained with α4β3 (328 ± 67 pA) and α6β3 (490 ± 125 pA) receptors were significantly smaller than those obtained with α1β2 (1863 ± 333 pA) and in particular with α3β3 (3924 ± 288 pA, $P < 0.01$; **Table 1**).

COMPARISON OF GABA POTENCIES AT EXTRASYNAPTIC-TYPE δ-CONTAINING RECEPTORS

The δ-containing receptors are generally considered to be extrasynaptic. By contrast with the limited distribution of α6βδ receptors

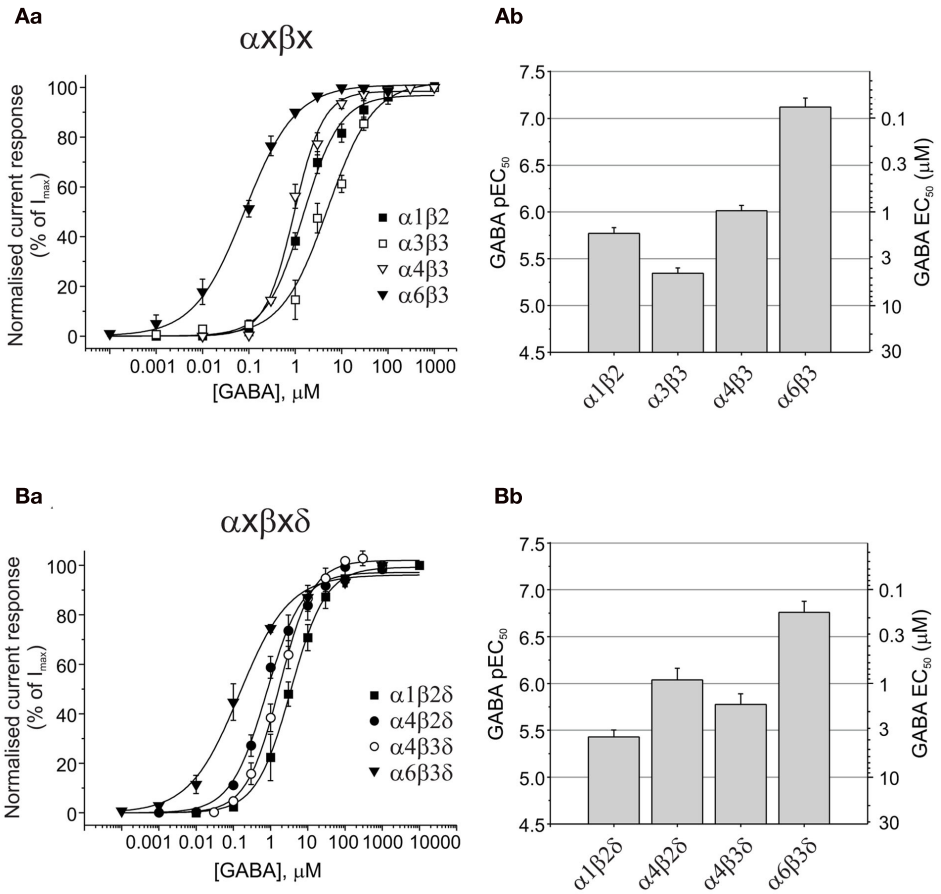

FIGURE 3 | GABA potencies at αβ and αβδ $GABA_A$ receptors. (Aa) GABA concentration response curves for α1β2, α3β3, α4β3, and α6β3 ($n = 20$). **(Ab)** Bar chart of pEC_{50} (mean ± SEM) and equivalent EC_{50} values for αβ receptors. **(Ba)** GABA concentration response curves for α1β2δ, α4β2δ, α4β3δ, and α6β3δ ($n = 20$). **(Bb)** Bar chart of pEC_{50} (mean ± SEM) and equivalent EC_{50} values for αβδ $GABA_A$ receptors expressed in HEK293 cells.

in cerebellar granule cells (Jechlinger et al., 1998), α4βδ is found in dentate granule cells and thalamic relay cells, and also expressed at lower levels in striatum and the cerebral cortex (Pirker et al., 2000). Current evidence indicates that the α4β2δ isoform predominates in dentate granule cells (Herd et al., 2008), whereas α4β2δ and α4β3δ are found in thalamic relay cells (Pirker et al., 2000). A novel δ-containing $GABA_A$ receptor, α1βδ, has also been proposed as a naturally expressed extrasynaptic receptor in hippocampal interneurons (Glykys et al., 2007).

GABA EC_{50} values revealed a higher potency for GABA at α6β3δ receptors [EC_{50}: 0.17 μM compared to α1β2δ (3.7 μM), α4β2δ (0.91 μM), and α4β3δ (1.7 μM); ANOVA: $P < 0.0001$; **Figures 3Ba,b**; **Table 1**]. GABA potency at α1β2δ was also significantly lower than that at α4β2δ and α4β3δ $GABA_A$ receptors ($P = 0.0028$ and $P = 0.0349$, respectively). The largest GABA I_{max} currents were observed for α4β2δ and α4β3δ (1544 ± 263 and 1224 ± 264 pA, respectively), which were significantly higher than those observed for α1β2δ (398 ± 147 pA; $P < 0.05$); α4β2δ also displayed higher currents than α6β3δ (706 ± 148 pA, $P = 0.024$; **Table 1**).

GABA POTENCIES AT α3β3 SUBUNIT-CONTAINING RECEPTORS WITH γ2, θ, OR ε

The potential co-expression of α3, θ, and ε subunits in noradrenergic cells of the locus coeruleus and also hypothalamic nuclei (primarily ventromedial and dorsomedial; Sinkkonen et al., 2000), further suggests the existence of neuronal $GABA_A$ receptor isoforms such as α3β3, α3β3γ2, α3β3θ, and α3β3ε. In accord with our previous observations of GABA displaying a higher potency at αβ compared to αβγ receptors, the GABA EC_{50} for α3β3 (4.5 μM) was significantly higher than that for α3β3γ2 (12.5 μM; $P = 0.0306$; **Figures 4A,B**; **Table 1**).

The mean GABA EC_{50} for α3β3θ (3.4 μM) was not significantly different from that determined with the α3β3 (4.5 μM) isoform, which could have been due to the θ subunit not being incorporated efficiently into the α3β3θ receptor. However, from the maximal GABA currents, it was clear that I_{max} was significantly reduced for α3β3θ compared with α3β3, indicative of θ being assembled into the receptor (1680 ± 508 and 3924 ± 288 pA, respectively; $P = 0.003$; **Figure 4C**; **Table 1**). For this group of receptors, GABA had the highest potency at α3β3ε (EC_{50}: 0.86 μM; $P < 0.01$; **Figures 4A,B**). Similarly to α3β3θ, the GABA I_{max} for

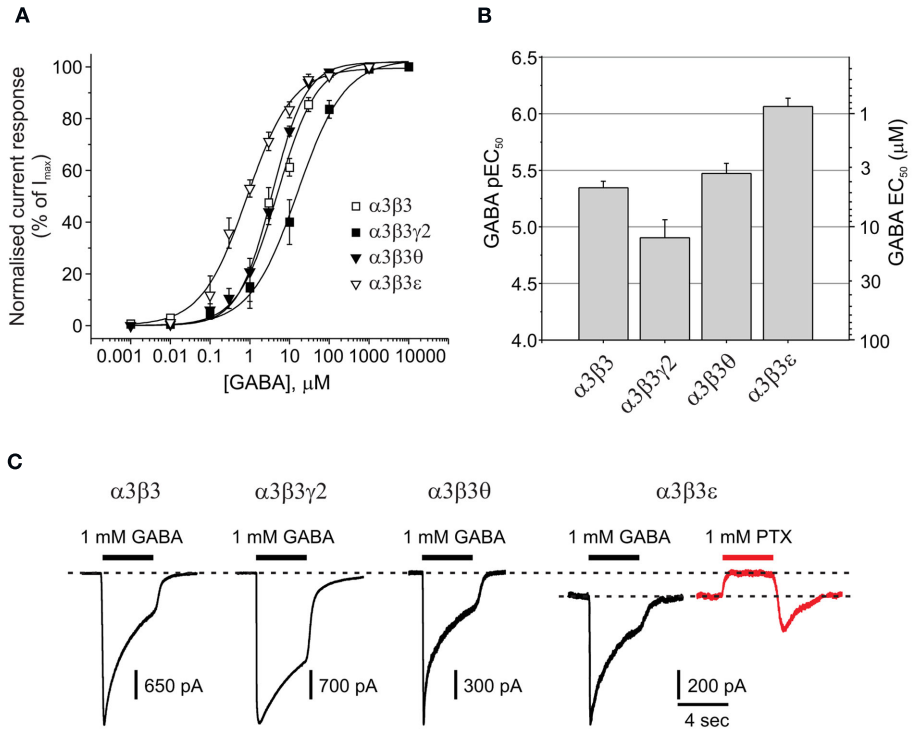

FIGURE 4 | Impact of θ or ε on GABA potency and spontaneous channel opening. (A) GABA concentration response curves for α3β3, α3β3γ2, α3β3θ, and α3β3ε $GABA_A$ receptors expressed in HEK293 cells ($n = 20$). **(B)** Bar chart of pEC_{50} (mean ± SEM) and EC_{50} values for α3β3x receptors. **(C)** Membrane currents activated by 1 mM GABA for α3β3, α3β3γ2, α3β3θ, and α3β3ε (black traces), including an example of outward current (red trace) generated by picrotoxin (PTX) blocking the spontaneous current of α3β3ε in the absence of GABA.

α3β3ε (811 ± 300 pA) was also reduced compared with that for α3β3 and α3β3γ2 ($P < 0.001$).

Of all the $GABA_A$ receptors that have been reviewed, the only isoform that displayed significant spontaneous channel activity in the absence of GABA was α3β3ε receptors. This was blocked by 1 mM picrotoxin (PTX; **Figure 4C**). The PTX-sensitive spontaneous current accounted for 24 ± 5% ($n = 8$) of the maximum total current that could be passed by these receptors.

DISCUSSION

The purpose of this review has been to consider the comparative potency data for GABA in activating 18 of the most likely isoforms of the $GABA_A$ receptor to be expressed in the CNS (Olsen and Sieghart, 2008). This catalog of neuronal $GABA_A$ receptor isoforms was accrued from *in situ* hybridization (Laurie et al., 1992; Wisden et al., 1992), immunocytochemical (Fritschy and Mohler, 1995; Pirker et al., 2000; Fritschy and Brunig, 2003), and immunoprecipitation (Khan et al., 1994) data, with supporting evidence from electrophysiological studies (Whiting et al., 1995; McKernan and Whiting, 1996). Of necessity however, the composition of the less common neuronal $GABA_A$ receptor isoforms is still subject to speculation given the difficulties of precisely determining native receptor subunit composition.

It is apparent that significant variations in potency can occur when comparing the same ligand against receptors expressed in different cell types. In particular, potencies measured with receptors expressed in *Xenopus* oocytes have yielded results that differ from similar determinations conducted in mammalian cell types (e.g., HEK293, COS, and NG108-15 cells). Variable determinations of potency can also arise from using different DNA transfection ratios, which might influence receptor subunit composition, and by using different speeds of drug application. This makes exact comparison of EC_{50} values from different isoforms of the receptor more difficult if they are not measured under exactly the same experimental conditions. In this review, we have ensured that experimental conditions are consistent, thereby enabling exact comparisons between neuronally relevant $GABA_A$ receptor isoforms.

Previous extensive and comparative studies of the effects of GABA and other $GABA_A$ receptor specific ligands on a range of $GABA_A$ receptor isoforms have been conducted on oocytes (Sigel et al., 1990; Ebert et al., 1994), but since this period, our understanding of $GABA_A$ receptor assembly and naturally occurring isoforms in the CNS has changed considerably.

Xenopus oocytes offer a robust expression system perfectly suited for screening the pharmacology of multiple receptor isoforms; however, due to their large size, drug application speeds are often slower than expected with mammalian cells, which may be the most likely reason why EC_{50} values from oocyte studies for a specific receptor isoform are usually notably higher than those determined with smaller immortalized cell lines (e.g., HEK293) more akin to a neuron (Verdoorn et al., 1990). Indeed, the EC_{50}

values, noted in this review obtained from HEK293 cells were consistently lower than those reported from oocyte studies.

It is clear from appraising GABA potency on different $GABA_A$ receptor isoforms that the identity of the α-subunit is most important with an almost 80-fold difference in EC_{50} values from the low potency α2β3γ2 and α3β3γ2 receptors, to the high potency isoform, α6β3γ2. The low potency of α2/α3βγ receptors would be suited to inhibitory synaptic compartments where the GABA concentration transient in the synaptic cleft will rise to >1 mM during vesicular release (Mozrzymas et al., 2003). For extrasynaptic receptors that are likely to be exposed to much lower basal and spillover GABA concentrations of ∼20–500 nM (Mortensen and Smart, 2006; Lee et al., 2010), a higher GABA potency is advantageous. The high potency of α6β3γ2 indicates that this isoform may be mainly located extrasynaptically in cerebellar granule cells and the cochlear nucleus, similar to α6βδ, although α6 subunit-containing receptors are also reported to access inhibitory synapses (Tia et al., 1996; Mellor et al., 2000; Santhakumar et al., 2006).

Interestingly, α1-, α4-, and α5-containing receptors assembled with βγ subunits displayed intermediate potencies for GABA suggesting these receptors could participate equally at inhibitory synapses as well as in extrasynaptic compartments. It has been previously demonstrated that α1βγ receptors are also located in extrasynaptic domains (Thomas et al., 2005). Similarly, it has been shown that α5-containing $GABA_A$ receptors play an important part in tonic inhibition in hippocampal pyramidal neurons, and that these can also contribute to synaptic inhibition (Caraiscos et al., 2004; Serwanski et al., 2006). By contrast, α4βδ receptors are regarded as important extrasynaptic receptors in the thalamus and the dentate gyrus, and there is also evidence for an α4βγ isoform, which has been reported to be located both within and outside inhibitory synapses (Peng et al., 2004).

Previous evidence suggests that extrasynaptic αβ receptors are present on hippocampal pyramidal cells and play a part (∼10%) in tonic inhibition (Mortensen and Smart, 2006). The presence of α6β receptors has similarly been observed in Thy1α6 transgenic mice with ectopic α6-expression, outside of the cerebellum (Sinkkonen et al., 2004). Although this is an abnormal expression pattern, it indicates that α6β receptors have the ability to be expressed and may, under normal conditions, be present in cerebellar granule cells where the expression of α6 is high. Similarly, there is evidence for the presence of α4β receptors in the brain (Bencsits et al., 1999) and this suggests the possibility that various αβ isoforms may be present in other brain areas could have been previously underestimated (e.g., α1β2 throughout the CNS, α4β2/3 in thalamus and dentate gyrus, α3β3 in the hypothalamus and locus coeruleus, and α6β2/3 in the cerebellum). The observation that αβ always displays a higher GABA potency than its αβγ counterpart, underlines the potential value of these γ-lacking αβ as extrasynaptic receptors, helping to set the level of tonic inhibitory tone. However, it is expected that their single channel conductances will be smaller compared with αβγ or αβδ counterparts (Moss et al., 1990; Angelotti and MacDonald, 1993; Mortensen and Smart, 2006).

Typically, prevalent forms of extrasynaptic $GABA_A$ receptors are those containing the δ-subunit that populate the dentate gyrus (α4β2δ), thalamus (α4β2δ and α4β3δ), and cerebellar granule cells (α6βδ). In addition, α1βδ receptors may also be expressed in the dentate gyrus (Glykys et al., 2007). Comparing the four δ-containing isoforms, GABA potency was highest at α6β3δ compared to α1β2δ, α4β2δ, and α4β3δ. These potency differences may reflect regional differences in ambient GABA concentrations, where the highly GABA sensitive α6β3δ would be ideally suited to an environment where the GABA concentration was lower.

The less abundant $GABA_A$ receptor subunits, ε and θ, which have defined expression patterns in the locus coeruleus, hypothalamus, tegmentum, and pontine nuclei, have been proposed to assemble as α3βε and α3βθ isoforms due to the co-localization of α3, ε, and θ on the same chromosome (Pape et al., 2009). These receptors have not previously been subject to a full characterization, but their relatively high potency for GABA (in particular α3β3ε) indicates that they would also be likely contributors to tonic inhibition and thus candidates for being located at extrasynaptic sites.

Of all the 18 $GABA_A$ receptor isoforms reviewed in this study, only α3β3ε receptors showed significant spontaneous activity. Spontaneous activity has been reported before for α1β3ε (Neelands et al., 1999; Mortensen et al., 2003) suggesting that the ε subunit is mainly responsible for spontaneity given that the α1 or β3 subunits do not impart this profile onto α1β3γ2 receptors.

In summary we have observed differences in GABA potency ranging up to 175-fold between $GABA_A$ receptor isoforms with GABA being most potent at extrasynaptic α6-containing receptors and least potent at synaptic-type α2β3γ2 and α3β3γ2 receptors. This range of GABA potency will clearly impact on the activation of $GABA_A$ receptors and influence the roles they play in controlling excitability from either synaptic or extrasynaptic locations.

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$GABA_A$ receptor β3 subunit expression regulates tonic current in developing striatopallidal medium spiny neurons

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The striatum is a key structure for movement control, but the mechanisms that dictate the output of distinct subpopulations of medium spiny projection neurons (MSNs), striatonigral projecting and dopamine D1 receptor- (D1+) or striatopallidal projecting and dopamine D2 receptor- (D2+) expressing neurons, remains poorly understood. GABA-mediated tonic inhibition largely controls neuronal excitability and action potential firing rates, and we previously suggested with pharmacological analysis that the $GABA_A$ receptor β3 subunit plays a large role in the basal tonic current seen in D2+ MSNs from young mice (Ade et al., 2008; Janssen et al., 2009). In this study, we demonstrated the essential role of the β3 $GABA_A$ receptor subunit in mediating MSN tonic currents using conditional β3 subunit knock-out (β3f/f^{Drd2}) mice. Cre-lox genetics were used to generate mice where Cre recombinase was expressed under the D2 receptor (Drd2) promoter. We show that while the wild-type MSN tonic current pattern demonstrates a high degree of variability, tonic current patterns from β3f/f^{Drd2} mice are narrow, suggesting that the β3 subunit is essential to striatal MSN GABA-mediated tonic current. Our data also suggest that a distinct population of synaptic receptors upregulate due to β3 subunit removal. Further, deletion of this subunit significantly decreases the D2+ MSN excitability. These results offer insight for target mechanisms in Parkinson's disease, where symptoms arise due to the imbalance in striatal D1+ and D2+ MSN excitability and output.

Keywords: GABA, tonic inhibition, striatum, patch-clamp, Cre-lox genetics

INTRODUCTION

Medium spiny neurons (MSNs) are GABAergic projection neurons of the striatum, and project either to the substantia nigra pars reticulata though a direct pathway or the globus pallidus through an indirect pathway (Gerfen et al., 1990). MSNs of the direct pathway express the dopamine D1 receptors (D1+); their activity is thought to facilitate movement. Dopamine D2 receptors (D2+) are expressed on indirect pathway MSNs that inhibit movement initiation.

Proper motor control relies on a complex balance of D1+ and D2+ MSN excitation. Further, MSN excitability and synchrony is thought to be altered in dopamine-depleted animal models of Parkinson's disease (Costa et al., 2006; Fino et al., 2007; Azdad et al., 2009; Burkhardt et al., 2009; Jáidar et al., 2010). Although overly simplified, it is thought that D2+ MSNs experience increased excitability in the disease state (Day et al., 2006, 2008; Mallet et al., 2006; Shen et al., 2007; Tian et al., 2010), while D1+ MSNs show little changes (Day et al., 2006; Tian et al., 2010). Indeed, optogenetic activation of striatal D2+ MSNs *in vivo* mimicked Parkinson's disease with decreased movement initiation and increased freezing behavior (Kravitz et al., 2010). Thus, the intrinsic mechanisms that control MSN output are of crucial interest in understanding the underlying factors that mediate altered neuronal excitability.

GABAergic inhibition is mediated by a combination of fast, phasic inhibition and a slower, tonic inhibition. Synaptic release of neurotransmitter mediates phasic inhibition through low-affinity synaptic $GABA_A$ receptors and is effective in generating neuronal rhythmic activity and synchronicity (Cobb et al., 1995). Tonic inhibition, due to GABA spillover, is mediated through high-affinity receptors located in the extrasynaptic space, and increases a cell's input conductance, affecting neuronal excitability (Brickley et al., 1996). Therefore, the subunits that mediate striatal MSN tonic inhibition are of crucial interest in understanding the underlying factors that control MSN output and altered neuronal excitability. Our previous study used pharmacology to identify the $GABA_A$ receptor β3 subunit as an important regulator of both striatal D1+ and D2+ MSN tonic current (Janssen et al., 2009).

In general, β3 subunit knock-out (KO) mice show an increase in seizure activity and serve as an animal model for Angelman's syndrome (DeLorey et al., 1998). Studies from global β3 subunit KO mice have illustrated the subunit's importance in synaptic transmission and oscillatory behavior or synchronicity (Huntsman et al., 1999; Nusser et al., 2001; Hentschke et al., 2009). Cortical cultures from β3 subunit KO animals further showed that this subunit is crucial for synaptic inhibition (Ramadan et al., 2003). To our knowledge, no study has investigated the role of the β3 subunit in striatal inhibition using a genetic approach. Therefore, we investigated the role of β3 $GABA_A$ receptor subunits on striatal MSN phasic and tonic currents by selectively excising the subunit from D2-expressing neurons (Drd2-Cre; Gong et al., 2007; i.e.,

striatal D2+ MSNs) using the Cre-lox system. This conditional KO approach allowed us to more accurately determine the importance of this subunit without more global compensatory mechanisms.

We found that the β3 $GABA_A$ receptor subunit is fundamental in both phasic and tonic inhibition as miniature inhibitory post-synaptic current (mIPSC) decay kinetics and tonic current were significantly altered in conditional KO animals. In addition, deletion of this subunit significantly decreased the excitability of MSNs, suggesting the $GABA_A$ receptor β3 subunit may be an important pharmacological target in the treatment of striatal disorders.

MATERIALS AND METHODS

ANIMALS

Bacterial artificial chromosome (BAC) mice that identify the indirect striatal output pathway (BAC-D2-EGFP; Gong et al., 2003) were crossed with BAC mice that identify the direct striatal output pathway (Drd1a-tdTomato; Shuen et al., 2008) so progeny (BAC-D2-EGFP; Drd1a-tdTomato) contained fluorescent markers for simultaneous visualization of both indirect and direct pathways.

Conditional β3 subunit knock-out (KO) mice were produced by crossing floxed β3 mice (β3f/f; Jackson Labs #008310; Ferguson et al., 2007) with transgenic mice that expressed Cre recombinase under the Drd2 promoter (GENSAT, ER44; Gong et al., 2007) to yield β3f/$+^{Drd2}$ mice. These mice were then crossed with β3f/f mice to generate β3f/f, β3f/$+^{Drd2}$, and β3f/f^{Drd2} mice. To determine the integrity of the Drd2-Cre expression, Drd2-Cre mice were crossed with a Cre reporter mouse expressing tdTomato (ROSA-tdTomato, Jackson Labs #007914; Madisen et al., 2010). Parvalbumin-expressing interneurons (zolpidem control experiments) were identified by a cross between Parv-Cre (Jackson Labs #008069; Murray et al., 2011) and ROSA-tdTomato reporter mice. All mice were genotyped through standard PCR procedures. The following primers were used to genotype tail DNA: *Cre* – F5′-GGATGAGGTTCGCAAGAACC-3′, R5′-CCATGAGTGAACGAACCTGG-3′ with a PCR product at 400 bp; *Floxed*β*3* – F5′-ATTCGCCTGAGACCCGACT-3′, R5′-GTTCATCCCCACGCAGAC-3′ with PCR products of ∼250 bp for wild-type β3 subunit allele and ∼300 bp for the floxedβ3 allele.

IMMUNOHISTOCHEMISTRY

Progeny of mouse matings between Drd2-Cre and ROSA-tdTomato reporter mice were used to assess the fidelity of the Drd2-Cre expression. Whole mouse brains (∼18 days) were fixed and coronally sliced at 100 μm using a Lancer Vibratome (Series 1000, Sherwood Medical, St. Louis, MO, USA). Free-floating tissue sections were blocked with 4% normal donkey serum in PBS for 1 h at room temperature and washed for 30 min in PBS/0.1% Triton-X100 (Tx). Rabbit α-DARPP-32 (19A3, Cell Signaling) 1° antibody (1:200) was diluted in PBS/Tx/1% BSA and slices were incubated at room temperature for approximately 18 h. Slices were washed for 30 min in PBS/Tx. Goat α-rabbit 2° antibody conjugated to FITC (Invitrogen, Carlsbad, CA, USA) was diluted 1:500 in PBS/Tx/BSA and exposed to tissue for 1.5 h at room temperature. The 2° antibody was washed with PBS/Tx, and sections were placed on microscope slides with VECTASHIELD H-1000 mounting media (Vector Labs, Burlingame, CA, USA) and sealed with glass coverslips. For imaging, a Nikon Eclipse E600 microscope (Nikon Instruments, Melville, NY, USA) was used to excite endogenous tdTomato and FITC fluorophores. Colocalization of immunostaining was performed by manually tracing regions of interest corresponding to cell bodies of tdTomato+ neurons and/or FITC+ neurons using MetaMorph software (Molecular Devices).

WESTERN BLOTS

Striata and cortex were dissected from 7 β3f/+ and β3f/f (Cre−) and 6 β3f/f^{Drd2} (Cre+) adult mice (>30 days) and flash-frozen on dry ice. Tissue homogenates were prepared in TEE buffer containing (in mM): Tris-HCl (10), pH 7.4, EDTA (1) and (1), centrifuged (30,000 × *g*) and crude membrane pellets were resuspended. Each sample was diluted down to a protein concentration of 1 μg/μL in TEE buffer. Equivalent amounts of protein (10 μg) were run on a sodium dodecyl sulfate-polyacrylamide gel (10% polyacrylamide) and transferred to the polyscreen polyvinylidene fluoride (New England Nuclear, Boston, MA, USA) membranes in transfer buffer containing (in mM): Tris-HCl (25), glycine (192), 20% methanol. Blots were blocked in PBST buffer containing (in mM): $NaPO_4$ (10), pH 7.4, NaCl (140), and 0.1% v/v Tween (20) containing 1% bovine serum albumin (Sigma) and incubated with α-β3 antibody (1:1000; NB300-119; Novus Biologicals, Littleton, CO, USA) overnight at 4°C. Blots were then washed with PBST buffer and incubated with α-rabbit horseradish peroxidase antibody (1:5000; Amersham, Piscataway, NJ, USA) for 30 min at room temperature. After several washes, individual bands were visualized on Hyperfilm (Amersham, Piscataway, NJ, USA) with enhanced chemiluminescence SuperSignal West Pico Enhancer Solution (Pierce, Rockford, IL, USA). Membranes were stripped of all antibodies for 1 h in elution buffer and incubated in α-β-actin antibody (Sigma) overnight at 4°C. Films were scanned and quantified using ImageJ software (http://rsb.info.nih.gov/ij/).

SLICE PREPARATION

Young male and female mice (15–23 days) were sacrificed by decapitation in agreement with the guidelines of the AMVA Panel on Euthanasia and the Georgetown University ACUC. Adult animals (>30 days) were used in some experiments, where noted. The whole brain was removed and placed in an ice-cold slicing solution containing (in mM): NaCl (85), KCl (2.5), $CaCl_2$ (1), $MgCl_2$ (4), NaH_2PO_4 (1), $NaHCO_3$ (25), glucose (25), sucrose (75; all from Sigma). Corticostriatal coronal slices (250 μm) were prepared using a Vibratome 3000 Plus Sectioning System (Vibratome, St Louis, MO, USA) in slicing solution. They were incubated in artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (124), KCl (4.5), Na_2HPO_4 (1.2), $NaHCO_3$ (26), $CaCl_2$ (2.0), $MgCl_2$ (1), and dextrose (10.0) at 305 mOsm at 32°C for 30 min. Slices recovered for an additional 30 min in aCSF, at room temperature, 22–24°C. All solutions were maintained at pH 7.4 by continuous bubbling with 95% O_2, 5% CO_2.

Slices were visualized under an upright microscope (E600FN, Nikon) equipped with Nomarski optics and an electrically insulated 60× water immersion objective with a long working distance (2 mm) and high numerical aperture (1.0). Recording pipettes were pulled on a vertical pipette puller from borosilicate glass capillaries (Wiretrol II; Drummond) and filled with cesium chloride (CsCl)- or potassium gluconate (Kgluc)-based internal

solutions. The CsCl-based internal solution contained (in mM): CsCl (145), HEPES (10), ATP–Mg (5), GTP–Na (0.2), EGTA (10), adjusted to pH 7.2 with CsOH. This high chloride internal solution enhanced the detection of GABAergic events, placing their reversal potential near 0 mV. In Kgluc-based internal solutions, CsCl was replaced with equimolar (145 mM) K gluconate and pH was adjusted with KOH. Kgluc internal solutions did not alter the chloride concentration and the reversal potential for GABAergic events was around −60 mV.

WHOLE-CELL RECORDINGS

In BAC-D2-EGFP; Drd1a-tdTomato mice, MSNs were classified as being either striatopallidal dopamine D2 receptor positive (D2+) or striatonigral dopamine D1 receptor positive (D1+) based on their expression of EGFP and tdTomato, respectively. In β3 subunit transgenic mice, MSNs were not tagged with fluorescent proteins (to blind the study), so the sample population likely contains both D1+ and D2+ MSNs.

All recordings were performed at room temperature, 22–24˚C. Voltage-clamp recordings were achieved using the whole-cell configuration of the patch-clamp technique at a holding voltage of −60 mV using the Axopatch 200B amplifier (Molecular Device Co., Sunnyvale, CA, USA). Access resistance was monitored during the recordings and experiments with >20% change were discarded. When Kgluc internal solutions were used, the baseline membrane potential for current-clamp recordings was set at −70 mV before each series of current step injection protocols. Rheobase current was defined as the first current step, within a series of 20 pA steps, that elicited an action potential. Recordings were not corrected for liquid junction potential.

Stock solutions of bicuculline methobromide (BMR), GABA (both from Sigma), and tetrodotoxin (TTX; Alomone Labs) were prepared in water. Etomidate, zolpidem, flumazenil, and diazepam (Sigma) were dissolved in dimethylsulfoxide (<0.01% final concentration). All stock solutions were diluted to the desired concentration in aCSF and applied locally through a Y tube (Murase et al., 1989) modified for optimal solution exchange in brain slices (Hevers and Lüddens, 2002).

Currents were filtered at 2 kHz with a low-pass Bessel filter and digitized at 5–10 kHz using a personal computer equipped with Digidata 1322A data acquisition board and pCLAMP10 software (both from Molecular Devices). Off-line data analysis, curve fitting, and figure preparation were performed with Clampfit10 software (Molecular Devices). Spontaneous and miniature inhibitory post-synaptic currents (sIPSCs and mIPSCs) were identified using a semi-automated threshold-based mini detection software (Mini Analysis, Synaptosoft Inc., Fort Lee, NJ, USA) and were visually confirmed. IPSC averages were based on more than 50 non-overlapping events, and decay kinetics were determined with averaged IPSC traces using double exponential curve fittings and reported as weighted time constants (Tw):

$$\mathrm{Tw} = \tau 1 \times A1/(A1 + A2) + \tau 2 \times A2/(A1 + A2)$$

where τx is the decay time constant for a particular component of the curve and Ax is the peak amplitude of the corresponding component. All detected events were used for event frequency analysis, but superimposing events were eliminated for the amplitude and decay kinetic analysis.

Tonic current was primarily measured with an all-points histogram that measured the mean holding current 10 s before and during BMR application (Janssen et al., 2009). Tonic current is represented as the change in baseline amplitude. When indicated, tonic current was also measured by changes in RMS noise before and during BMR application (Glykys and Mody, 2007). These analyses were based on an all-points histogram that was fitted to the Gaussian function (Fleming et al., 2007):

$$(x) = A \bullet \exp\left[-(x-\mu)^2/2\sigma^2\right]$$

where σ represents the RMS noise during the 10 s period before and during drug application and μ represents the mean holding current. Tonic noise was measured as a difference in RMS noise between two conditions.

STATISTICS

Box and whisker plots were generated for more accurate representation of critical data. The whiskers include the minimum and maximum values, while the box outlines the 25th and 75th percentile of data points. The median value is represented by a bar inside the box. Scatterplots of individual data points are also included in these plots to further show data spread.

Cumulative probability plots were constructed by averaging the single cell probability distribution, as calculated in Mini Analysis, for amplitudes and frequencies across all cells. Dotted lines denote the SEM for the data set.

Statistical significance was determined using the paired two-tailed Student's t-test to compare pre-drug conditions with recordings made under drug conditions of the same cell population. Unpaired two-tailed Student's t-test was used for comparisons across cell groups as well as for comparisons of striata and cortex from β3f/+ and β3f/f (Cre−) and β3f/f^{Drd2} (Cre+) mice for β3 subunit and actin protein in Western blots. Significance criteria was set at $p < 0.05$, and all values in text and figures are expressed as mean ± SEM. In text and figures, $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$, unless noted otherwise.

RESULTS

GENERATION OF THE CONDITIONAL β3 GABA$_A$ RECEPTOR SUBUNIT KNOCK-OUT IN D2+ MSNs

To study the role of the β3 GABA$_A$ receptor subunit in striatal MSN tonic current, the subunit was rendered non-functional by deleting exon 3 (Ferguson et al., 2007). We deleted this sequence by crossing floxed β3 subunit (β3f/f) mice with Drd2-Cre mice, where Cre recombinase is selectively expressed in dopamine receptor 2 (D2+) neurons. β3f/f^{Drd2} mice had the β3 subunit selectively deleted from all Drd2-expressing neurons, while β3f/+^{Drd2} and β3f/f mice had the incomplete combination of alleles and served as controls.

To verify the integrity of Drd2-Cre expression, we crossed this animal with a ROSA-tdTomato reporter mouse; any cell that expresses Cre recombinase will also express tdTomato. Counterstaining to DARPP-32 (a general marker for MSNs) showed that

all tdTomato+ cells were also DARPP-32+ (**Figure 1A**). Further, approximately half of the DARPP-32+ cells were also positive for tdTomato, consistent with previous reports that show similar numbers of striatal D2+ and D1+ MSNs (Shuen et al., 2008). The adjacent cortex showed minimal tdTomato and DARPP-32 expression (data not shown).

To examine the degree to which the β3 subunit was reduced in the striatum of β3f/f^{Drd2} mice, western blot analysis of cortex and striatum were performed using an antibody specific for the $GABA_A$ receptor β3 subunit (**Figures 1B–D**). As shown in **Figure 1B**, β3 subunit protein was significantly reduced in striatum of Cre positive mice compared to Cre negative controls (31% reduction). This reduction was expected as striatal D2+ MSNs constitute approximately half of the striatal MSN population (Shuen et al., 2008; personal observations). Since the cortex does not contain Drd2-expressing neurons, it serves as an ideal control. Indeed, β3 subunit protein levels were not reduced in cortical tissue from Cre positive mice (**Figure 1C**).

To investigate the consequences of β3 $GABA_A$ receptor subunit ablation in Drd2-expressing neurons, we first examined whether this deletion was affecting litter size. The floxed β3 $GABA_A$ receptor subunit mice were originally developed (Ferguson et al., 2007) to avoid neonatal lethality associated with global knock-out (KO) of the β3 subunit (Homanics et al., 1997). To determine whether our conditional KO animals experienced enhanced lethality, the number of β3f/+^{Drd2} mice surviving to p7 was compared to the number of β3f/f^{Drd2}mice from several litters. The ratio of β3f/+^{Drd2} to β3f/f^{Drd2} mice was similar to the 1:1 ratio ($n = 10$)

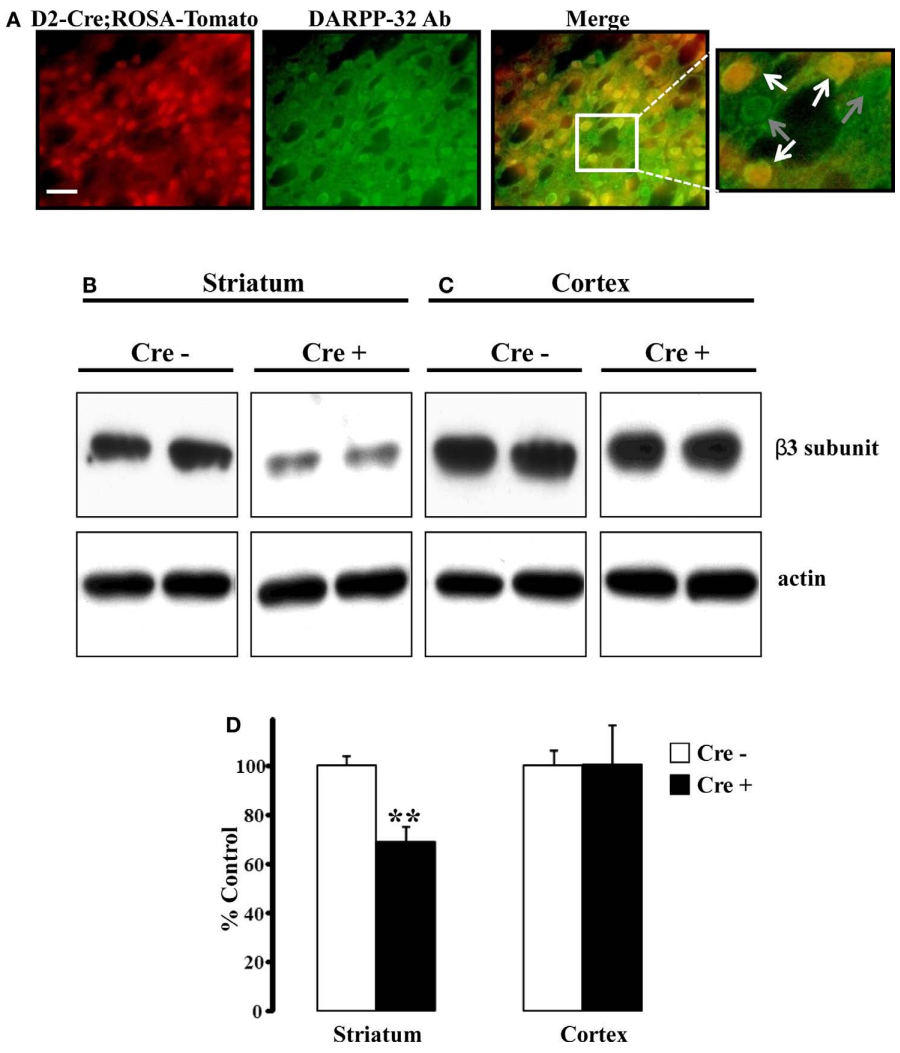

FIGURE 1 | Striatal β3 subunit protein reduced conditional KO mice. **(A)** Fluorescent images demonstrating colocalization between endogenous tdTomato fluorescence and DARPP-32 expression. Approximately half of the DARPP-32+ cells expressed endogenous tdTomato, indicative of Cre expression. Scale bar is 25 μm. **(B)** Representative western blot analysis of the $GABA_A$ receptor β3 subunit from individual 30-day-old mice revealed reduced amounts of β3 subunit protein in striatum **(B)** from Cre positive (β3f/f^{Drd2}) animals compared to Cre negative (β3f/+ and β3f/f) control animals. The amount of β3 protein in cortex **(C)** did not differ between genotypes. Blots were reprobed for β-actin. **(D)** Summary graph of western blot analysis demonstrating a significant reduction in β3 protein in striatum, but not cortex. Data are expressed as percent change in band intensity relative to Cre negative controls following normalization to actin.

predicted by Mendelian genetics with our breeding scheme, suggesting that β3f/f^{Drd2} mice do not experience premature death. All mice lacked any abnormal phenotypes like cleft palate or hyperactivity, which were observed in global β3 subunit KOs (Homanics et al., 1997), but not pan-neuronal KOs (Ferguson et al., 2007). Under general observation, β3f/f^{Drd2} animals did not display gross behavioral abnormalities compared to control littermates. Further tests need to be conducted to determine more subtle behavioral abnormalities.

CONDITIONAL β3 SUBUNIT KO MICE LACK TYPICAL MSN TONIC CURRENT EXPRESSION PATTERN

Because our previous work suggested that striatal MSN GABAergic tonic current may be mediated (at least in part) through the β3 $GABA_A$ receptor subunit (Janssen et al., 2009), it was important to determine MSN tonic current expression patterns in these conditional β3 subunit KO mice. Although dopamine receptor expression was not identified, many cells were sampled, and therefore the sample population likely contains both D1+ and D2+ MSNs. Because cell identity was not known, box and whisker plots are presented to demonstrate the tonic current expression spread and pattern. Averages were used to determine significance in these cells.

Striatal MSN GABAergic tonic inhibition was measured and compared between wild-type BAC-D2-EGFP; Drd1a-tdTomato mice, β3f/f, β3f/+^{Drd2}, and β3f/f^{Drd2} mice to verify that any differences observed in tonic current were due to the lack of the β3 subunit. It is possible that the floxed β3 allele alters β3 subunit function, also affecting tonic current expression in neurons. Therefore, MSN GABA-mediated tonic currents were measured from mice that lacked Cre recombinase expression, but were homozygous for the floxed β3 allele (β3f/f). Suggesting inclusion of unaltered D1+ and D2+ MSNs in the sample population, β3f/f mice showed quite varied tonic current amplitudes (0–34 pA; **Figure 2B**), similar to the varied tonic current amplitudes in BAC-D2-EGFP; Drd1a-tdTomato wild-type mice (**Figure 2B**, right). These functional data support western blot analysis which suggested that β3 subunits are not affected by the inclusion of loxP sites (**Figure 1B**; Ferguson et al., 2007). Further, the presence of the wide box representing the 25th and 75th percentile, shown in **Figure 2B**, shows a high degree of variability in tonic current amplitudes (0–25 pA, $n = 14$) from striatal MSNs in β3f/+^{Drd2} mice. On the other hand, β3f/f^{Drd2} mice had tonic current amplitudes that were confined to a much more narrow range (0–10 pA, $n = 29$). The average GABA-mediated tonic current amplitude was significantly smaller in conditional β3 subunit KO mice compared to both types of control mice (β3f/f^{Drd2}: 3.1 ± 0.5 pA, $n = 29$; β3f/f: 11.3 ± 2.7 pA, $n = 13$, $p < 0.0005$; β3f/+^{Drd2}: 11.0 ± 2.3 pA, $n = 14$, $p < 0.0005$). In addition, RMS noise was significantly lower in β3f/f^{Drd2} mice (0.8 ± 0.1 pA, $n = 24$) compared to β3f/+^{Drd2} (1.4 ± 0.2 pA, $n = 14$; $p < 0.05$) and β3f/f littermates (1.9 ± 0.5 pA, $n = 11$, $p < 0.005$). Because results from these two control mice did not differ, the data were pooled for **Figures 3–5** (although labeled in text and figures as β3f/f for clarity). Since all MSNs in the sample population showed little to no tonic current, these results suggest D2+ MSN tonic current expression is largely dependent upon β3 subunit expression.

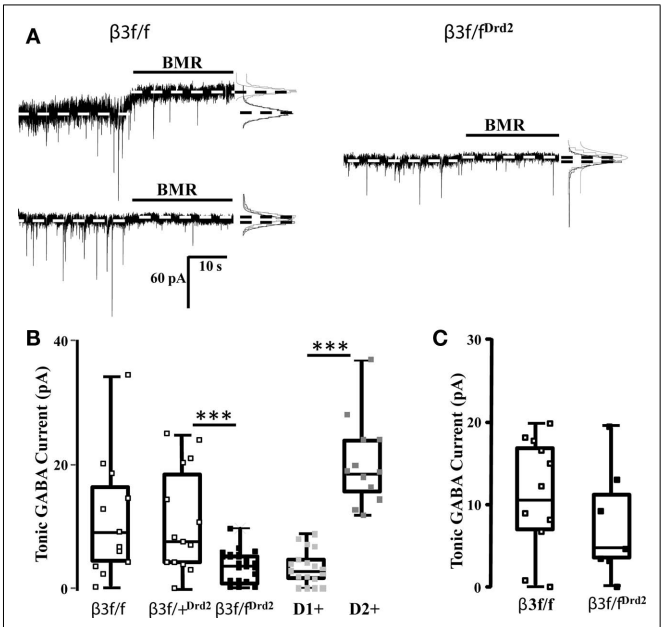

FIGURE 2 | Conditional β3 subunit KO mice show reduced GABA-mediated tonic current in MSNs. (A) Individual representative current traces displaying MSN BMR-sensitive (25 μM) tonic current in control β3f/f mice (left) and β3f/f^{Drd2} mice (right). The dichotomous pattern of tonic current was not present in β3f/f^{Drd2} mice. **(B)** Summary box and whisker plots displaying the tonic current patterns in β3f/f (open square, left; $n = 13$), β3f/+^{Drd2} (open square, middle; $n = 14$), β3f/f^{Drd2} (black square, middle; $n = 29$), and wild-type BAC-D2-EGFP;Drd1a-tdTomato D1+ (light gray, right $n = 15$) and D2+ (dark gray, right $n = 10$) MSNs from at least three mice in each group. **(C)** Summary box and whisker plots displaying the tonic current patterns in adult β3f/f (open square, left; $n = 12$), β3f/f^{Drd2} (black square, right; $n = 7$) from at least three mice in each group.

Our immunohistochemistry and western blot data suggest that the β3 subunit was deleted from a select group of striatal MSNs, but does not address the subtype. Because the MSN GABAergic tonic current pattern shifts through development such that adult D1+ MSNs express the majority of the GABAergic tonic conductance (Janssen et al., 2009; Santhakumar et al., 2010), we recorded tonic current from adult β3f/f^{Drd2} and β3f/f mice (>30 days). Indicative of a population of D1+ and D2+ MSNs, BMR-sensitive tonic current from β3f/f MSNs was highly variable (0–18 pA), with an average of 10.9 ± 2.0 pA ($n = 12$; **Figure 2C**). Tonic current expression from adult β3f/f^{Drd2} MSNs showed a similar, variable pattern (0–19 pA), with an average of 7.6 ± 2.8 pA ($n = 7$; $p = 0.3$). Both data sets were significantly different from tonic current averages from young β3f/f^{Drd2} mice (adult β3f/f $p < 0.0005$; β3f/f^{Drd2} $p < 0.05$). These data further suggest that the β3 subunit was only deleted from D2+ MSNs since adult striatal tonic current from KO mice resembled that of the controls.

CONDITIONAL DELETION OF β3 SUBUNIT ALTERS MSN ETOMIDATE AND GABA RESPONSE

To functionally verify that the β3 subunit was successfully excised from striatal D2+ MSNs, the effects of etomidate in TTX were tested on β3f/f (and β3f/+^{Drd2} with pooled data) and β3f/f^{Drd2} mice. This general anesthetic is a selective pharmacological agent

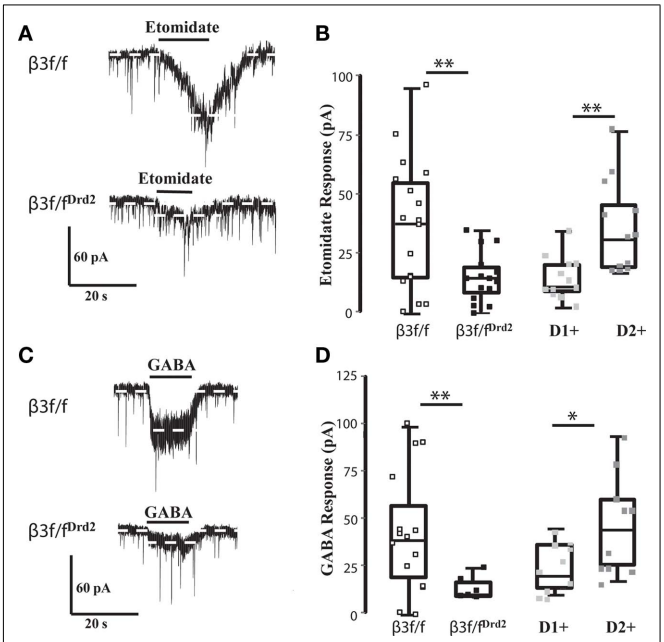

FIGURE 3 | β3 GABA$_A$ receptor subunit affects GABA sensitivity. (A) Representative traces of etomidate (3 μM) responses in TTX (0.5 μM) from β3f/f (top) and β3f/f^{Drd2} (bottom) mice. **(B)** Summary box and whisker plots showing effects of etomidate in TTX from 3 β3f/f (open square; $n = 17$), 5 β3f/f^{Drd2} (black; $n = 15$), and 6 BAC-Drd2-EGFP; Drd1a-tdTomato mice with D1+ (light gray; $n = 12$) and D2+ (dark gray; $n = 12$) neurons. **(C)** Representative traces of GABA (1 μM) responses in TTX (0.5 μM) from β3f/f (top) and β3f/f^{Drd2} (bottom) mice. **(D)** Summary box and whisker plots showing effects of GABA in TTX from 5 β3f/f (open square; $n = 15$), 3 β3f/f^{Drd2} (black; $n = 6$), and 6 wild-type BAC-D2-EGFP; Drd1a-tdTomato mice from D1+ (light gray; $n = 10$) and D2+ (dark gray; $n = 11$) neurons.

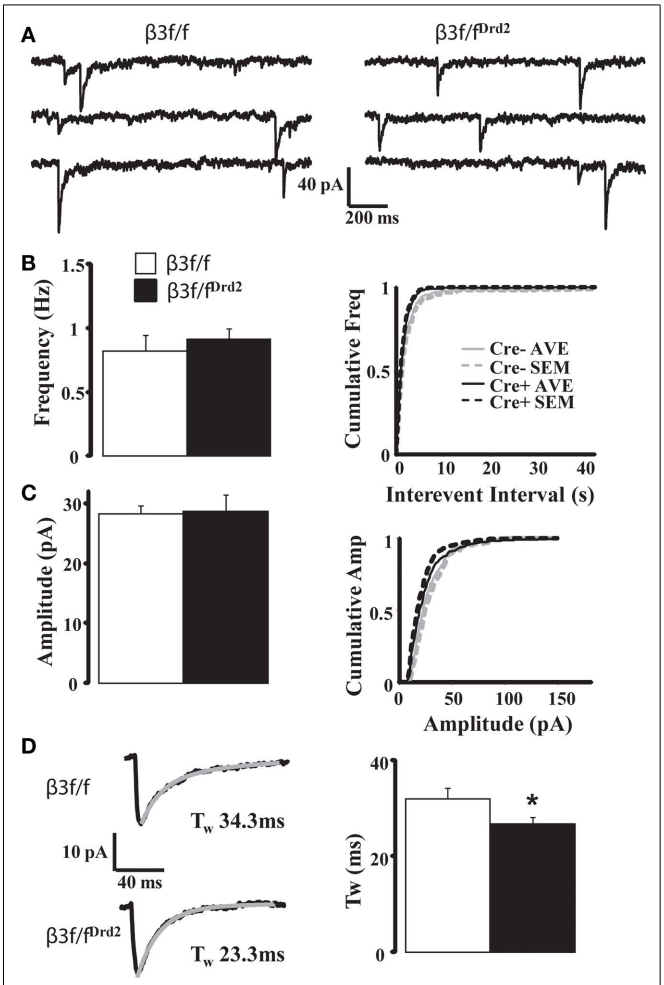

FIGURE 4 | Synaptic decay is altered in MSNs from conditional KO mice. (A) Representative mIPSC traces from β3f/f and β3f/f^{Drd2} mice. **(B,C)** Summary mIPSC frequency and amplitude graphs (left) and averaged cumulative distribution plots for all cells (right) in β3f/f and β3f/f^{Drd2} mice. **(D)** Representative individual mIPSCs (left) with double exponential decay fittings in gray from β3f/f and β3f/f^{Drd2} mice. Summary of decay fittings, right. Data derive from 16 to 17 cells from at least three mice in each group.

at β2/3-containing GABA$_A$ receptors (Hill-Venning et al., 1997). In striatal MSNs, however, etomidate identifies β3 subunit-containing GABA$_A$ receptors (Janssen et al., 2009) due to their lack of β2 subunit expression (Flores-Hernandez et al., 2000).

Responses to etomidate (3 μM) in TTX averaged 37.7 ± 6.8 pA ($n = 17$) in β3f/f mice, with a wide range of amplitudes (1–95 pA), suggesting that this population contains both relatively etomidate-insensitive D1+ MSNs and etomidate-sensitive D2+ MSNs, as seen in wild-type BAC-D2-EGFP; Drd1a-tdTomato mice (**Figure 3B**). The range of etomidate responses was more narrow from β3f/f^{Drd2} mice (0–35 pA), and the average was significantly smaller (14.9 ± 2.7 pA, $n = 15$, $p < 0.005$; **Figure 3B**), and was similar to etomidate currents recorded from wild-type D1+ MSNs. These data functionally confirm that the β3 subunit was deleted in etomidate-sensitive D2+ MSNs.

Post-synaptic GABA responses were also assessed in β3f/f^{Drd2} mice (in the presence of TTX) to determine if the β3 subunit plays a role in the differential D1+ and D2+ MSN GABA sensitivity (Ade et al., 2008) seen in wild-type mice. The average response to GABA (1 μM) in control β3f/f mice was 44.2 ± 8.5 pA ($n = 15$) with a range of 1–101 pA. The wide range of GABA responses were similar in current amplitude to identified D2+ and D1+ MSNs from BAC-D2-EGFP; Drd1a-tdTomato mice (**Figure 3D**). In contrast, β3f/f^{Drd2} mice expressed an average inward GABA current of just 14.0 ± 2.7 pA ($n = 6$, $p < 0.05$), with a more narrow range of amplitudes (9–24 pA; **Figure 3D**). These data suggest that the absence β3 subunit may affect GABA$_A$ receptor number and agonist sensitivity at D2+ MSN receptors, as previously shown in HEK293 cells (Janssen et al., 2009).

DELETION OF THE β3 SUBUNIT AFFECTS SYNAPTIC RECEPTOR COMPOSITION

Although D1+ and D2+ MSNs do not differ in their mIPSC profile (Ade et al., 2008), mIPSC characteristics from unidentified MSN subtypes were compared between β3f/f^{Drd2} and control β3f/f mice to evaluate changes in the composition of synaptically located GABA$_A$ receptors. As shown in **Figure 4A** and quantified in **Figures 4B,C** and **Table 1**, mIPSC frequency and amplitude did not change with β3 subunit deletion. However, mIPSC decay kinetics from β3f/f^{Drd2} mice were significantly faster than those from β3f/f mice (**Figure 4D**).

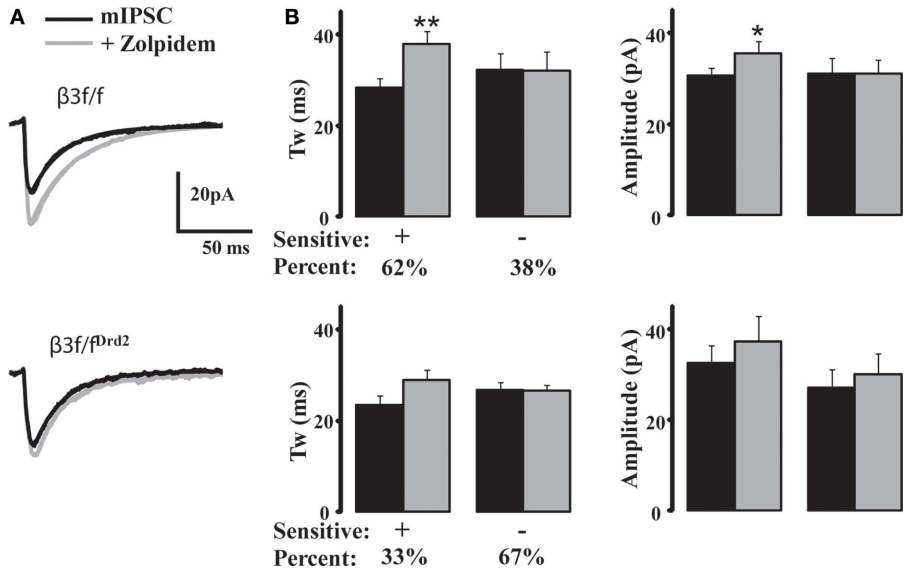

FIGURE 5 | Zolpidem fails to modulate majority of mIPSCs in MSNs from conditional KO mice. (A) Averaged representative mIPSC traces from a β3f/f (top) and a β3f/f^{Drd2} (bottom) neuron with zolpidem (1 μM). **(B)** Summary graphs showing zolpidem sensitivity on β3f/f (top) and β3f/f^{Drd2} (bottom) MSN kinetics (left) and amplitude (right). Cells were determined to be responders if their mIPSC decay increased at least 110% of control. The majority of neurons from control β3f/f mice showed a significant mIPSC prolongation ($n = 15/24$), while the majority of neurons from β3f/f^{Drd2} mice did not ($n = 6/9$). Cells derive from at least two mice in each group.

Table 1 | mIPSC characteristics of MSNs from β3f/f and β3f/f^{Drd2} mice.

	mIPSC Frequency (Hz)	mIPSC Amplitude (pA)	mIPSC Tw (ms)
β3f/f	0.99 ± 0.1 ($n = 13$)	28.2 ± 1.5 ($n = 13$)	31.9 ± 2.3 ($n = 14$)
β3f/f^{Drd2}	0.82 ± 0.1 ($n = 17$)	28.4 ± 2.6 ($n = 17$)	26.2 ± 1.0 ($n = 15$)*

**Denotes significance to control β3f/f mice.*

Since decreased numbers of synaptic receptors are reflected by decreased mIPSC amplitude, the data presented here suggest homeostatic changes of $GABA_A$ receptors at MSN inhibitory synapses upon β3 subunit deletion. Further, the faster decay of mIPSCs in β3f/f^{Drd2} animals suggests that this subunit deletion uncovers a distinct pool of synaptic receptors and/or causes a compensatory upregulation of synaptic $GABA_A$ receptors with faster kinetic properties.

Because α subunits largely determine synaptic receptor decay properties (Picton and Fisher, 2007), we used the imidazopyridine zolpidem (1 μM), effective at $GABA_A$ receptor α1–3 subunits, to determine $GABA_A$ receptor subunit differences between β3f/f and β3f/f^{Drd2} mice (**Figure 5A**). Cells that displayed decay prolongation more than 110% control were defined as "zolpidem sensitive." Zolpidem prolonged the average mIPSC decay kinetics in 15 of 24 (62%) neurons from β3f/f mice (134 ± 5% control; **Figure 5B**). The remaining nine neurons did not respond (97.9 ± 3% control). mIPSC potentiation in zolpidem sensitive cells was suppressed with co-application of the benzodiazepine antagonist flumazenil (10 μM), bringing mIPSC decay kinetics to their original states (zolpidem: 142.3 ± 8% control; flumazenil + zolpidem: 107.6 ± 14% control, $n = 6$). Flumazenil application alone failed to prolong the kinetics of zolpidem sensitive and insensitive mIPSCs (85 ± 5%, $n = 14$ and 68.9 ± 2%, $n = 2$), suggesting a minimal contribution from α4 subunits (Wafford et al., 1996). Diazepam (5 μM) prolonged the kinetics in all cells (sensitive: 138.4 ± 11%, $n = 12$; insensitive: 141.5 ± 11%, $n = 3$), supporting previous findings (Ade et al., 2008) suggesting the presence of α5-containing synaptic receptors in MSNs.

Zolpidem affected fewer neurons from β3f/f^{Drd2} mice (three of nine, 33%); the increased decay kinetics in these cells did not reach significance (124.1 ± 1% control; **Figure 5B**). Most cells (six of nine, 67%) from β3f/f^{Drd2} mice showed no response to zolpidem application (100.6 ± 5% control). Therefore, zolpidem failed to affect the majority of MSNs from β3f/f^{Drd2} mice, suggesting the upregulation of a zolpidem-insensitive α subunit.

To test the sensitivity of zolpidem on a cell-type that is known to express an abundance of synaptic α1-containing $GABA_A$ receptors, we recorded from parvalbumin- (PV+) expressing striatal interneurons (Riedel et al., 1998). The PV+ interneuron mIPSC decay was faster (15.9 ± 1.5 ms, $n = 4$) than MSNs, and was prolonged 221.7 ± 50% control with zolpidem (1 μM) application.

β3 SUBUNIT RESPONSIBLE FOR DIFFERENCES IN EXCITABILITY BETWEEN D1+ AND D2+ MSNs

Our previous work suggested that GABA-mediated tonic current affects striatal MSN excitability (Ade et al., 2008; Janssen et al., 2009), and therefore it is possible that by modulating GABAergic tonic current, β3 subunit expression also contributes to differences in D1+ and D2+ MSN excitability (Ade et al., 2008; Cepeda et al., 2008; Gertler et al., 2008; Janssen et al., 2009). Thus, we compared MSN excitability in identified D1+ and D2+ MSNs in

BAC-D2-EGFP; Drd1a-tdTomato mice to MSNs from β3f/f^{Drd2} mice. For these experiments, a Kgluc-based internal solution was used with the current-clamp technique. Cells were held at a pre-determined membrane potential (−70 mV), and subjected to 1 s, 20 pA steps of hyperpolarizing and depolarizing current injections.

As previously shown (Ade et al., 2008; Janssen et al., 2009), D2+ MSNs were significantly more excitable than D1+ MSNs (**Figure 6**). A substantial MSN sample size from β3f/f^{Drd2} mice showed that these cells were significantly less excitable than wild-type MSNs (**Figure 6**). The input–output curves and rheobase measurements from this mutant mouse were similar to wild-type D1+ MSNs and support the finding that the β3 $GABA_A$ receptor subunit regulates and contributes to the increased excitability of striatal D2+ MSNs.

DISCUSSION

Pharmacological evidence suggested that β3 $GABA_A$ receptor subunits contribute to differences in GABA-mediated tonic currents between D2+ and D1+ MSNs (Janssen et al., 2009). The present study sought to complement and confirm those results with a genetic approach: the β3 subunit was conditionally removed from Drd2-expressing neurons using the Cre-lox system (β3f/f^{Drd2}).

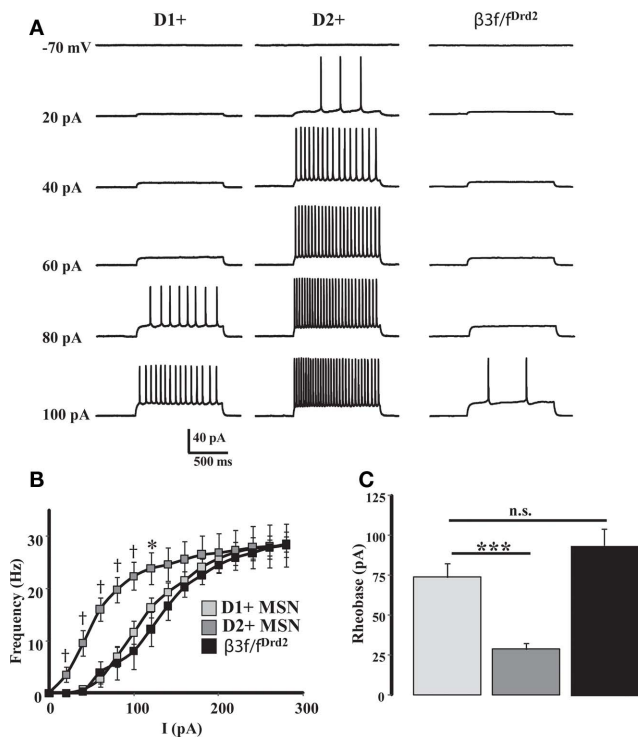

FIGURE 6 | β3 subunit determines cell excitability. (A) Representative current-clamp recordings from a D1+ MSN (left), D2+ MSN (middle), β3f/f^{Drd2} MSN (right), illustrating the responses to a series of depolarizing current injections (20 pA steps) from a set potential of −70 mV. **(B)** Summary of action potential firing frequency in response to increasing depolarizing current injections in D1+ ($n = 10$), D2+ ($n = 9$), and β3f/f^{Drd2} ($n = 10$) MSNs. * Denotes $p < 0.05$ and † denotes $p < 0.005$ for clarity. **(C)** Summary graph showing the averaged rheobase current in D1+, D2+, and β3f/f^{Drd2} MSNs. Data derive from the same cells in **(B)**, from at least three mice in each group.

Western blot data and etomidate-mediated currents from β3f/f^{Drd2} mice verified that the β3 subunit was reduced in a subpopulation, presumably D2+, of MSNs. The most significant, yet anticipated, finding with β3f/f^{Drd2} mice was the dramatic reduction in tonic current amplitude and spread, a consequence of $GABA_A$ receptor β3 subunits deletion in D2+ MSNs.

β3f/f^{Drd2} mice also showed substantially less GABA-mediated inward currents in response to exogenous GABA application than β3f/f littermates. Therefore, it is likely that high-affinity β3 subunit-containing $GABA_A$ receptors contribute to the greater GABA-sensitivity of D2+ MSNs (Ade et al., 2008). This reduced GABA sensitivity may be due to a reduction of highly sensitive α5β3-containing extrasynaptic receptors (Homanics et al., 1997; Ade et al., 2008).

Synaptic current properties in β3f/f^{Drd2} MSNs suggest that β3 subunit deletion affects α subunit composition; receptors with faster decay kinetics were uncovered or upregulated. Previous studies of IPSCs in distinct neuronal populations from β3 subunit KO mice (Huntsman et al., 1999; Ramadan et al., 2003; Hentschke et al., 2009) showed stronger changes in IPSCs than observed here. Faster decay and increased zolpidem sensitivity of mIPSCs in cortical neurons of primary cultures from β3 subunit KO mice suggested that the β3 subunit preferentially assembles with α2/3 subunits (Ramadan et al., 2003) with slower decay properties, as has been shown through immunoprecipitation (Benke et al., 1994). It has been hypothesized that removal of the β3 subunit leaves α1β2 subunit-containing synaptic receptors with faster decay and greater zolpidem sensitivity (Ramadan et al., 2003). All available data, however, suggest β3 subunit-containing $GABA_A$ receptors are characterized by slow synaptic decay (**Figure 4**; Huntsman et al., 1999; Ramadan et al., 2003; Hentschke et al., 2009).

We previously showed that MSN synaptic receptors do not differ between D1+ and D2+ MSNs, are comprised of α2 and α5 subunits, and are slightly sensitive to zolpidem (Ade et al., 2008). As zolpidem had no effect on the majority of rapidly decaying mIPSCs from β3f/f^{Drd2} mice, we suggest that deletion of the β3 subunit uncovered or upregulated synaptic receptors that contain the α4 or α5 subunit, characterized by faster decay kinetics than those with α2 or α1 subunits (Picton and Fisher, 2007). It is plausible that removal of the β3 subunit uncovers or upregulates α4/5β1-containing synaptic receptors since the β2 subunit is not expressed in MSNs (Flores-Hernandez et al., 2000).

Our data also support a homeostatic upregulation of $GABA_A$ receptors upon β3 subunit deletion since mIPSC amplitude remained unchanged. A select population of sIPSCs in the hippocampus of β3 subunit KO mice also showed unaltered amplitude with faster decay although receptor upregulation was not specifically investigated (Hentschke et al., 2009). Yet, it appears that β3 subunit deletion does not result in receptor upregulation in many brain regions (Homanics et al., 1997; Huntsman et al., 1999; Nusser et al., 2001; Wong et al., 2001; Ramadan et al., 2003; Ferguson et al., 2007). Receptor upregulation may occur in the striatum following β3 subunit deletion because receptor composition is highly dependent upon the abundance of both β1 and β3 subunits.

MSN excitability was significantly lower in MSNs from β3f/f^{Drd2} mice, suggesting that D2+ MSN output was decreased to levels that are normally characteristic of D1+ MSNs. This data indicates that disruption of β3 subunit expression, and therefore MSN $GABA_A$-mediated tonic current, significantly affects MSN output, as previously reported and discussed (Ade et al., 2008; Janssen et al., 2009).

This study verifies that D2+ MSN tonic current is mediated through the β3 $GABA_A$ receptor subunit, but offers no direct indication that the subunit plays an essential role in D1+ MSN current since the subunit was selectively deleted from D2+ MSNs. However, our previous study suggested that the β3 subunit was also essential in modulating D1+ MSN tonic current in both young and adult animals. Because tonic current from both cell-types could be pharmacologically modulated with similar conditions, the β3 subunit may mediate tonic current in both D1+ and D2+ MSNs, albeit in different situations and developmental stages.

This study primarily focuses on GABA-mediated tonic current in young mice, yet it is important to note the developmental changes in $GABA_A$ receptor composition (Laurie et al., 1992) and their potential impact on striatal MSN tonic current. The developmental regulation of tonic current is clearly demonstrated by the shifts observed in MSN tonic current patterns (Janssen et al., 2009; Santhakumar et al., 2010). The α5 subunit mediates tonic current in young D2+ MSNs (Ade et al., 2008), but pharmacological manipulation of this subunit did not affect D1+ or D2+ MSN tonic current in adult mice (Santhakumar et al., 2010). On the other hand, the δ subunit has only been found to mediate striatal tonic current in adult animals (Janssen et al., 2009; Santhakumar et al., 2010). mRNA expression studies have shown that young striatal tissue primarily expresses α2, α5, β3, and γX $GABA_A$ receptor subunits, while adult tissue abundantly expresses α2, α4, β3, and δ subunits (Laurie et al., 1992). Importantly, striatal β subunit expression remains relatively intact throughout striatal development, although it is more developmentally regulated in other brain regions (Laurie et al., 1992). Based on the available data, MSN tonic current in the young animal is predominately mediated through α5β3γ2 receptors, while tonic current in older MSNs is likely mediated through αXβ3δ receptors.

Many studies have noted the crucial importance of the $GABA_A$ receptor β3 subunit; this subunit has been linked to many autism spectrum disorders (Buxbaum et al., 2002) and childhood absence epilepsy (Feucht et al., 1999; Urak et al., 2006). Although the mechanism that links the β3 subunit with these developmental disorders is unknown, the data presented here suggest that the $GABA_A$ receptor β3 subunit largely regulates both phasic and tonic striatal GABAergic inhibitory currents and neuronal output, a plausible target mechanism for some aspects of these disorders. Our data also provide significant insight into the etiology and treatment of movement disorders like Parkinson's disease that manifest due to the imbalance of output from D2+ and D1+ MSNs. Pharmacological manipulation aimed at the β3 subunit may restore proper MSN output and alleviate movement disturbances.

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$GABA_A$ receptors: post-synaptic co-localization and cross-talk with other receptors

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γ-Aminobutyric acid type A receptors ($GABA_A$Rs) are the major inhibitory neurotransmitter receptors in the central nervous system, and importantly contribute to the functional regulation of the nervous system. Several studies in the last few decades have convincingly shown that GABA can be co-localized with other neurotransmitters in the same synapse, and can be co-released with these neurotransmitters either from the same vesicles or from different vesicle pools. The co-released transmitters may act on post-synaptically co-localized receptors resulting in a simultaneous activation of both receptors. Most of the studies investigating such co-activation observed a reduced efficacy of GABA for activating $GABA_A$Rs and thus, a reduced inhibition of the post-synaptic neuron. Similarly, in several cases activation of $GABA_A$Rs has been reported to suppress the response of the associated receptors. Such a receptor cross-talk is either mediated via a direct coupling between the two receptors or via the activation of intracellular signaling pathways and is used for fine tuning of inhibition in the nervous system. Recently, it was demonstrated that a direct interaction of different receptors might already occur in intracellular compartments and might also be used to specifically target the receptors to the cell membrane. In this article, we provide an overview on such cross-talks between $GABA_A$Rs and several other neurotransmitter receptors and briefly discuss their possible physiological and clinical importance.

Keywords: $GABA_A$R, cross-talk, ionotropic receptors, metabotropic receptors, targeting

INTRODUCTION

GABA (γ-aminobutyric acid) is the major inhibitory transmitter in the brain. Depending on the brain region, about 20–50% of all synapses use GABA as a transmitter. GABA, released from the pre-synaptic terminal can exert its action via two types of receptors, ionotropic $GABA_A$ ($GABA_A$Rs) and metabotropic $GABA_B$ receptors ($GABA_B$Rs). Most of the actions of GABA are mediated via $GABA_A$Rs. These are chloride ion channels composed of five subunits that can belong to different subunit classes. In mammalian brain there are a total of 19 different subunits derived from 8 different subunit classes (6α, 3β, 3γ, δ, ε, π, θ, 3ρ), giving rise to a multiplicity of different $GABA_A$R subtypes with distinct subunit composition and pharmacology (Sieghart, 1995; Olsen and Sieghart, 2009). The majority of $GABA_A$Rs, however, is composed of 2α, 2β, and 1γ subunit. Like other neurotransmitter receptors, $GABA_A$Rs are located at synapses as synaptic clusters (Moss and Smart, 2001; Renner et al., 2008; Bannai et al., 2009) where they are stabilized by scaffold proteins such as gephyrin (Kneussel et al., 1999; Tretter et al., 2008). Recent evidence, however, indicates that "clusters" of these receptors are also present at extrasynaptic locations (Lévi et al., 2008; Kasugai et al., 2010; Kneussel, 2010; Shrivastava et al., 2011). Synaptic as well as extrasynaptic clusters presumably consist of multi-molecular aggregates localized in the plane of the plasma membrane, which can contain not only multiple $GABA_A$Rs and interacting proteins, but also other types of receptors. In fact, several of such interacting receptors from different transmitter classes have been described to be closely associated with $GABA_A$R clusters. Interestingly, most if not all of the studies addressing $GABA_A$R interactions with other receptors have observed a suppression of GABAergic inhibition upon co-activation of the associated receptors. Additionally, in several studies activation of $GABA_A$Rs has been reported to suppress the response of the associated receptors. Such a functional inhibition of one receptor activity by activation of a second receptor is often termed as "cross-talk" between these receptors.

Here we summarize what is known on the post-synaptic co-localization and cross-talk of $GABA_A$Rs with other receptors. Such a cross-talk can be mediated by a direct physical contact of the receptors and subsequent allosteric modulation of their properties on binding of the one, or the other, or of both ligands, by

Abbreviations: 5-HT_3Rs, serotonin type 3 receptors; A1, A2A, A2B, A3 receptors, adenosine receptors; AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; D1-5 receptors, dopamine D1-5 receptors; GABA, γ-aminobutyric acid; $GABA_A$Rs, GABA type A receptors; $GABA_B$Rs, GABA type B receptors; GAD, glutamic acid decarboxylase; GlyR, glycine receptors; IPSC, inhibitory post-synaptic currents; MAPK, mitogen-activated protein kinase; nAChRs, nicotinic acetylcholine receptors; NMDARs, *N*-methyl-D-aspartate receptors; P2XRs, purinergic P2X receptors; PKA, protein kinase A; PKC, protein kinase C; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter.

indirect modulation of receptor properties via a second messenger system activated by the associated receptor, or by both mechanisms together. A more remote interaction between different receptors, in which one receptor modulates the release of the transmitter activating the other receptor, will not be systematically considered in this review, because each receptor located pre-synaptically can

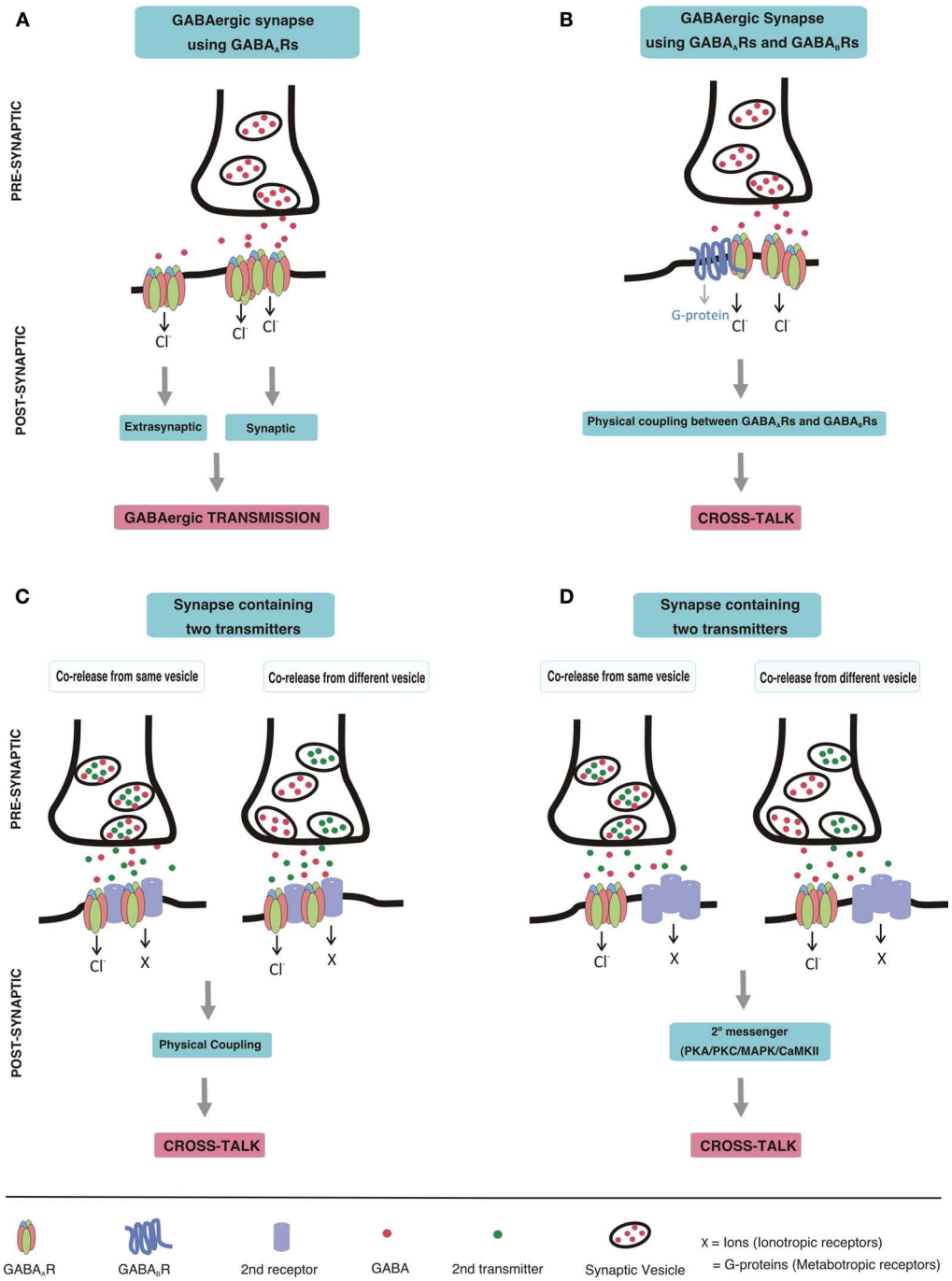

FIGURE 1 | Schematic drawing depicting GABAergic transmission and postsynaptic cross-talk between GABA$_A$Rs and other receptors (A) GABAergic transmission. GABA, released from a pre-synaptic terminal, acts on post-synaptic GABA$_A$R clusters resulting in phasic transmission. Spillover GABA can also act on extrasynaptic GABA$_A$Rs, thereby eliciting tonic currents. **(B)** Cross-talk between GABA$_A$Rs and GABA$_B$Rs. Direct physical coupling between GABA$_A$Rs and GABA$_B$Rs results in cross-talk at GABAergic synapses expressing both of these receptors. **(C,D)** Cross-talk of GABA$_A$Rs with other receptors. Co-release of neurotransmitters from pre-synaptic terminal may occur either from the same vesicles or from different vesicles. Co-released transmitters, then may act on post-synaptically co-localized receptors leading to cross-talk. The cross-talk could be mediated by direct physical coupling of GABA$_A$Rs with other receptors **(C)**, or by second messenger pathways **(D)**.

modulate the release of a transmitter. It only will be mentioned when a possible additional direct cross-talk between a receptor pair seems to exist. Furthermore, modulation of GABAergic transmission by second messenger mechanisms elicited via other synapses will also not be discussed here, because many receptors located in the post-synaptic neuron can cause such modulation. Accordingly, we use the following criteria as supportive arguments for receptor cross-talk: (i) their neurotransmitters are either co-released or can simultaneously interact with both receptors at the post-synaptic membrane, (ii) the receptors are co-localized in certain synapses of the brain, (iii) they physically interact with each other (iv) simultaneous activation of the two receptors produces a non-additive current (current occlusion; Figure 1).

The review has been split into the following chapters to separately address individual receptor pairs:

i Cross-talk of GABA$_A$ with GABA$_B$ receptors
ii Cross-talk of GABA$_A$ with glycine receptors
iii Cross-talk of GABA$_A$ with nicotinic acetylcholine receptors
iv Cross-talk of GABA$_A$ with serotonin receptors
v Cross-talk of GABA$_A$ with dopamine receptors
vi Cross-talk of GABA$_A$ with purinergic P2X receptors
vii Cross-talk of GABA$_A$ with adenosine receptors
viii Cross-talk of GABA$_A$ with NMDA receptors

CROSS-TALK OF GABA$_A$ WITH GABA$_B$ RECEPTORS

Pre-synaptically released GABA not only acts on GABA$_A$Rs but also on GABA$_B$Rs. GABA$_B$Rs are metabotropic G-protein coupled receptors, which exist as heteromers of GABA$_{B1}$ and GABA$_{B2}$ subunits (Schwenk et al., 2010). These receptors are localized both pre- and post-synaptically (Pinard et al., 2010). Several studies have demonstrated that activation of pre-synaptic GABA$_B$Rs reduced post-synaptic GABAergic transmission by reducing GABA release (Deisz and Prince, 1989; Davies et al., 1991; Mott and Lewis, 1991; Kardos et al., 1994). Other studies, however, are consistent with an additional cross-talk of post-synaptic GABA$_A$ and GABA$_B$Rs. Immuno-localization studies in the basal ganglion of the human brain indicated that the GABA$_A$α1-subunit and the GABA$_{B1}$ subunit are co-localized on GABAergic striatal interneurons and on neurons in the globus pallidus and substantia nigra pars reticulata but not on those of substantia nigra pars compacta (Waldvogel et al., 2004). In addition, both receptors were found to be co-localized at symmetrical synapses and possibly also at extrasynaptic sites (Smith et al., 2001). Another study demonstrated a direct interaction of GABA$_{B1}$ subunits with γ2S subunits of GABA$_A$Rs in rat brain lysate (Balasubramanian et al., 2004) and results indicated that this subunit regulates GABA$_B$R trafficking in multiple ways. This study also demonstrated that GABA$_{B1}$ subunit co-expression with GABA$_A$Rs increased the potency of GABA for activating GABA$_A$Rs.

In addition, earlier studies provide some evidence that the two receptors indeed exhibit cross-talk. Thus, Kardos et al. (1994), reported that the inhibitory action of GABA on K^+-evoked glutamate release in cultured cerebral granule cells was similar to that observed by the concerted action of the GABA$_A$R agonist isoguvacine and the GABA$_B$R agonist, baclofen. Surprisingly this effect was smaller than the sum of the inhibitory actions of isoguvacine and baclofen added separately. These results for the first time proposed that the two GABA receptors may functionally interact with each other in such a way that the final outcome results in a reduced inhibition (=disinhibition; Kardos et al., 1994; Schousboe, 1999). The functional inhibition of GABA$_A$Rs by activation of GABA$_B$Rs was additionally observed using Ca^{2+}-imaging in developing hypothalamic neurons, in which GABA$_A$Rs are excitatory and raise cytosolic Ca^{2+} level on activation. Dose-dependent administration of baclofen depressed GABA$_A$R-mediated Ca^{2+}-influx in these cells (Obrietan and van den Pol, 1998). Although the authors confirmed a stronger involvement of pre-synaptic GABA$_B$Rs on electrically evoked GABA release, they could additionally observe depression of muscimol-evoked GABA$_A$R-mediated Ca^{2+}-influx by baclofen when blocking pre-synaptic activity by tetrodotoxin, suggesting a possible involvement of post-synaptic GABA$_B$Rs too. Whether these effects were caused by a direct interaction of GABA$_A$ and GABA$_B$ receptors or by a second messenger mechanism, however, was not investigated.

These studies seem to indicate that pre-synaptically released GABA can act on post-synaptically co-localized GABA$_A$ and GABA$_B$Rs resulting in a reduction of GABAergic signaling. However, more studies have to be performed to understand the possible mechanism of this cross-talk. Additionally, whether GABA$_A$R can also suppress GABA$_B$Rs mediated signaling, and whether any second messenger system is involved, still needs to be clarified. As GABA$_B$Rs are highly abundant in the central nervous system (CNS), their simultaneous activation and cross-talk with GABA$_A$Rs may play an important role in fine tuning of inhibition in the nervous system. Thus, it might limit hyperpolarization caused by excess of GABA release, and simultaneously overcome a failure of inhibition caused by a local change of the Cl^- reversal potential (Segal and Barker, 1984; Staley et al., 1995) by GABA$_B$R activated K^+ channels.

CROSS-TALK OF GABA$_A$ WITH GLYCINE RECEPTORS

Glycine receptors (GlyRs), like GABA$_A$Rs, are members of the cys-loop receptor family and share the load of inhibition with GABA$_A$Rs in the CNS. GlyRs are highly abundant in spinal cord, brain stem and retina, where GABA$_A$Rs are also expressed. The neurotransmitters GABA and glycine are co-localized in many synapses, and can be co-released from them. Actually, co-release of GABA and glycine was demonstrated to occur from the same vesicle in interneuron–motoneurons synapses in spinal cord slices (Jonas et. al, 1998). Co-release of GABA and glycine has additionally been reported in brain stem slice preparations at the hypoglossal motoneuron synapse (O'Brien and Berger, 1999) and the abducens motoneuron synapse (Russier et al., 2002). Other studies not only demonstrated the co-existence of GABA and glycine, but also a co-localization of GABA$_A$Rs and GlyRs at synapses in spinal cord neurons (Triller et al., 1987; Bohlhalter et al., 1994; Todd et al., 1996; Shrivastava et al., 2011). Another recent study provided a quantitative estimate of such mixed GABAergic/glycinergic synapses (Lévi et al., 2008) suggesting that a substantial proportion of spinal cord inhibitory synapses house both of these inhibitory receptors.

Surprisingly, even before the co-release of these two neurotransmitters was demonstrated, a partial inhibition of the GABA response by glycine was reported in rat medullary neurons (Lewis and Faber, 1993). Later, Trombley et al. (1999), using whole-cell voltage clamp recordings, studied this in more detail in rat olfactory bulb neurons, where they observed that in the majority of these neurons, glycine inhibited GABA-evoked currents and GABA inhibited glycine-evoked currents. They additionally observed that on co-application of saturating concentrations of GABA and glycine to these neurons the current amplitude was less than the sum of the currents evoked by GABA and glycine alone, demonstrating current occlusion. Later, similar cross-inhibition was reported in neurons from rat sacral dorsal commissural nucleus (Li et al., 2003). The authors additionally observed that this cross-inhibition was asymmetric (GABA-evoked currents were more affected by glycine than glycine-evoked responses by GABA). In addition, it was reversible, and depended on the phosphorylation of GABA$_A$Rs. This indicates that more complex signaling pathways might be involved in this cross-talk. Taken together, all these results suggest that the two receptors are in close proximity, but whether they also are physically interacting remains to be clarified.

Recent findings, however, offer alternative explanations for a possible cross-talk of GABA$_A$Rs and GlyRs, that does not require direct physical interaction of these receptors. Thus, it has been demonstrated that at physiological concentrations GABA acts as an endogenous ligand (weak partial agonist) for synaptic GlyRs (Jonas et al., 1998; De Saint Jan et al., 2001; Lu et al., 2008; Singer, 2008). GABA thus not only activates GABA$_A$Rs but also to a lesser extent GlyRs. The GlyR activation by GABA, however, would be gone as soon as glycine is simultaneously applied, resulting in non-additive effects. In addition it was reported that GlyRs activated by very high concentrations of GABA have deactivation times 10 times faster than receptors activated by glycine (Fucile et al., 1999). Thus, by directly activating GlyRs, GABA can narrow the time window for effective glycinergic inhibition (Lu et al., 2008) resulting in an additional mechanism of cross-talk of the GABAergic and glycinergic system. Finally, strong simultaneous activation of GABA$_A$Rs and GlyRs could cause an increase of the local chloride concentration in the cell, thus reducing further chloride influx (Segal and Barker, 1984; Staley et al., 1995). Further experiments will have to analyze whether such mechanisms could explain the observed receptor "cross-talk."

In any case, GABA$_A$R and GlyR mediated cross-talk in spinal cord and brain stem may precisely regulate the time course of the post-synaptic conductance during development. This may play an important role in motor coordination and the generation of locomotor patterns (Jonas et al., 1998; O'Brien and Berger, 1999), however this possibility so far has not been investigated. The interaction possibly also helps in shaping odor information processing in developing olfactory bulb (Trombley et al., 1999) and acoustic information processing in auditory synapses (Lu et al., 2008). Finally, it provides a mechanism that ensures efficient synaptic inhibition even in the case of a mutation in the one or the other transmitter receptor types.

CROSS-TALK OF GABA$_A$ WITH NICOTINIC ACETYLCHOLINE RECEPTORS

Nicotinic acetylcholine receptors (nAChRs) are other major members of the cys-loop receptor family. In contrast to GABA$_A$Rs, that are ligand-gated anion channels, nAChRs are ligand-gated cation channels. Co-localization of GABA and the ACh-synthesizing enzyme choline acetyltransferase has been demonstrated in rodent cortical neurons (Hallanger et al., 1986), chick retina amacrine-like cells (O'Malley and Masland, 1989; Santos et al., 1998) and in the nerve fibers innervating adrenal gland (Iwasa et al., 1999). Moreover, several studies have convincingly demonstrated that GABA can be co-released with ACh from cholinergic terminals (O'Malley and Masland, 1989; O'Malley et al., 1992; Lee et al., 2010). Unlike co-release of GABA and glycine, which involves release from the same vesicles, all these studies suggested the involvement of different vesicle pools in the co-release of GABA and ACh (Santos et al., 1998; Lee et al., 2010). Even though it is well established that GABA can be co-released at cholinergic nerve terminals, very few studies have focused on post-synaptic co-localization of GABA$_A$ and nACh receptors. There is some evidence that the α7-subtype containing nAChRs co-localize with GABA$_A$Rs in interneurons of embryonic hippocampus (Kawai et al., 2002; Zago et al., 2006). It remains to be elucidated, however, if other subtypes of nAChRs apart from the α7-subtype also co-localize with GABA$_A$Rs in brain regions where GABA and acetylcholine are co-released.

Direct evidence of a post-synaptic cross-talk between GABA$_A$Rs and α7-nAChRs comes from recent studies on rodent hippocampal interneurons and on chick ciliary ganglion neurons (Wanaverbecq et al., 2007; Zhang and Berg, 2007). Using whole-cell voltage clamp recording, it was demonstrated that activation of post-synaptically localized α7-nAChRs reversibly inhibited GABAergic inhibitory post-synaptic currents (IPSCs) by generating a calcium influx, resulting in Ca^{2+}/calmodulin-dependent kinase II (CaMKII), Mitogen-activated protein kinase (MAPK; Zhang and Berg, 2007), and protein kinase C (PKC; Wanaverbecq et al., 2007) dependent phosphorylation of GABA$_A$Rs. This inhibition was observed in autonomic neurons where α7-nAChRs are concentrated on the soma along with GABA$_A$Rs, and on hippocampal interneurons where α7-nAChRs often co-cluster with GABA$_A$Rs on dendrites and filopodia (Zhang and Berg, 2007). It however remains to be determined if Ca^{2+} influx via α7-nAChRs may additionally result in phosphorylation and/or internalization of synaptic GABA$_A$Rs. In any case, as hippocampal interneurons are spontaneously active by generating rhythms (Cobb et al., 1995) even when glutamatergic transmission is blocked, their regulation by α7-nAChRs, which are highly enriched in interneurons, possibly plays an important role in modulating inhibitory neurotransmission.

In addition, pre-synaptic nAChRs depending on their subunit composition are known to facilitate action-potential dependent GABA release in hippocampal interneurons (Alkondon et al., 1997, 1999; Ji and Dani, 2000; Buhler and Dunwiddie, 2002), superficial superior colliculus neurons (Endo et al., 2005), and ventral tegmental area dopamine neurons (Yang et al., 2011). Surprisingly, Wanaverbecq et al. (2007), in their study, also observed an increase

in pre-synaptic GABA release along with post-synaptic suppression of GABA$_A$R-mediated IPSCs, effects that should oppose each other. In any case, cholinergic input can both facilitate as well as suppress GABAergic neurotransmission via complex pre-synaptic as well post-synaptic mechanisms. Further studies addressing regulation of GABA$_A$Rs by both pre- and post-synaptic nAChRs are required to provide clues on the mechanism and function of this fine tuning.

CROSS-TALK OF GABA$_A$ WITH SEROTONIN RECEPTORS

Serotonin (5-Hydroxytryptamine, 5-HT) is a monoamine transmitter that exerts its action by binding to serotonin receptors (5-HTRs) that are widely expressed in the nervous system as well as in non-neuronal tissue. Apart from the 5-HT$_3$R subtype, which is a ligand-gated ion channel and member of the cys-loop receptor family, all other serotonin receptors (5-HT$_{1,\,2,\,4–7}$) belong to the G-protein coupled receptor family (Hoyer et al., 2002; Kitson, 2007). So far, the only evidence for a co-localization of the transmitters GABA and serotonin comes from a recent study in the early vertebrate sea lamprey, where serotonin and GABA was demonstrated to co-exist in several brain regions (Barreiro-Iglesias et al., 2009a). Whether such a co-existence of these neurotransmitters and their receptors is also present in the mammalian nervous system, still needs to be determined. Furthermore, a co-release of serotonin with GABA (along with ATP) has only been reported in non-neuronal rat pancreatic beta cells (Braun et al., 2007), and thus, a possible co-release in the CNS still needs to be investigated.

However, a recent study for the first time described a cross-talk between 5-HT$_3$Rs and GABA$_A$Rs in myenteric neurons using whole-cell recordings (Miranda-Morales et al., 2007), by providing evidence for current occlusion on simultaneous activation of these two receptors. The cross-talk as observed at saturating concentration of agonists for both receptors occurred immediately after the application of the two agonists, possibly suggesting that the two receptors are located very close to each other. The authors provided additional evidence for an allosteric interaction as they observed current occlusion even in the absence of Ca^{2+} and also in the presence of staurosporine, a general protein kinase inhibitor. Further experiments will have to investigate the mechanism of interaction of these receptors. As 5-HT$_3$R have a preferential localization on a sub-population of GABAergic interneurons in hippocampus and cortex, cross-talk between 5-HT$_3$Rs and GABA$_A$Rs possibly may play a crucial role in the control of the balance between excitation and inhibition in the CNS (Chameau and van Hooft, 2006). It may also be important to note that the co-localization as reported for nACh/GABA$_A$ and 5-HT$_3$/GABA$_A$ receptors have been primarily observed in interneurons suggesting that nAChRs and 5-HT$_3$Rs possibly participate in fine tuning of inter-neuron functioning.

So far, there is no evidence of any post-synaptic cross-talk between GABA$_A$Rs and G-protein coupled serotonin receptors. However, pre-synaptic potentiation of GABA release by serotonin, as well as by 5-HT$_2$ and 5-HT$_3$ receptors has been documented in spinal cord (Xu et al., 1998; Li et al., 2000; Fukushima et al., 2009) and hippocampal neurons (Dorostkar and Boehm, 2007). Whether these receptors additionally cross-talk with GABA$_A$Rs receptors post-synaptically, still remains to be clarified.

CROSS-TALK OF GABA$_A$ WITH DOPAMINE RECEPTORS

Dopamine receptors are G-protein coupled receptors consisting of five different subtypes, namely D1–D5 receptors. These receptors act via adenylyl cyclase mediated second messenger systems and are thus responsible for long-term regulation of brain functioning (Girault and Greengard, 2004). Even though there are only very few dopaminergic neurons, they have been described to actively regulate post-synaptic GABAergic signaling. Virtually all dopaminergic pre-synaptic terminals in glomerulus were reported to be glutamic acid decarboxylase (GAD) positive (Gall et al., 1987), suggesting a possible co-existence and co-release of the transmitters dopamine and GABA. This conclusion was supported by demonstrating co-localization of tyrosine hydroxylase and GAD in periglomerular cells in mouse brain slices (Maher and Westbrook, 2008). Additional evidence for the co-existence of dopamine and GABA came from a study on the brain of early vertebrate sea lamprey, where these two neurotransmitters were observed to co-exist in several brain regions (Barreiro-Iglesias et al., 2009b). Finally, Maher and Westbrook, in their study also reported the co-release of GABA and dopamine from dopaminergic nerve terminals of periglomerular cells.

Several studies have demonstrated that most, if not all members of the dopamine receptor family modulate post-synaptic GABA$_A$R-mediated neurotransmission in different regions of the brain. However, so far only dopamine D5 receptors have been demonstrated to directly bind to and cross-talk with GABA$_A$Rs (Liu et al., 2000). It was demonstrated that D5, but not D1 receptor antibodies precipitated hippocampal GABA$_A$Rs, indicating complex formation between these two receptors *in vivo*. Additionally, it was shown that the C-terminus of dopamine D5, but not D1 receptors could directly interact with the intracellular loop between the third and fourth transmembrane domain of the γ2-subunit of GABA$_A$Rs. In addition, the authors reported that co-activation of GABA$_A$ and D5 receptors suppressed dopamine induced cAMP accumulation and resulted in current occlusion through GABA$_A$Rs. In agreement with the absence of co-precipitation with D1 receptors, the authors observed no functional cross-talk. Actually, this was one of the first studies demonstrating that cross-talk could indeed occur via direct coupling between two receptors; however an additional involvement of adenylyl cyclase induced intracellular signaling following the activation of D1 receptors cannot be ruled out. Surprisingly, most of the other studies investigating dopamine/GABA$_A$ receptor interaction only dealt with this second possibility.

Although Liu et al. (2000), observed no interaction and cross-talk of GABA$_A$R with D1 receptors in hippocampus, three other studies using whole-cell patch-clamp recordings, reported a suppression of GABA$_A$R-mediated chloride influx following activation of dopamine D1 receptors in olfactory bulb neurons (Brünig et al., 1999) and in the striatum (Flores-Hernandez et al., 2000; Goffin et al., 2010). Using radiolabeling and immunoprecipitation experiments, it was initially demonstrated that activation of D1 receptors increased the phosphorylation of β1/β3 subunit of GABA$_A$Rs via PKA/DARPP-32/PP1 (protein kinase A/Dopamine- and cAMP-regulated phosphoprotein/Protein phosphatase 1) signaling cascade, which in turn leads to reduced GABA-evoked currents in neostriatal medium spiny neurons (Flores-Hernandez

et al., 2000). Recently, Goffin et al. (2010) additionally showed that 10 min to 7 days treatment of striatal neurons with D1 receptor agonist resulted in a decreased number of GABA$_A$Rs at the cell surface, eventually leading to a reduction in the number of GABAergic synapses and in GABA$_A$R cluster size. Surprisingly, they observed that the reduction in number of GABA$_A$Rs was due to endocytosis followed by dephosphorylation of β-subunits by a protein phosphatase 2A-dependent pathway (PP2A; Goffin et al., 2010). Thus it seems likely, that the activation of D1 receptors results in rapid PKA-dependent phosphorylation of GABA$_A$Rs, which over time, gets dephosphorylated by PP2A and eventually endocytosed, leading to sustained inhibition. The effect of GABA$_A$R activation on D1 receptor signaling and whether there is a direct interaction between the two receptors in striatal neurons was not investigated in these studies.

In similar lines, Goffin et al. (2010), additionally observed a D2 receptor-mediated down-regulation of surface GABA$_A$Rs following dephosphorylation of β-subunits involving protein phosphatase 1 dependent pathway, eventually resulting in a smaller inhibitory response in the medium spiny neurons of the striatum (Goffin et al., 2010). In contrast, Brünig et al. (1999), had previously reported that D2 receptor activation resulted in an enhancement of GABAergic transmission due to a phosphorylation of GABA$_A$Rs involving the protein kinase C pathway in mitral/tufted cells in the olfactory bulb. These discrepancies may possibly suggest a cell-type-specific modulation by dopamine receptors. More studies in this direction have to be performed to clarify this discrepancy. The reciprocal suppression of D2 receptor function by activation of GABA$_A$Rs has been demonstrated in another study using dopamine D2 receptor radio-ligand binding assays in membrane preparation from rat neostriatum. It was demonstrated that the activation of GABA$_A$Rs significantly increased the dissociation constant of the high affinity selective D2 receptor antagonist, raclopride, (Pérez de la Mora et al., 1997) which could be reversed by use of the GABA$_A$R antagonist bicuculline. Altogether, these results suggest a high probability of reciprocal cross-talk and possibly direct interaction between GABA$_A$/D2 receptors in similar lines as proposed for GABA$_A$/D5 receptors. However, this needs to be investigated further.

Finally, using whole-cell patch-clamp recordings in various brain regions, the last two members of the dopamine receptor family, the D3 and D4 receptors, have also been demonstrated to reduce GABAergic inhibition by stimulating endocytosis of GABA$_A$Rs via the PKA/protein phosphatase I pathway. D3 receptors seem to reduce GABA$_A$R-mediated current by inactivating PKA activity, resulting in dephosphorylation of β-subunits, followed by clathrin/activator protein 2-mediated endocytosis of GABA$_A$Rs in the nucleus accumbens (Chen et al., 2006) and hippocampus (Swant et al., 2008). Similarly, PKA/protein phosphatase I pathway-dependent rundown of GABA$_A$Rs by D4 receptors has been reported in prefrontal cortex (Wang et al., 2002) and globus pallidus neurons (Shin et al., 2003). Functional experiments on dopamine receptors will clarify whether D3 or D4 receptors are also reciprocally inhibited followed by GABA$_A$R activation.

Based on all these studies, it seems clear that complex intracellular signaling pathways can be activated by dopamine receptors, which then determines the fate of nearby localized GABA$_A$Rs. In addition, a direct coupling and a reciprocal cross-talk between dopamine and GABA$_A$ receptors cannot be ruled out. In any case, it seems that dopamine receptors are important regulators of surface GABA$_A$R expression. As GABA$_A$Rs are highly mobile and continuously exchange between synaptic and extrasynaptic sites as well as by exocytosis and endocytosis, it is apparent that interactions with dopamine receptors may be one of the determinants for such rapid transition and therefore directly defining the strength of inhibitory synapse. Loss of coordination between the GABAergic and dopaminergic system could contribute to psychomotor and neuropsychiatric diseases in striatum and cortex, which affects both these pathways (Liu et al., 2000). Inhibition of GABA$_A$R-mediated IPSCs by dopamine receptors could enhance oscillations between globus pallidus neurons and subthalamic nucleus, thereby ameliorating Parkinson's disease (Shin et al., 2003). Additionally the action of dopamine on GABAergic inhibitory circuit in olfactory bulb could assist in odor detection and olfactory learning (Brünig et al., 1999).

CROSS-TALK OF GABA$_A$ WITH P2X RECEPTORS

P2X receptors (P2XRs) are ligand-gated ion channels that are activated by adenosine-5′-triphosphate (ATP). Seven different subtypes of these receptors are now known (P2X$_1$–P2X$_7$) which assemble to form predominantly homo-trimeric but also hetero-trimeric receptors (Burnstock, 2006). Even though ATP got late recognition as a neurotransmitter, it is now well established that it acts as a fast excitatory neurotransmitter and co-transmitter in several regions of the CNS. Synaptic co-release of ATP with GABA but not glutamate has been demonstrated in the rat spinal cord and mouse lateral hypothalamus (Jo and Schlichter, 1999; Jo and Role, 2002). Moreover, co-localization studies have clearly demonstrated the co-existence of P2X$_2$R-subtype with GABA$_A$Rs in dorsal root ganglion (Labrakakis et al., 2003) and spinal cord neurons (Shrivastava et al., 2011), suggesting a possible physical association between these receptors.

A direct cross-talk between GABA$_A$Rs and P2XRs was first demonstrated using whole-cell patch-clamp recording in rat dorsal root ganglion cells. It was demonstrated that co-application of the agonists of GABA$_A$Rs and P2XRs produced a total current much smaller than the predicted linear summation of individual responses (Sokolova et al., 2001). This interaction was shown to be dependent on Ca^{2+} influx through endogenous P2XRs expressed in dorsal root ganglion neurons. Another study, investigated the mechanism of this cross-inhibition in more detail in a recombinant system after the co-expression of GABA$_A$Rs and P2X$_2$Rs in *Xenopus* oocytes (Boué-Grabot et al., 2004b). Surprisingly, the authors observed the cross-talk to be independent of Ca^{2+}influx via P2X$_2$Rs. Additionally, they demonstrated that the intracellular C-terminus of P2X$_2$Rs and of the intracellular loop (between transmembrane domain III and IV) of GABA$_A$R β-subunit is essential for the interaction, which was additionally verified in our recent study (Shrivastava et al., 2011). In agreement with the previous study, Ca^{2+}-independent cross-talk was also reported in myenteric neurons (Karanjia et al., 2006). Finally, a similar cross-talk between P2X$_3$Rs/GABA$_A$Rs and P2X$_4$Rs/GABA$_A$Rs was reported recently (Toulmé et al., 2007; Jo et al., 2011).

We studied this interaction in more detail by employing co-immunoprecipitation and FRET imaging in transiently transfected HEK cells, in combination with single particle tracking and quantitative immunocytochemistry in primary neurons of spinal cord. We demonstrated that GABA$_A$Rs and P2X$_2$Rs interact with each other already intracellularly possibly within the endoplasmic reticulum and are then co-transported to the cell membrane where they are primarily co-localized extrasynaptically in mice spinal cord neurons (Shrivastava et al., 2011). Additionally we observed that upon activation of P2X$_2$Rs by ATP or P2X$_2$R agonists, this transient complex gets dissociated. This agonist-induced dissociation seems to be both Ca^{2+} dependent and thus mediated via signaling mechanisms and Ca^{2+} independent and thus mediated via a conformational change. Although this cannot explain the cross-talk observed by electrophysiological studies due to the much longer time course used in our studies, it appears that following activation of one of the two receptors, an initial Ca^{2+}-independent (Boué-Grabot et al., 2004b; Karanjia et al., 2006) conformational change results in current occlusion, followed by a Ca^{2+}-dependent (Sokolova et al., 2001; Shrivastava et al., 2011) dissociation of this receptor–receptor complex. Whether this Ca^{2+}-influx additionally leads to activation of Ca^{2+}-dependent intracellular signaling pathways, as reported for nACh and dopamine receptors, remains a highly intriguing question to be answered.

The importance of cross-talk involving members of P2XR family can be estimated by the fact that P2XRs not only cross-talk with GABA$_A$Rs, but are now known to also cross-talk with most of the other members of cys-loop receptor family, possibly in a similar way (Zhou and Galligan, 1998; Khakh et al., 2000, 2005; Sokolova et al., 2001; Boué-Grabot et al., 2004a,2004b; Karanjia et al., 2006; Decker and Galligan, 2009). P2XR-mediated regulation of GABA$_A$R number at the cell surface can have direct implications on modulating pain transmission in spinal cord (Zeilhofer et al., 2009; Shrivastava et al., 2011) or on epileptic seizures in hippocampus (Kang et al., 2003).

CROSS-TALK OF GABA$_A$ WITH ADENOSINE RECEPTORS

Adenosine receptors are members of the G-protein coupled purine receptor family, with adenosine being their natural ligand. Four different adenosine receptors are known, namely A1, A2A, A2B, and A3 receptors (Burnstock, 2006). ATP released from presynaptic terminals rapidly degrades to ADP and adenosine by ectonucleotidases that are present in the synaptic cleft (Abbracchio et al., 2009). Thus, ATP co-released from GABAergic terminals (Jo and Schlichter, 1999) can also give rise to adenosine. An additional source of ATP and hence adenosine are astrocytes surrounding the neurons. Thus, it is highly probable that even in the absence of a direct co-release with GABA, adenosine may modulate GABA$_A$R signaling if adenosine receptors are in the vicinity of GABA$_A$Rs. Although to the best of our knowledge no co-localization study of adenosine receptors and GABA$_A$Rs has been published so far, there have been several reports suggesting a possible post-synaptic cross-talk of adenosine receptors and GABA$_A$Rs.

Cross-talk between GABA$_A$ and A1 receptors has been reported in dorsal root ganglion neurons and sacral dorsal commissural nucleus neurons where a suppression of post-synaptic GABA$_A$R-mediated chloride current was observed in the presence of adenosine and the A1 receptor agonist N6-cyclohexyladenosine but not the A2A receptor agonist 2-[*p*-(2-carboxyethyl)-phenethylamino]-5'-*N*-ethylcarboxamidoadenosine (Hu and Li, 1997; Li et al., 2004) using voltage-clamped whole-cell patch recordings. The suppression effect of adenosine could be blocked specifically by the A1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine. Interestingly, this inhibition of GABA$_A$R-mediated chloride flux was observed to occur via a Ca^{2+}-independent–PKC-dependent pathway and also did not involve PKA-dependent pathways. Although the involvement of PKC strongly suggests that post-synaptic A1 receptors were mediating this response, a possible inhibition of GABA release by presynaptic A1 receptors cannot be ruled out. Surprisingly, no study was published in the last few years investigating this cross-talk further. However, a couple of recent studies suggest an important functional role of adenosine receptors in the regulation of GABAergic transmission under pathological conditions. After injecting neurosurgically resected epileptic human brain tissue in *Xenopus* oocytes, the oocytes use the mRNAs present in the tissue to synthesize the respective proteins and receptors that then can be investigated electrophysiologically by whole-cell recordings. When GABA$_A$Rs expressed by these oocytes were investigated, the authors observed a rundown of the GABA-induced current on repetitive application of GABA, which could be significantly reduced by antagonists of A2A, A2B, and A3 receptors and surprisingly not by those of A1 receptors. A similar effect was found in rodent brain (Roseti et al., 2008, 2009). The mechanism behind this effect remains to be elucidated, however the authors speculate on the possible involvement of MAPK and/or PKA-dependent pathway regulating the rundown of GABA$_A$R-mediated current. Adenosine receptor-mediated regulation of GABA$_A$R functioning has additionally been implicated in hypoxia, ischemia, and pain, which is supported by that fact that under the conditions of tissue injury and inflammation, an increase in the levels of purines in the neuronal system is observed (Gourine et al., 2007). It is highly interesting to speculate that a functional cross-talk between GABA$_A$Rs and adenosine receptors may play an important role under conditions of neuro-inflammation.

CROSS-TALK OF GABA$_A$ WITH N-METHYL-D-ASPARTATE RECEPTORS (NMDA) RECEPTORS

Several studies have demonstrated that glutamate and GABA can be co-released from inhibitory as well as excitatory nerve terminals. This is not surprising because glutamate is the precursor for the synthesis of GABA. In one of the first studies, the presence and release of glutamate and aspartate, in addition to GABA, was demonstrated from the anti-GAD immune-purified GABAergic synaptosomal preparations from brain cortex (Docherty et al., 1987). These GABAergic synaptosomes released glutamate in response to depolarizing treatments with either potassium or veratrine, a sodium channel activator. However, whether these synaptosomal preparations were in fact purely GABAergic and not contaminated with glutamatergic synaptosomes cannot be answered retrospectively. Recent studies have identified the type 3 vesicular glutamate transporter (VGLUT3) to be co-localized extensively with vesicular GABA transporter (VGAT) around unstained pyramidal cells and granule cells in the hippocampus

(Boulland et al., 2004) and in developing auditory medial nucleus of the trapezoid body (Gillespie et al., 2005). Boulland et al. (2004), additionally observed a dramatic increase in co-localization of VGAT and VGLUT3 in GABAergic nerve terminals during early stages of development suggesting co-release of glutamate along with GABA plays a crucial role during development. In fact, a very recent work demonstrated that the co-transmission of glutamate in developing GABA/glycinergic sound localization pathway is crucial for synaptic reorganization of inhibitory circuits and disruption of glutamate co-transmission prevented the strengthening of inhibitory connections that would normally occur with maturation (Noh et al., 2010). In a very recent study, Fattorini et al. (2009), observed that GABAergic synapses can express VGLUT1 and glutamatergic synapses can express VGAT. This was further supported by immunoisolation experiments demonstrating that anti-VGAT immunoisolated vesicles contained VGLUT1 and anti-VGLUT1 immunoisolated vesicles contained VGAT (Fattorini et al., 2009). A more recent work using postembedding immunogold double labeling additionally revealed that VGLUT1, VGLUT2, and VGAT coexist in mossy fiber terminals of the hippocampal CA3 area and cerebellar mossy fiber terminals. The presence of VGLUT2 was also demonstrated in cerebellar GABAergic basket cells in this study (Zander et al., 2010).

The co-localization of pre-synaptic transporters for co-released glutamate and GABA suggests a possible co-localization of their respective receptors at post-synaptic terminals. So far, co-localization of glutamate receptors at GABAergic synapses has not been extensively investigated. Recently, however, the existence of NMDA receptors in hippocampal CA1 and CA3c GABAergic synapses has been demonstrated (Szabadits et al., 2011). In these synapses NMDA receptors triggered a retrograde nitric oxide-cGMP cascade, thus modulating GABAergic inhibition in an activity-dependent manner. Since GABAergic inhibition plays a central role in the control of pyramidal cell ensemble activities, such a regulation is able to fine-tune network patterns.

In contrast, localization of GABA$_A$Rs at the post-synaptic membrane of glutamatergic synapses has been demonstrated in several studies. Thus, using electron microscopic immunogold labeling, it was demonstrated that the α6, but not the α1 subunit of GABA$_A$Rs was also concentrated in glutamatergic cerebral mossy fiber synapses (Nusser et al., 1996). More functional evidence on the existence of GABA$_A$Rs at glutamatergic synapses comes from a very recent study where the authors observed that at puberty, expression of inhibitory α4 and δ subunits of GABA$_A$Rs increased by 700% on the plasma membrane perisynaptically to asymmetric glutamatergic synapses of hippocampal CA1 pyramidal neurons (Shen et al., 2010). Among other subunits, the primarily extrasynaptically localized α5 subunit has also been detected at excitatory synapses (Crestani et al., 2002). The existence of post-synaptic GABA$_A$Rs at excitatory synapses may serve to regulate the time course of excitation and to limit glutamatergic overexcitation and subsequent excitotoxicity.

Post-synaptically, glutamate exerts its action via ionotropic NMDARs, AMPARs, and kainate receptors, or metabotropic glutamate receptors (Dingledine et al., 1999). Among these, NMDA receptors have been best studied and shown to directly modulate GABA$_A$R functioning in a Ca^{2+} dependent manner. NMDA receptors are highly permeable to $Ca^{2+}$and are localized to post-synaptic densities, where they are structurally organized in characteristic spines (Lau and Zukin, 2007). Several studies demonstrate that activation of NMDARs resulted in the suppression of GABA$_A$R function due to calcium dependent activation of phosphatase 2B/calcineurin and consecutive dephosphorylation of GABA$_A$Rs in hippocampus (Stelzer and Shi, 1994; Chen and Wong, 1995; Marsden et al., 2007; Bannai et al., 2009) and cerebellum granule cells (Robello et al., 1997). Following dephosphorylation of serine-327 in the intracellular loop of GABA$_A$Rs γ2-subunit, synaptic GABA$_A$Rs then escape to extrasynaptic sites (Wang et al., 2003; Bannai et al., 2009; Muir et al., 2010). In contradiction to all these observations, one study reported that NMDAR activation resulted in the removal of AMPARs from the membrane while simultaneously increased expression of surface GABA$_A$Rs (Marsden et al., 2007). However, this pathway was dependent on Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). This discrepancy possibly could be explained on the basis of the Ca^{2+} level in the cell followed by activation of NMDARs. NMDAR activation of CaMKII is generally associated with high levels of Ca^{2+} influx (Silva et al., 1992), so depending on the level of Ca^{2+} influx through NMDARs, phosphatases (calcineurin), or kinases (CaMKII) may be activated resulting in a decrease or increase of surface GABA$_A$Rs, respectively. Thus, it seems clear that glutamate primarily acting via NMDARs can modulate the expression of GABA$_A$Rs, thus modulating inhibition. It would be interesting to see whether GABA acting via GABA$_A$Rs can also modulate NMDAR expression or function. However a direct cross-talk of NMDARs and GABA$_A$Rs has not been investigated in these studies.

CONCLUSION

Recently it was demonstrated that several members of the G-protein coupled receptors can heteromerize, thus producing functional entities that possess different biochemical characteristics with respect to the individual components of the heteromer. In addition, the qualitative or quantitative aspects of the signaling generated by stimulation of any of the individual receptor units in the heteromer are different from those obtained during co-activation, they thus exhibit "cross-talk" (Ferré et al., 2007). A similar heteromerization obviously can also occur between different types of ligand-gated ion channels and ligand-gated ion channels and G-protein coupled receptors. These interactions allow cross-talk between different signaling pathways, but to date, their molecular nature and functional implications are poorly understood. Here we provide an overview on the current knowledge on the interactions of GABA$_A$R with other ligand-gated ion channels or metabotropic G-protein coupled receptors at the post-synaptic membrane.

GABA$_A$Rs have been clearly demonstrated to heteromerize with GABA$_B$Rs (Balasubramanian et al., 2004), dopamine D5 receptors (Liu et al., 2000), and purinergic P2XRs (Jo et al., 2011; Shrivastava et al., 2011). All these receptors have also been demonstrated to cross-talk with GABA$_A$Rs, resulting in a decrease in GABAergic neurotransmission. As other members of the dopamine receptor family are also known to cross-talk with GABA$_A$Rs, other dopamine receptors (D1–D4) may

also heteromerize with GABA$_A$Rs. Additional experiments will have to be performed to investigate this possibility. Although there is strong evidence of a functional interaction and co-localization of GABA$_A$Rs and GlyRs, a direct coupling between them so far has not been demonstrated. In cross-talk studies involving serotonin 5-HT$_3$Rs, the authors proposed a very close proximity with GABA$_A$Rs, as they observed current occlusion even in the absence of Ca^{2+}or protein kinase inhibitor. As the cross-talk reported was similar to the one described for GABA$_A$/P2X receptors, a possible direct coupling remains a challenging issue to be addressed. Among other receptors known to cross-talk with GABA$_A$Rs are nAChRs and adenosine receptors. However, so far evidence is too weak to speculate on a direct interaction of these receptors with GABA$_A$Rs. Lastly, NMDAR mediated down-regulation of GABA$_A$Rs and thus disinhibition of the respective neuron possibly involves only intracellular signaling mechanisms.

Whereas heteromerization of GABA$_A$R with other receptors in most cases has been demonstrated at synapses, such interactions may also occur at extrasynaptic sites, as observed for GABA$_A$/P2X$_2$ receptor interactions regulating tonic inhibition. In addition, it was demonstrated that these receptors already interact with each other in intracellular compartments, possibly providing a mechanism of specifically targeting the heteromer to extrasynaptic sites. A similar intracellular interaction followed by co-trafficking to the cell membrane has also been observed for GABA$_A$R and GABA$_B$Rs (Balasubramanian et al., 2004). Similar studies have not been performed so far with other receptors that heteromerize with GABA$_A$Rs.

From most of these studies it is evident that a direct or indirect interaction of GABA$_A$R with other receptors resulted in a decrease in GABAergic neurotransmission and in several cases, also in a decreased function of the interacting receptor. Receptor heteromers thus integrate signals from two different neurotransmitter systems, enabling the one to control the effects of the other on neurotransmission (Ferré et al., 2007). So far, no evidence exists for a change in the pharmacological properties of individual receptors of a directly interacting receptor heteromer. But changes in pharmacology elicited by directly interacting proteins are quite plausible (Olsen and Sieghart, 2009; Zhang et al., 2010). Novel drugs addressing the changed pharmacology of these receptor heteromers might thus be developed that could exhibit quite selective properties. In addition, development of drugs interfering with receptor–receptor interactions might be another strategy for modulating not only the function of GABA$_A$Rs, but also that of associated receptors. Overall, it might well be that cross-talk between receptors is a general phenomenon in the nervous system that provides an additional complexity in the regulation of the post-synaptic response. Further studies will have to clarify the mechanism involved and the possible importance of such receptor heteromerizations in health and disease.

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Understanding the molecular diversity of GABAergic synapses

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GABAergic synapses exhibit a high degree of subcellular and molecular specialization, which contrasts with their apparent simplicity in ultrastructural appearance. Indeed, when observed in the electron microscope, GABAergic synapses fit in the symmetric, or Gray's type II category, being characterized by a relatively simple postsynaptic specialization. The inhibitory postsynaptic density cannot be readily isolated, and progress in understanding its molecular composition has lagged behind that of excitatory synapses. However, recent studies have brought significant progress in the identification of new synaptic proteins, revealing an unexpected complexity in the molecular machinery that regulates GABAergic synaptogenesis. In this article, we provide an overview of the molecular diversity of GABAergic synapses, and we consider how synapse specificity may be encoded by selective *trans*-synaptic interactions between pre- and postsynaptic adhesion molecules and secreted factors that reside in the synaptic cleft. We also discuss the importance of developing cataloguing tools that could be used to decipher the molecular diversity of synapses and to predict alterations of inhibitory transmission in the course of neurological diseases.

Keywords: GABAergic synapse, $GABA_A$ receptor, gephyrin, dystroglycan, neuroligin, neurexin, synapse specificity

INTRODUCTION

One of the most striking aspects of inhibitory synaptic circuits is the remarkable diversity of GABAergic systems. GABAergic interneurons occur in many different subtypes, that play exquisitely precise functions in neural networks (Markram et al., 2004). Each type of interneuron is highly selective, making synapses with particular populations of target cells and only with specific subcellular compartments (Huang et al., 2007). Moreover, the diversity of GABAergic interneurons matches a corresponding multiplicity of synaptic and extrasynaptic $GABA_A$ receptor ($GABA_AR$) subtypes, that support neural circuit operations under an extensive range of behavior-dependent brain states (Monyer and Markram, 2004; Freund and Katona, 2007; Klausberger and Somogyi, 2008; Olsen and Sieghart, 2009). For example, in hippocampal and cortical circuits, basket cells containing either parvalbumin (PV) or cholecystokinin (CCK) target the cell body of pyramidal cells at synapses containing mainly α1β2/3γ2 $GABA_ARs$, which display fast kinetics of deactivation, or α2β2/3γ2 $GABA_ARs$, which have slower kinetics compared to receptors containing the α1 subunit (Klausberger et al., 2002; Klausberger and Somogyi, 2008). While PV-basket cells appear to control the temporal coordination of principal neurons and network oscillations, CCK-positive interneurons modulate synchronous activities by integrating subcortical and local modulatory signals (Freund and Katona, 2007; Sohal et al., 2009; Thomson and Jovanovic, 2010). Notably, these differences can be extended to the level of pathology, as the two types of $GABA_ARs$ appear to be implicated in different types of neurological and psychiatric disorders (Lewis et al., 2005; Freund and Katona, 2007). Similarly, genetic manipulations of individual $GABA_AR$ subunits in mice produce selective alterations in behavior, reinforcing the idea that specific neuronal networks characterized by respective $GABA_AR$ subtypes are involved in the regulation of defined behavioral patterns (McKernan et al., 2000; Möhler, 2006, 2007; Whiting, 2006).

This extraordinary diversity contrasts with the apparent simplicity in the ultrastructural appearance of GABAergic synapses. When observed in the electron microscope, GABAergic synapses fit in the symmetric, or Gray's type II category (Gray, 1959; Colonnier, 1968; Peters and Palay, 1996). These synapses are characterized by a thin postsynaptic density (PSD), similar in size to the presynaptic active zone. In contrast, glutamatergic synapses belong to the asymmetric, or Gray's type I group, which is distinguished by a prominent PSD. Asymmetric synapses also show a conspicuous electron dense material in the synaptic cleft, which is less obvious in symmetric synapses. Not surprisingly, the morphological differences between type I and type II synapses are proportional to their molecular complexity. Proteomic studies of the excitatory PSD have revealed a number of proteins greater than 1000 (Collins et al., 2005; Bayés et al., 2011), which exceeds by two orders of magnitude the number of molecules that have been found in the inhibitory PSD (Lüscher and Keller, 2004; Charych et al., 2009). It would be hazardous, however, to conclude that all GABAergic synapses share the same basic molecules and mechanisms to sustain their exquisite specificity. In this essay, we review recent work that points to a remarkable heterogeneity of the molecular machinery that regulates GABAergic synaptogenesis *in vivo* and we provide a personal view of the mechanisms that may underlie synapse specificity during the development of neural circuits.

Understanding these mechanisms is of interest for both basic and clinical neuroscience, as disruption of inhibitory synapse development is now regarded as a major cause of brain disease (Lewis et al., 2005; Südhof, 2008; Charych et al., 2009). A detailed description of the molecular organization of GABAergic synapses falls outside the scope of this paper and can be found in other excellent review articles (Lüscher and Keller, 2004; Tretter and Moss, 2008). Here we rather focus on the evidence that the diversity of GABAergic synapses may be generated by the interplay of multiple molecular mechanisms with partially overlapping functions. We also discuss the importance of a classification scheme that could be used to identify distinct types of synapses based on the differential expression of groups of interacting proteins.

POSTSYNAPTIC SCAFFOLDS OF INHIBITORY SYNAPSES

One of the main achievements in the field of synapse research has been the characterization of the molecular components of the PSD. Molecular investigations of glutamatergic synapses have shown that the PSD is a specialized microdomain characterized by core scaffolding proteins, such as PSD-95, that link glutamate receptors to the subsynaptic cytoskeleton and also interact with different types of regulatory proteins and with cell adhesion molecules via specific PDZ domains (Kennedy, 1997; Ziff, 1997; O'Brien et al., 1998; Garner et al., 2000; Kim and Sheng, 2004; Boeckers, 2006). Importantly, there is increasing evidence that protein–protein interactions within the PSD are not static and that dynamic modulation of the PSD provides a mechanism for the regulation of synaptic plasticity (Scannevin and Huganir, 2000; Kim et al., 2007; Steiner et al., 2008). The inhibitory PSD cannot be readily isolated, and progress in understanding its molecular composition has lagged behind that of excitatory synapses. The multi-domain, 93 kDa protein gephyrin has emerged as a major scaffolding molecule of the inhibitory PSD (Kneussel and Betz, 2000; Fritschy et al., 2008). Here we briefly summarize the proposed functions of gephyrin and we describe other scaffolding molecules that may contribute to assemble postsynaptic specializations in at least some subtypes of inhibitory synapses.

GEPHYRIN

Gephyrin was originally copurified with the glycine receptor (GlyR; Pfeiffer et al., 1982), and was later found also at postsynaptic sites of GABAergic synapses (Sassoè-Pognetto et al., 1995; Sassoè-Pognetto and Fritschy, 2000). This molecule lacks PDZ domains, but can form aggregates by spontaneous oligomerization, although the precise mechanisms by which gephyrin forms postsynaptic scaffolds is still unresolved (for review, see Fritschy et al., 2008). Gephyrin binds a cytoplasmic loop of the GlyR β subunit (Meyer et al., 1995), and is essential for postsynaptic clustering of GlyRs (Kirsch et al., 1993; Feng et al., 1998). This scaffold protein also contributes to stabilize postsynaptic $GABA_A$Rs, as its knockdown in cultured neurons causes a disruption of $GABA_A$R clusters (Essrich et al., 1998; Yu et al., 2007). Similarly, knockout of the gephyrin gene in mice results in an extensive loss of postsynaptic $GABA_A$R aggregates (Kneussel et al., 1999, 2001; Fischer et al., 2000; Lévi et al., 2004).

The precise mechanisms by which gephyrin clusters $GABA_A$Rs are poorly understood, although there is evidence that this molecule restrains the lateral mobility of the receptors in the plasma membrane (Jacob et al., 2005; Thomas et al., 2005). Most likely, this function involves interactions with the cytoskeleton, as gephyrin binds with high affinity polymerized tubulin (Kirsch et al., 1991; Kirsch and Betz, 1995) and serves as an adaptor for regulators of microfilament dynamics (Mammoto et al., 1998; Giesemann et al., 2003; Bausen et al., 2006). Interestingly, the clustering of gephyrin and $GABA_A$Rs are to some extent mutually dependent on each other, since synaptic gephyrin clusters are disrupted after deletion of $GABA_A$Rs (Essrich et al., 1998; Schweizer et al., 2003; Li et al., 2005; Kralic et al., 2006; Studer et al., 2006). Alldred et al. (2005) have identified the fourth transmembrane domain of the $GABA_A$R γ2 subunit as essential to mediate postsynaptic clustering of $GABA_A$Rs, whereas the major γ2 cytoplasmic loop is required for recruitment of gephyrin to $GABA_A$R clusters. Direct interactions between gephyrin and the $GABA_A$R α2 and α3 subunits have emerged only recently (Tretter and Moss, 2008; Saiepour et al., 2010). The binding of gephyrin to the α subunits appears to be detergent sensitive, which may explain why it has been remarkably difficult to reveal these interactions using biochemical approaches. However, whether gephyrin binding to $GABA_A$Rs involves multiple interactions with distinct α and γ subunits is presently unclear. The relevance of the numerous gephyrin isoforms generated by alternative splicing is also not understood (Paarmann et al., 2006; Fritschy et al., 2008). Moreover, gephyrin function may depend on post-translational modifications. Indeed, recent studies indicate that phosphorylation of gephyrin at specific residues contributes to regulate the anchoring of GlyRs and $GABA_A$Rs at postsynaptic sites (Zita et al., 2007; Charrier et al., 2010; Tyagarajan et al., 2011).

A still unresolved issue is whether gephyrin contributes equally to the clustering of all major subtypes of synaptic $GABA_A$Rs (that is receptors that are highly concentrated in the postsynaptic membrane and mediate phasic inhibition). Extensive experimental evidence indicates that receptors containing a γ2 subunit in association with two α and two β subunits (α1β2/3γ2, α2β2/3γ2, and α3β2/3γ2) are the predominant types of synaptic $GABA_A$Rs (for review, see Lüscher and Keller, 2004; Farrant and Nusser, 2005). The analysis of spinal cord sections, retina organotypic cultures, and cultured hippocampal neurons derived from gephyrin knockout mice lead to the idea that gephyrin mediates the postsynaptic accumulation of $GABA_A$Rs containing the α2 or the α3 subunit, and suggested the existence of additional clustering mechanisms (Fischer et al., 2000; Kneussel et al., 2001; Lévi et al., 2004). However, in the brain gephyrin colocalizes with all major types of postsynaptic $GABA_A$Rs containing either the α1, α2, or α3 subunit (Sassoè-Pognetto et al., 2000), indicating that its function is not restricted to α2 and α3-containing synapses. Because gephyrin knockout mice die at birth (Feng et al., 1998), a better appreciation of gephyrin function at distinct types of GABAergic synapses may derive from the study of mouse models with conditional deletion of this protein in selected populations of neurons characterized by the expression of distinct $GABA_A$R subtypes. The complex situation regarding the role of gephyrin in the clustering of different $GABA_A$Rs may also be explained by redundancy between multiple clustering factors with partially overlapping synaptic expression profiles (see below).

THE DYSTROPHIN–GLYCOPROTEIN COMPLEX

Another postsynaptic scaffold that is present in some GABAergic synapses is dystrophin. This protein belongs to the dystrophin–glycoprotein complex (DGC), a large, membrane-spanning protein complex that links the cytoskeleton to the extracellular matrix (Ervasti and Campbell, 1991; Blake and Kröger, 2000; Waite et al., 2009). Dystrophin is derived from a large gene with at least seven internal promoters that enable the expression of several distinct isoforms (for review see Perronnet and Vaillend, 2010). Interestingly, the full-length (Dp427) isoform is derived from three independent promoters with differential expression in muscle, forebrain, and cerebellar Purkinje cells. The N-terminal domain of dystrophin binds to filamentous actin, whereas the C-terminal domain interacts with dystrobrevins (α and β) and syntrophins (α, β1–2, γ1–2), which are also cytoplasmic constituents of the DGC. Several dystrobrevin-binding elements have been identified, including dysbindin, a protein that has been associated with schizophrenia (Benson et al., 2001; Talbot et al., 2009). However, immunohistochemical analyses suggest that dysbindin is not enriched at GABAergic synapses (unpublished observations). Syntrophins are adaptor proteins each containing a PDZ domain and two pleckstrin homology (PH) domains mediating interactions with several other proteins, including kinases, ion and water channels, and nNOS (Waite et al., 2009). Syntrophin colocalizes with $GABA_A$Rs in cultured hippocampal neurons (Brünig et al., 2002), however the synaptic localization of endogenous syntrophins in unknown. Of particular interest is the fact that the γ2-syntrophin isoform has been reported to interact with the PDZ binding motif of the adhesion molecules neuroligin 3 and neuroligin 4 (Yamakawa et al., 2007), which are present at inhibitory synapses (see below). It is therefore possible that interactions between syntrophins and neuroligins may help organize postsynaptic scaffolds at inhibitory synapses (**Figure 1**).

A core component of the DGC is dystroglycan, which is composed of an extracellular α subunit and a transmembrane β subunit, derived by proteolytic cleavage from a single precursor protein (Ibraghimov-Beskrovnaya et al., 1992). The β-dystroglycan subunit contains a single transmembrane domain and its cytoplasmic tail binds dystrophin, whereas α-dystroglycan is a secreted glycoprotein that binds to LNS (laminin G, neurexins, and sex hormone-binding globulin)-domain-containing proteins, such as laminin, perlecan, agrin, neurexin, and pikachurin (Bowe et al., 1994; Gee et al., 1994; Talts et al., 1999; Sugita et al., 2001; Sato et al., 2008). The fact that α-dystroglycan binds neurexin is particularly intriguing, as it suggests that the DGC may mediate *trans*-synaptic interactions between pre- and postsynaptic specializations.

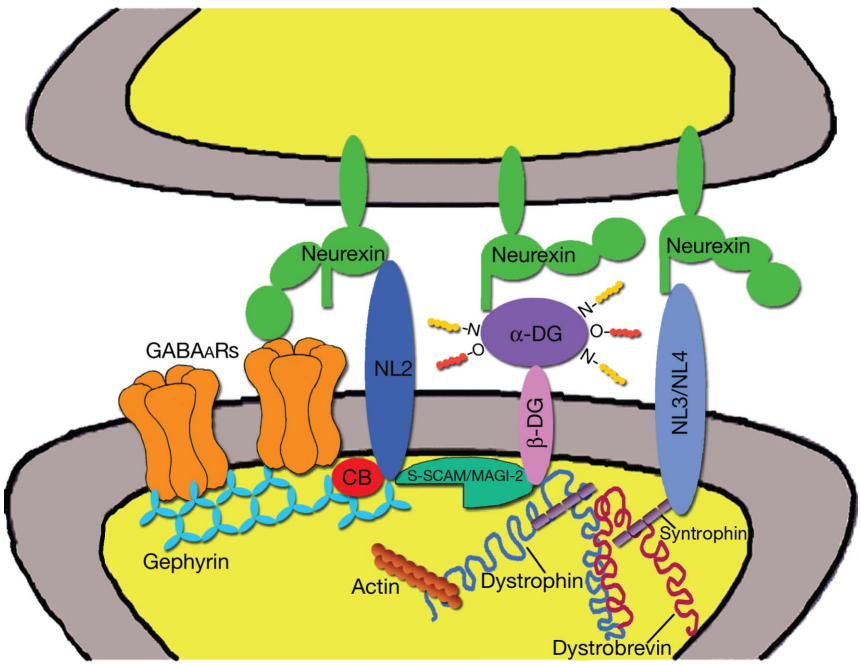

FIGURE 1 | Postsynaptic scaffolds and adhesion molecules of GABAergic synapses. The diagram is based on reported molecular interactions (see text), some of which remain to be confirmed *in vivo*. Gephyrin, S-SCAM/MAGI-2, and dystrophin are shown in the same postsynaptic specialization, although dystrophin is present in only a subset of GABAergic synapses and the *in vivo* distribution of S-SCAM/MAGI-2 has not been characterized. Gephyrin trimers are believed to aggregate into a submembranous lattice that provides stability to postsynaptic $GABA_A$Rs. Gephyrin also binds collybistin (CB) and neuroligin 2 (NL2), that has been proposed to function as a specific activator of collybistin. Cytoskeletal proteins associated with gephyrin, such as Mena/VASP and microtubules, are not shown. Neuroligin 2 bridges the synaptic cleft and binds to neurexins on the presynaptic terminal. Reported interactions between neurexins and $GABA_A$Rs are also indicated. Neurexins may also interact with α-dystroglycan (α-DG), thus establishing a link with the dystrophin–glycoprotein complex (DGC). One component of the DGC, syntrophin, has been reported to bind neuroligin 3 and neuroligin 4. Finally, there is evidence that S-SCAM/MAGI-2 may establish a link between neuroligin 2 and the DGC by interacting with the intracellular domain of β-dystroglycan (β-DG).

Immunohistochemical analyses have shown that the DGC is present in a subset of GABAergic synapses, specifically in cerebellar Purkinje cells and in forebrain pyramidal neurons (Knuesel et al., 1999; Sekiguchi et al., 2009; Briatore et al., 2010). Interestingly, both *in vitro* and *in vivo* analyses have shown that the synaptic localization of dystrophin and dystroglycan are independent of $GABA_A$Rs and gephyrin, suggesting that the DGC has the capacity to self-assemble at postsynaptic sites (Brünig et al., 2002; Lévi et al., 2002; Patrizi et al., 2008a). The studies that have investigated the function of the DGC at GABAergic synapses have generated partially conflicting results. Deletion of dystroglycan in cultured hippocampal neurons caused a loss of postsynaptic dystrophin, but did not affect the localization of gephyrin and $GABA_A$Rs (Lévi et al., 2002). In contrast, *in vivo* analyses of *mdx* mice, that lack the full-length version of dystrophin, revealed a selective deficit in the synaptic clustering of $GABA_A$Rs, but not gephyrin, in cerebellum, hippocampus, and amygdala (Knuesel et al., 1999; Sekiguchi et al., 2009; Vaillend et al., 2010). Similar results have been reported in the cerebellum of double knockout mice lacking both α and β dystrobrevins (Grady et al., 2006). The selective loss of $GABA_A$R clusters, but not gephyrin clusters in *mdx* mice is surprising, also considering that deletion of $GABA_A$Rs from Purkinje cells causes a severe defect in the clustering of gephyrin (Kralic et al., 2006) without affecting dystrophin and dystroglycan (Patrizi et al., 2008a). However, there is extensive electrophysiological evidence indicating that an intact DGC is required for normal GABAergic inhibition (Anderson et al., 2003; Kueh et al., 2008; Sekiguchi et al., 2009). In short, the available data indicate that the DGC is involved in modulating synaptic function in a subset of GABAergic synapses. However, the precise organization of the synaptic DGC and the specific contribution of its molecular constituents require further investigations.

S-SCAM/MAGI-2

According to a recent study, the synaptic scaffolding molecule (S-SCAM)/membrane-associated guanylate kinase with inverted organization (MAGI)-2 localizes at inhibitory synapses in rat primary hippocampal neurons (Sumita et al., 2007). This is surprising, because S-SCAM/MAGI-2 has a molecular organization similar to PSD-95, harboring multiple PDZ domains, a guanylate kinase, and two WW domains. S-SCAM/MAGI-2 is also present at glutamatergic synapses, where it interacts with NMDA receptors, neuroligin 1, and β-catenin (Hirao et al., 1998; Iida et al., 2004). At GABAergic synapses, it has been reported that S-SCAM/MAGI-2 can interact with β-dystroglycan and neuroligin 2, suggesting that this scaffold molecule may provide a link between the DGC and the neurexin–neuroligin adhesion system (Sumita et al., 2007). Thus it appears that S-SCAM/MAGI-2 and syntrophins may mediate selective interactions between the DGC and, respectively, neuroligin 2 and neuroligins 3 and 4 (**Figure 1**). Research on S-SCAM/MAGI-2 is still at its beginnings, but it may lead to the discovery of new mechanisms underlying the assembly of the inhibitory PSD. It will be important to characterize the spatio-temporal profile of S-SCAM/MAGI-2 expression in relation to the other molecular constituents of GABAergic postsynapses. In addition, it will be of primary interest to investigate the possible redundancy of *trans*-synaptic signals mediated by the DGC and the neurexin–neuroligin adhesion system. More in general, *in vivo* analyses are needed to get a clear picture of the endogenous distributions of these different scaffolding systems and to help dissecting their specific roles in different populations of synapses.

VARIABILITY IN THE MECHANISMS THAT REGULATE THE POSTSYNAPTIC ACCUMULATION OF GEPHYRIN

While gephyrin is expressed almost ubiquitously at inhibitory synapses, recent studies have evidenced an unexpected variability in the mechanisms that control its synaptic localization. For example, in cerebellar Purkinje cells gephyrin is expressed initially at all GABAergic synapses, however during postnatal development gephyrin clusters disappear from perisomatic synapses and remain exclusively at axodendritic contacts (Viltono et al., 2008). While the reasons for this differential regulation are unclear, the loss of gephyrin is mirrored by a structural reorganization of perisomatic synapses, consisting in a reduction in the size of $GABA_A$R clusters and in the length of synaptic appositions.

One protein implicated in the recruitment of gephyrin to postsynaptic specializations is the GEF (guanine nucleotide exchange factor) collybistin, that was identified as a gephyrin-binding protein in a two-hybrid screening (Kins et al., 2000). Collybistin, like other GEFs, is characterized by an N-terminal *src* homology 3 (SH3) domain, a catalytic tandem Dbl homology (DH) domain and a PH domain. It is believed that collybistin participates in the membrane targeting of gephyrin by binding membrane lipids through its PH domain (Harvey et al., 2004; Reddy-Alla et al., 2010). Different collybistin isoforms (CB1-3) have been identified, which are created by alternative splicing of exons encoding the SH3 domain and three alternate C termini (Kins et al., 2000; Harvey et al., 2004). Interestingly, expression studies have shown that the SH3 domain negatively regulates collybistin function (Kins et al., 2000; Harvey et al., 2004). However, most endogenous collybistin isoforms harbor this region, suggesting that collybistin activity requires protein–protein interactions at the SH3 domain (Kins et al., 2000; Harvey et al., 2004). Indeed, recent investigations have shown that the synaptic adhesion molecules neuroligin 2 and neuroligin 4 can bind to and activate collybistin by relieving the SH3-mediated inhibition (Poulopoulos et al., 2009; Hoon et al., 2011). In addition, all neuroligin isoforms have in their cytoplasmic domain a conserved gephyrin-binding motif that contributes to recruit gephyrin to synapses. It has been proposed that by interacting with gephyrin and collybistin neuroligin 2 can act as a nucleation site for the formation of postsynaptic gephyrin scaffolds that recruit $GABA_A$Rs at postsynaptic sites (Poulopoulos et al., 2009). On the other hand, knockout of neuroligin 2 has only a relatively small effect on the clustering of gephyrin and $GABA_A$Rs, specifically at perisomatic synapses of hippocampal neurons (Poulopoulos et al., 2009). In contrast, deletion of collybistin or $GABA_A$Rs causes an extensive loss of gephyrin clusters (Essrich et al., 1998; Li et al., 2005; Kralic et al., 2006; Studer et al., 2006; Papadopoulos et al., 2007; Patrizi et al., 2008a). These observations question the importance of neuroligin 2 as a major physiological clustering factor for gephyrin and collybistin and suggest that there could be multiple pathways capable of activating collybistin with differential cellular and subcellular specificity.

Mouse genetic studies have revealed that *in vivo* collybistin is required for the initial localization and maintenance of gephyrin

and $GABA_AR$ clusters in a subset of inhibitory synapses in selected brain regions, particularly in the hippocampus and basolateral amygdala (Papadopoulos et al., 2007, 2008). Surprisingly, deletion of collybistin did not affect the organization of GABAergic synapses in other regions, nor that of glycinergic synapses. These findings indicate that the mechanisms that control the assembly of the inhibitory PSD are region and synapse-specific. This selectivity may be explained by the fact that the expression of collybistin at inhibitory synapses is highly heterogeneous. In the retina, collybistin is preferentially colocalized with α2-$GABA_ARs$, and shows limited localization at synapses containing other $GABA_AR$ subtypes or GlyRs (Saiepour et al., 2010). Similarly, in brain circuits collybistin has been found in only a subset of gephyrin-positive synapse, although no specific association with particular $GABA_AR$ subtypes was found (unpublished observations). These observations suggest that other GEFs or unknown clustering factors may also contribute to cluster gephyrin at postsynaptic sites. Interestingly, a recent study has revealed that SynArfGEF (also known as BRAG3 or IQSEC3), a member of the A-resistant Arf-GEF/IQSEC3 family, localizes at postsynaptic specializations of GABAergic and glycinergic synapses and can interact with dystrophin and S-SCAM/Magi-2 (Fukaya et al., 2011). Thus, SynArfGEF is another GEF expressed at inhibitory synapses, although its precise function remains to be determined. It will be important to understand whether SynArfGEF, like collybistin, is associated with selected subtypes of inhibitory synapses, and whether these two GEFs have differential or partially overlapping distributions.

SYNAPTIC SPECIFICITY DEPENDS ON MULTIPLE MECHANISMS

A crucial and still open question in developmental neurobiology is to decipher the mechanisms that ensure the formation of functional connections between appropriate synaptic partners characterized by distinct molecular signatures. A large number of studies in both vertebrate and invertebrate nervous systems have shown that the specificity of synapses depends on multiple mechanisms, including homophilic and heterophilic interactions between adhesion molecules, secreted synaptic organizers, anti-synaptogenic molecules, interactions with guidepost and/or glial cells, temporally restricted expression of transcription factors, and defined patterns of neuronal activity (for a recent review see Margeta and Shen, 2010). To illustrate the remarkable variety of the mechanisms that give rise to connectional specificity, we refer to recent work on cerebellar Purkinje cells. These neurons receive GABAergic inhibition mainly from basket cells, that target the cell body and the axon initial segment (AIS), and from stellate cells, that innervate exclusively the dendritic shafts (Palay and Chan-Palay, 1974). Both types of synapse express the same $GABA_AR$ subtype containing the α1 subunit (Fritschy et al., 2006). Studies in transgenic mouse models have shown that stellate and basket cells use different molecular cues to innervate distinct subcellular domains of Purkinje cells. The targeting of basket axons to the AIS depends on a subcellular gradient of neurofascin 186, a cell adhesion molecule of the L1 immunoglobulin family (Ango et al., 2004). This gradient requires ankyrinG, a membrane adaptor protein that is restricted to the AIS and recruits neurofascin. In ankyrinG-deficient Purkinje cells, the neurofascin gradient is abolished, and basket axons lose their directional growth along Purkinje cells, resulting in impaired synapse formation. On the other hand, the formation of stellate cell synapses depends on close homolog of L1 (CHL1), another member of the same family of adhesion molecules, localized along Bergmann glia fibers (Ango et al., 2008). Thus, different members of the L1 family of cell adhesion molecules contribute to axon patterning and subcellular synapse organization in different types of interneurons, although it seems that these molecules are more directly involved in axon guidance rather than in mediating synapse formation.

There is also evidence that $GABA_ARs$ play a remarkably selective role in the refinement of perisomatic and axodendritic synapses in Purkinje cells. Deletion of $GABA_ARs$ from Purkinje cells causes a selective decrease in the density of axodendritic synapses without altering the number of perisomatic synapses (Fritschy et al., 2006; Patrizi et al., 2008a). Notably, the reduced axodendritic innervation is accompanied by the appearance of numerous heterologous contacts between GABAergic axon terminals and Purkinje cell spines, which retain an asymmetric PSD typical of glutamatergic synapses (Fritschy et al., 2006).

These examples highlight two important aspects of synaptic specificity. First, the selectivity of connections does not depend on a hard-wired process based on exclusive cellular interactions, but rather results from a mechanism of selection among potential synaptic partners. In Purkinje cells, the silencing of GABAergic transmission is sufficient to boost ectopic synapses on spines, suggesting that in this specific case activity-dependent competition is a major determinant of synaptic specificity. Second, different synapses are subject to different regulation, implying that mutations that perturb synapse development in some populations of synapses may leave other synapses unaffected. This heterogeneity must be understood before common principles of synaptogenesis can be defined.

COMPLEX ORGANIZATION OF ADHESION MOLECULES AT INHIBITORY SYNAPSES

Once a synaptic adhesion has been established, it is essential that synaptic specializations recruit the correct complement of pre- and postsynaptic molecules, including the correct types of neurotransmitter receptors and their anchoring proteins. In the case of GABAergic synapses, it is still unknown how postsynaptic neurons cluster distinct types of $GABA_ARs$ at synapses that can be located only a few micrometers apart. Selective interactions between pre- and postsynaptic adhesion molecules have been invoked to explain the selectivity in the segregation of different $GABA_ARs$ (Thomson and Jovanovic, 2010). Indeed, there seems to be enough variability in the different families of synaptic adhesion molecules to support this function (Scheiffele, 2003; Yamagata et al., 2003; Washbourne et al., 2004; Craig et al., 2006; Piechotta et al., 2006; Dalva et al., 2007; Arikkath and Reichardt, 2008; Biederer and Stagi, 2008; Brose, 2009; Siddiqui and Craig, 2010; Tallafuss et al., 2010), although none of the known adhesion proteins appears to have a selective localization that would be compatible with a role in segregating distinct $GABA_ARs$ to different synapses. Here we discuss the possibility that synapse diversity may result from the differential co-expression of multiple adhesion molecules with partially overlapping distributions.

Neuroligin 2 is arguably the best characterized adhesion molecule of GABAergic synapses. This molecule belongs to a family of four (in rodents) isoforms, which appear to segregate into distinct types of excitatory and inhibitory synapses (Song et al., 1999; Craig and Kang, 2007). Neuroligin 2 is localized at postsynaptic sites of inhibitory synapses throughout the brain (Varoqueaux et al., 2004; Patrizi et al., 2008a), and promotes GABAergic synaptogenesis *in vitro* (Graf et al., 2004; Chih et al., 2006; Chubykin et al., 2007). Moreover, neuroligin 2 knockout mice exhibit selective deficits in GABAergic neurotransmission, indicating that this adhesion molecule is required for normal synapse development (Chubykin et al., 2007; Hoon et al., 2009; Jedlicka et al., 2011).

Recent studies have evidenced a complex organization of neuroligins at inhibitory synapses. While NL2 is present in practically all inhibitory synapses throughout the brain, other neuroligin isoforms have a more restricted distribution. Thus, NL4 is mainly associated with glycinergic synapses (Hoon et al., 2011), whereas NL3 is coexpressed with NL2 in subsets of GABAergic synapses (Budreck and Scheiffele, 2007; Patrizi et al., 2008b). These observations indicate that differential expression of neuroligins may confer specific functional properties to individual synapses, although the contribution of each individual neuroligin isoform remains unclear. Interestingly, recent research has indicated that NL2 has quite selective functions at GABAergic synapses, despite its broad distribution. In hippocampal pyramidal neurons, deletion of NL2 decreases the amplitude of IPSCs evoked from PV-positive interneurons, but has no effect on IPSCs evoked from somatostatin-positive cells (Gibson et al., 2009). A similar level of selectivity has been reported also for other cell adhesion molecules. For example, perturbation of the neural cell adhesion molecule (NCAM) produces selective effects on GABAergic synapses in frontal and cingulate cortex and in the amygdala, but not in hippocampus (Pillai-Nair et al., 2005). Together, these data reveal an unexpected variability in the synaptic properties conferred by individual cell adhesion molecules and provide support to the idea that synaptic specificity may be encoded by multiple interactions between selective combinations of synaptogenic proteins.

It is generally assumed that neuroligins promote synapse maturation by interacting with presynaptic neurexins (Ushkaryov et al., 1992; Graf et al., 2004; Chih et al., 2005; Kang et al., 2008). Neurexins occur in six different isoforms (three longer α-neurexins and three shorter β-neurexins), that are further subject to alternative splicing, giving rise to several distinct variants that can bind with different affinities to multiple types of postsynaptic partners, including neuroligins, LRRTMs (leucine-rich repeat transmembrane neuronal proteins), neurexophilins, and the Cbln1–GluD2 (cerebellin 1-glutamate receptor δ2) complex (Ullrich et al., 1995; Missler and Südhof, 1998; Koehnke et al., 2010; Siddiqui and Craig, 2010; Uemura et al., 2010; Wright and Washbourne, 2011). In particular, the presence or the absence of an insert at splice site 4 (S4) appears to be an important determinant of binding partner selectivity (for review, see Craig and Kang, 2007; Siddiqui and Craig, 2010). Co-culture studies support a preferential role of α-neurexins(+S4) in mediating GABAergic synaptogenesis (Boucard et al., 2005; Chih et al., 2006; Kang et al., 2008). Likewise, knockout of all α-neurexins decreases considerably the density of GABAergic synapses in cortex (Missler et al., 2003). Interestingly, α neurexins can also interact with α-dystroglycan (Sugita et al., 2001), which is present in a subset of GABAergic synapses as discussed above. Neurexins have also been reported to interact directly with $GABA_A$Rs, although the importance of these interactions for synapse development is still unclear (Zhang et al., 2010). In summary, although there is clear evidence that both neuroligin 2 and α-neurexins(+S4) promote GABAergic synaptogenesis *in vitro*, the extensive alternative splicing and numerous binding partners of neurexins suggest that these molecules may regulate synapse development by multiple, and not necessarily shared, mechanisms. As novel studies evidence that neuroligins can regulate glutamatergic synaptogenesis by neurexin independent mechanisms (Ko et al., 2009) and, *vice versa*, that neurexins induce the formation of glutamatergic synapses by interacting with postsynaptic molecules other than neuroligins (Uemura et al., 2010; Matsuda and Yuzaki, 2011), it appears reasonable to re-evaluate the relevance of neurexin–neuroligin interactions in the context of inhibitory synapse development. It will also be of primary interest to understand what is the expression profile of the different neurexin isoforms in distinct types of excitatory and inhibitory synapses.

SYNAPTIC CLEFT PROTEINS PROVIDE A FURTHER LEVEL OF COMPLEXITY

An emerging concept is that proteins localized in the synaptic cleft may act bi-directionally to coordinate selective interactions between the pre- and postsynaptic compartments. By itself, this is not a new idea, as it is well established that at the neuromuscular junction secreted proteins, such as agrin and laminin, serve as synaptic organizers (Kummer et al., 2006; Witzemann, 2006; Rushton et al., 2009). Recently, a novel class of secreted molecules that link pre- and postsynaptic specializations has been characterized in the cerebellar cortex. Specifically, it has been shown that Cbln1 acts as a crucial synaptic organizer that is required for the formation and maintenance of glutamatergic synapses made by parallel fibers with Purkinje cell spines (Yuzaki, 2010). Cbln1 is a glycoprotein of the C1q family that is secreted from cerebellar granule cells. Mice lacking Cbln1 are ataxic and show a surprising similarity to mice lacking the δ2 glutamate receptor (GluD2), which is expressed selectively in Purkinje cells. Both these mutants have a remarkable (∼50%) reduction in the number of parallel fiber-Purkinje cell synapses, with the remaining synapses showing a mismatch between PSDs and presynaptic active zones, as well as impaired LTD (Kashiwabuchi et al., 1995, Kurihara et al., 1997, Hirai et al., 2005). The similarities in the structural and functional abnormalities observed in *Cbln1*-null and *GluD2*-null mice have suggested that these two molecules are engaged in a common signaling pathway. Indeed it has been demonstrated that Cbln1 binds to the N-terminal domain of GluD2 (Matsuda et al., 2010), and that the Cbln1–GluD2 complex mediates synapse formation by interacting selectively with neurexins(+S4) (Uemura et al., 2010; **Figure 2**).

These new exciting findings show that secreted proteins can act as divalent ligands linking pre- and postsynaptic transmembrane components. Accordingly, the synaptic cleft can be regarded as the site in which secreted factors and cell adhesion molecules

mediate *trans*-synaptic interactions that may contribute to encode synaptic specificity. For example, the ternary interaction between neurexin(+S4), Cbln1 and GluD2 may represent a "protein code" specific for parallel fiber-Purkinje cell synapses (Uemura et al., 2010). Notably, there is evidence that Cbln1 and the closely related Cbln2 can interact with neurexins and mediate synapse formation not only in cerebellum but also in forebrain regions (Matsuda and Yuzaki, 2011). Co-culture analyses have shown that Cbln1 and Cbln2 induce preferentially inhibitory presynaptic differentiation by interacting with neurexin variants containing S4, although the postsynaptic partners remain unknown (Joo et al., 2011). It is therefore reasonable to assume that differential interactions of neurexin variants with neuroligins, Cbln and LRRTMs may be involved in specifying distinct types of excitatory and inhibitory synapses. By analogy, we suggest that *trans*-synaptic interactions mediated by α-dystroglycan may constitute a molecular code for a specific subset of GABAergic synapses (**Figure 2**).

In short, cross-interactions between synaptic cleft proteins and cell adhesion molecules provide an additional level of complexity that could be exploited by neurons to functionally specify synapses. It is now a key task to increase our understanding of the synaptic extracellular matrix or synaptomatrix (Vautrin, 2010). Ultrastructural analyses have shown that this material is particularly dense, even denser than the neuronal cytosol, and is characterized by periodically organized complexes, suggesting a regular arrangement of cleft proteins (Zuber et al., 2005). Resolving the molecular interactions that occur in the synaptomatrix is likely to provide important insights into the mechanisms that underlie the formation and specificity of synapses.

A MOLECULAR CATALOG OF INHIBITORY SYNAPSES

The studies revised above have revealed an unexpected complexity in the molecules and mechanisms that control the assembly and specificity of inhibitory synapses during the formation of neural circuits. An important insight that has emerged from these investigations is that not all synaptic proteins are expressed equally at all inhibitory synapses, suggesting that synapse diversity is produced by unique combinations of synaptic molecules with

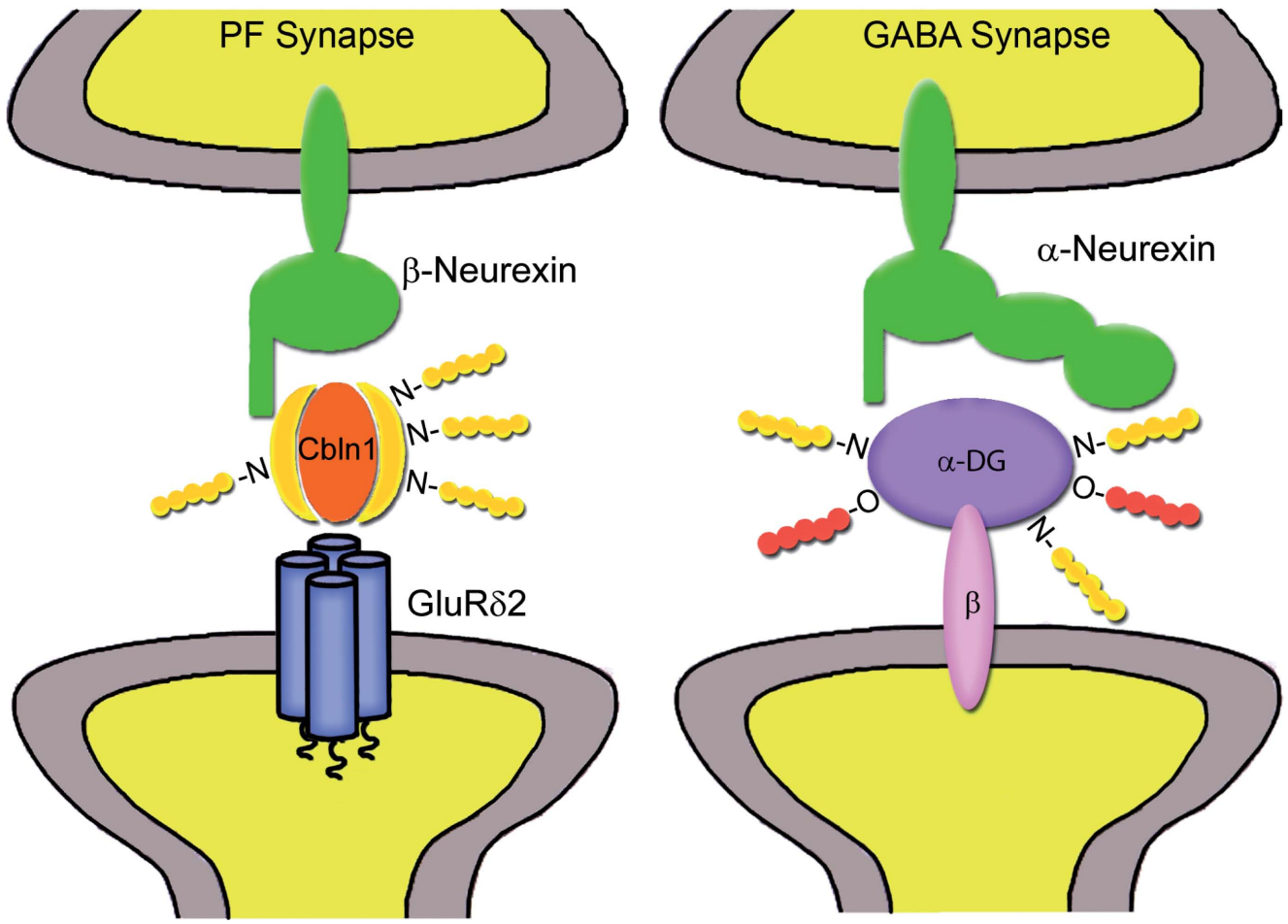

FIGURE 2 | Proposed *trans*-synaptic interactions mediated by synaptic cleft glycoproteins. At parallel fiber-Purkinje cell synapses (left), Cbln1 forms a ternary complex with neurexin variants containing the S4 insert and postsynaptic GluRδ2 receptors (modified from Uemura et al., 2010). At some GABAergic synapses (right), α-dystroglycan (α-DG) may establish a link between α-neurexins and the postsynaptic dystrophin–glycoprotein complex through the transmembrane β-dystroglycan isoform (β-DG). Glycan side chains of Cbln1 and α-DG may also mediate multiple interactions with extracellular matrix molecules.

partially overlapping localizations and functions. The combinatorial expression of distinct sets of synaptic molecules may be regarded as a signature that identifies individual synapses and could be used to generate a molecular-based system of synapse categorization (Grant, 2007). A molecular catalog could be used to identify distinct types of synapses based on the differential expression of groups of interacting proteins, each contributing to specific aspects of synapse organization and physiology.

While the tremendous complexity of glutamatergic synapses poses a formidable challenge toward the accomplishment of a synapse catalog (Grant, 2007), a molecular characterization of inhibitory synapses appears to be within reach. A main obstacle is that our current list of synaptic proteins is not yet complete. Therefore a crucial step will be the identification of all proteins, including their splice variants and post-translational modifications. Expression screenings in co-culture systems represent a useful method for the identification of novel families of synaptogenic molecules (Paradis et al., 2007; Linhoff et al., 2009). On a larger scale, existing proteomic methods, such as mass spectrometry and protein array tools, can be applied to reveal protein–protein interactions (Husi et al., 2000; Schweitzer et al., 2003; Yuk et al., 2004; Collins and Choudhary, 2008). Bioinformatics tools could also be employed to generate hypothesis of interactions that could be verified experimentally and to construct interaction maps and models that could be used to predict the effects of mutations of single proteins (Armstrong et al., 2006).

Ideally, a categorization of synapses based on molecular markers should be combined with detailed knowledge of synaptic connectivity *in situ*, the ultimate goal being a convergence of synaptic proteomics with connectomics (Lichtman and Sanes, 2008). Considering the diversity and specificity of synapses, a full appreciation of their molecular complexity can be achieved only by microscopic analyses aimed at individual synapses. In this context, immunofluorescence methods are of particular interest because they combine high sensitivity with adequate resolution, and because they are suitable for large-scale analyses of protein distribution (Schneider Gasser et al., 2006; Sassoè-Pognetto, 2011). Moreover, labeling with multiple antibodies allows to determine whether two or more synaptic proteins colocalize at specific synapses. Recent developments, such as the advent of super-resolution light microscopy (Gustafsson, 2005; Hell, 2007; Nägerl et al., 2008), have considerably expanded the analytical power of immunofluorescence microscopy. In particular, array tomography is a new proteomic imaging method that exploits a combination of light and electron microscopic approaches (Micheva and Smith, 2007). This method consists in immunolabeling and imaging ordered arrays of ultrathin (50–200 nm), resin-embedded serial sections on glass microscopic slides, resulting in the acquisition of very large volume images at high resolution. Moreover, antibodies can be eluted and the sections restained a number of times thus allowing the detection of a large number of antigens in the same sample. Because of its proteomic capabilities and high resolution, array tomography represents a useful method for large-scale exploration of synaptic diversity. This method has been recently used to determine the composition of glutamatergic and GABAergic synapses in somatosensory cortex of Line-H-YFP Thy-1 transgenic mice (Micheva et al., 2010).

The potential of array tomography and other immunohistochemical methods is limited by the availability of antibodies that can be used to stain brain sections. In many cases the extensive sequence homology between related protein isoforms precludes the generation of specific antibodies. These technical difficulties may be overcome by labeling proteins directly by recombinant fusion protein technologies. For example, in a recent study pH-sensitive pHluorin tagging was used to distinguish the membrane vs. intracellular pools of engineered neurexin 1α and neurexin 1β in cortical organotypic cultures (Fu and Huang, 2010). In this study, the pHluorin-tagged neurexin isoforms were expressed in PV-positive interneurons, allowing the visualization of their subaxonal localization and dynamics in a specific subset of GABAergic synapses. In another study, PSD-95–GFP was transfected by *in utero* electroporation in a specific population of cortical pyramidal neurons to monitor *in vivo* the dynamics of PSD-95 clusters using two-photon microscopy (Gray et al., 2006). While the expression of a tagged protein in isolated neurons may facilitate the visualization of its subcellular localization, the molecular cataloguing of synapses would require that the tagged proteins are expressed *in vivo* and replicate precisely the distribution patterns and expression levels of the endogenous proteins. Although the technology to perform this is potentially available, there have been no systematic analyses of synaptic protein distribution using this approach.

A full appreciation of the molecular diversity of synapses may help to uncover relationships between molecular composition and functional properties. In this context, the analysis of molecular organization should be complemented by gain or loss-of-function studies aimed at individual synaptic proteins in genetic model organisms. Likewise, a detailed knowledge of synaptic molecular composition may provide an interpretation key for the results obtained in knockout mutants, where the effects of the mutation are often confounded by the co-existence of multiple redundant molecular pathways (Piechotta et al., 2006). A synapse catalog could also be used to predict the consequences of mutations in the context of brain pathology and to identify populations of synapses that are likely to be affected in a particular disease (Grant, 2007). Finally, it will be of prime interest to consider how synapses vary over time, in particular by comparing synapse organization during the period of development and in mature circuits. As the last two decades have witnessed an impressive advancement in the identification of the molecular constituents of synapses (Südhof and Malenka, 2008), a big challenge ahead is to define the spatio-temporal expression profile of the endogenous synaptic proteins, to understand how this large array of molecules assemble into functional units, and to link the molecular data sets with a characterization of the anatomical and physiological diversity of synapses.

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Gephyrin, the enigmatic organizer at GABAergic synapses

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$GABA_A$ receptors are clustered at synaptic sites to achieve a high density of postsynaptic receptors opposite the input axonal terminals. This allows for an efficient propagation of GABA mediated signals, which mostly result in neuronal inhibition. A key organizer for inhibitory synaptic receptors is the 93 kDa protein gephyrin that forms oligomeric superstructures beneath the synaptic area. Gephyrin has long been known to be directly associated with glycine receptor β subunits that mediate synaptic inhibition in the spinal cord. Recently, synaptic $GABA_A$ receptors have also been shown to directly interact with gephyrin and interaction sites have been identified and mapped within the intracellular loops of the $GABA_A$ receptor α1, α2, and α3 subunits. Gephyrin-binding to $GABA_A$ receptors seems to be at least one order of magnitude weaker than to glycine receptors (GlyRs) and most probably is regulated by phosphorylation. Gephyrin not only has a structural function at synaptic sites, but also plays a crucial role in synaptic dynamics and is a platform for multiple protein-protein interactions, bringing receptors, cytoskeletal proteins and downstream signaling proteins into close spatial proximity.

Keywords: $GABA_A$ receptors, gephyrin, receptor clustering, synapse formation, inhibitory synapse

INTRODUCTION

The impressive performance of the central nervous system is rendered possible by neuronal networks that form an uncountable number of flexible synaptic contacts passing on information from one cell to many others. Information processing is primarily achieved by fast acting signals, i.e., neurotransmitters acting on synaptic ligand-gated ion channels and slower mechanisms like extrasynaptic receptors and G-protein coupled receptors (GPCRs). The main counter-acting neurotransmitters, glutamate and GABA (γ-amino butyric acid), both exert their actions through ligand-gated ion channels and GPCRs. Synapses are highly complex structures, where a large number of proteins control neurotransmitter release on the presynaptic site and the effects of neurotransmitters at the postsynaptic site.

The glutamatergic synapse has been investigated in great detail over the past 20 years and these studies have revealed a large number of postsynaptic proteins that interact in a controlled manner to keep glutamate receptors in place but also allow for a highly regulated dynamic insertion and removal of receptors, which is a prerequisite for synaptic plasticity. Sub-membrane adaptor and scaffold proteins are crucial players in this process. The intracellular C-termini of glutamate receptors (**Figure 1A**) strongly interact with modular intracellular scaffold and signaling proteins (like PSD-95) via well-characterized protein-protein interaction motifs (like PDZ, SH3, and other domains). It was, therefore, experimentally easier to find intracellular interaction partners using the classical yeast-two-hybrid system and to finally assemble the concept of the complex dynamic structure of the excitatory postsynaptic density as we understand it today (Sheng and Lin, 2001).

The postsynaptic structure of inhibitory synapses turned out to be more difficult to investigate. In $GABA_A$ receptors ($GABA_A$Rs), which belong to the Cys-loop receptor family, the N- and C-termini are both extracellular and possible intracellular interactions can only be mediated by a small intracellular loop between transmembrane helices (TM) 1 and 2, or the large intracellular loop between TM3 and 4 of individual subunits. (**Figure 1A**). A further difficulty is the complexity of the $GABA_A$R composition. There are a total of 19 subunits from eight subunit classes (α, β, γ, δ, ε, π, ρ, θ) that come together in pentameric assemblies with different compositions and form a central pore that is permeable to chloride ions (**Figure 1B**) (Sarto-Jackson and Sieghart, 2008). Depending on cell type, the individual synapses and the developmental state, the subunit composition of the pentameric receptor is predominantly recruited from six different α subunits, 3β subunits, and 3γ subunits (some of which also occur in alternatively spliced forms). These subunit categories were defined by the degree of their sequence similarity with 30–40% sequence identity between members of different subunit classes and 60–80% identity between members of the same subunit class (Barnard et al., 1998). Interestingly, the highest variability between members within a class and between classes is found in the large intracellular loop between TM3 and 4 (Olsen and Sieghart, 2008).

The most common synaptic receptors are formed by $\alpha_{1/2/3/6}$-β_x-γ_2 subunit combinations. They respond to the short-lived bursts (<1 ms) of high concentrations of GABA (mM range) during neurotransmitter release into the synaptic cleft with receptor kinetics characterized by a fast rise time, high amplitude, and fast inactivation. Receptors composed of αβ or $\alpha_{4/6}$βδ subunits

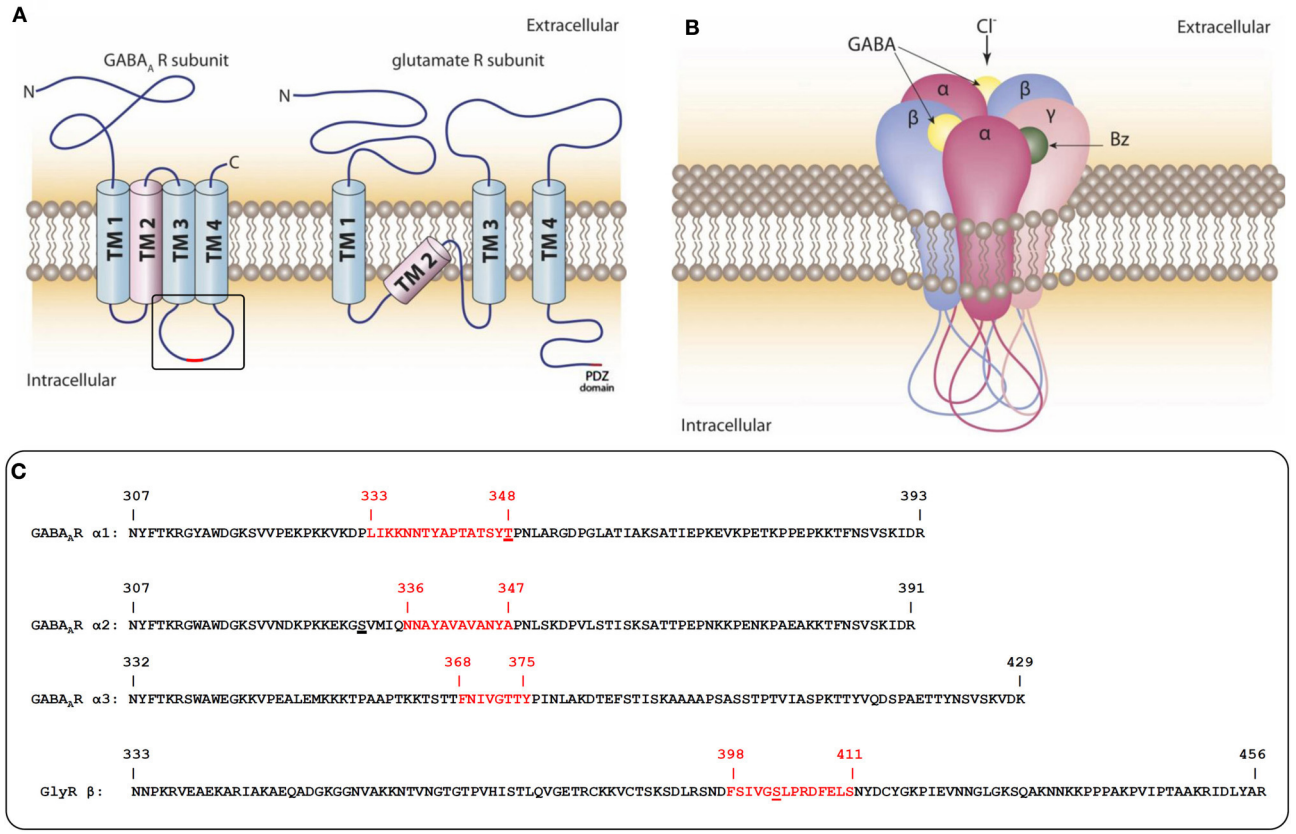

FIGURE 1 | Domain-structure of GABA$_A$R- and GluR-subunits and the pentameric assembly of GABA$_A$Rs. (A) Schematic representation of the transmembrane topology of subunits from GABA$_A$R and AMPA/NMDA (glutamate) receptors. The GABA$_A$R intracellular loop between TM3 and 4 and the intracellular C-terminus of glutamate receptors are the main sites of interaction with intracellular scaffold and signaling proteins. **(B)** Pentameric assembly of subunits within the GABA$_A$R: subunit interfaces of the extracellular domains form binding pockets for many therapeutically used drugs. The intracellular loops of all five subunits are substrates for post-translational modifications that dynamically regulate interactions with other proteins. Some protein interactions might require more than one cytoplasmic loop. **(C)** Sequences of intracellular loops of GABA$_A$R α1, α2, and α3 subunits and the GlyR β subunit from rat. The sequences identified as gephyrin interaction sites are highlighted in red. Identified phosphorylation sites within or near the gephyrin interaction sites are underlined.

are believed to form a specialized population of extrasynaptic receptors. They have a higher agonist affinity and longer open times than their synaptic counterparts and respond to lower ambient GABA concentrations (μM range) or to GABA spillover from synapses and provide the respective neuron with a constant tonic inhibition that elevates the threshold of action potential firing (Farrant and Nusser, 2005). Those receptors are either distributed in a dispersed manner on the cell surface or form micro-clusters with intracellular scaffold proteins that are different from those of synaptic receptors. Of particular pharmacological relevance is the sensitivity of the large number of GABA$_A$R subtypes toward allosteric modulators such as benzodiazepines and neurosteroids (Rudolph and Mohler, 2004).

To search for downstream effectors that are involved in the construction of the inhibitory synapse it is important to keep in mind that there are five large intracellular loops per single receptor complex as potential interaction sites with intracellular molecules (**Figures 1A,B**). These loops also contain multiple sites for post-translational modifications (like phosphorylation, palmitoylation, sumoylation, and ubiquitylation). These modifications dynamically regulate the interactions with other proteins and are involved in synaptic stability and trafficking (Moss and Smart, 2001).

Similar to glutamate receptors yeast-two-hybrid screens were used intensively to identify intracellular interaction partners of GABA$_A$R subunits, but turned out to be more laborious than with glutamate receptors. The first proteins to be identified by this technique were proteins involved in the assembly (chaperones) and trafficking (like MAP-1B, PLIC, GABARAP) (Hanley et al., 1999; Wang et al., 1999; Bedford et al., 2001). Kinases and phosphatases were also among the first proteins to be shown to interact with GABA$_A$Rs (Brandon et al., 2002, 2003). These enzymes usually bind to their substrate upon phosphorylation or de-phosphorylation either directly or via an adaptor protein like AKAP-79/150 for protein kinase A (PKA). GABA$_A$R interacting proteins have been extensively reviewed in Jacob et al. (2008); Tretter and Moss (2008) and Luscher et al. (2011).

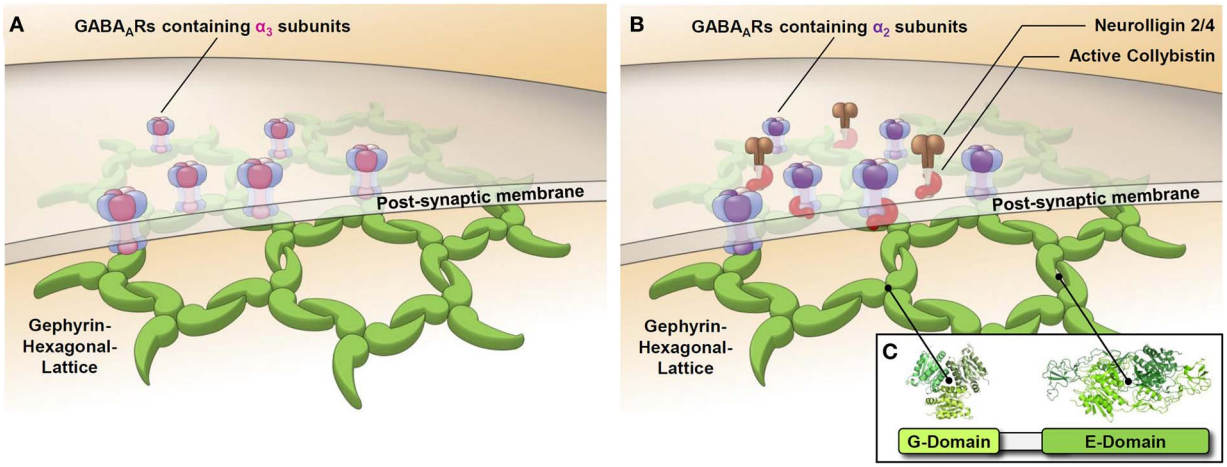

FIGURE 2 | Synaptic GABA$_A$Rs containing the α2 or α3 subunit are clustered by different mechanisms. Schematic representations of GABAergic post-synapses containing the GABA$_A$R α3 subunit **(A)** or the GABA$_A$R α2 subunit **(B)** show the postulated differential role of collybistin in synaptic cluster formation. GABA$_A$Rs containing the α3 subunit directly bind to gephyrin, while GABA$_A$Rs containing the α2 subunit use collybistin as an accessory factor to enhance the affinity of binding to gephyrin. Isoforms of the cell adhesion molecule neuroligin (NL–2/4) have a preference for GABAergic synapses and have been shown to activate collybistin.

One particular protein, gephyrin, soon gained a lot of scientific attention as a GABA$_A$R associated protein. It was originally identified to interact with GlyRs that mediate inhibition in the spinal cord and are also members of the Cys-loop receptor family. During receptor purification, GlyRs remain strongly associated with this prominent intracellular anchoring protein, which has been named gephyrin after the Greek word for "bridge" *gephyra* (Langosch et al., 1992; Prior et al., 1992). The observation that GABA$_A$Rs also colocalize to a large degree with gephyrin in the brain was made soon after this protein was identified, but it was neither possible to co-precipitate or co-purify gephyrin with GABA$_A$R nor was it found in yeast-two-hybrid screens using many kinds of brain mRNA libraries and GABA$_A$R intracellular loops as baits (Sassoè-Pognetto et al., 1995; Betz, 1998; Essrich et al., 1998). It only turned out recently that, similar to GlyRs, there is indeed a direct interaction between GABA$_A$Rs and gephyrin, but a co-purification using classical protocols has not been reported.

GABA$_A$ RECEPTOR INTERACTIONS WITH GEPHYRIN

Gephyrin clusters are very abundant in the brain, where GlyRs are a minor receptor population compared to GABA$_A$Rs. The colocalization of GABA$_A$Rs and gephyrin in clusters on the neuronal surface implied that GABA$_A$Rs are associated with this scaffold protein, either directly or indirectly (via a linker protein). The GABA$_A$R γ2 subunit was originally found to be important for synaptic localization of GABA$_A$Rs (Essrich et al., 1998). Knockout mice with a deletion of the γ2 subunit die within a few weeks after birth and were found to lack GABA$_A$R clusters (Günther et al., 1995). Transfection of neuronal cultures from these mice with γ2 cDNA restored clustering (Baer et al., 2000). On the other hand, transfecting cultured hippocampal neurons with shRNAi constructs against gephyrin reduces the number of γ2 containing GABA$_A$R clusters in cultured hippocampal neurons (Yu et al., 2007). Similarly, cultures from gephyrin knock-out mice lack GABA$_A$R clusters (Kneussel et al., 1999).

One of the early hits from yeast-two-hybrid screens, the protein GABARAP (GABA$_A$R associated protein), was identified to interact with the γ2 subunit as well as with gephyrin (Wang et al., 1999; Kneussel et al., 2000). This finding led to the hypothesis, that GABARAP might be the linker protein that connects GABA$_A$Rs to gephyrin clusters. As a consequence GABARAP was intensively investigated and found to be important for receptor insertion into the cell surface membrane (Kittler et al., 2001; Bavro et al., 2002). GABARAP is widely distributed in multiple cell types and its relatively low abundance at synaptic sites raised doubts about its role as a linker protein. Finally, GABARAP knock-out mice turned out to be viable and neuronal cultures from these mice exhibited strong postsynaptic co-clustering of gephyrin and GABA$_A$Rs (O'Sullivan et al., 2005). Soon, the discovery of other GABARAP-interacting proteins, PRIP1/2 (Phospholipase C-Related Inactive Protein) and NSF (N-ethylmaleimide Sensitive Fusionprotein) supported its function during synaptic delivery of receptors (Kittler et al., 2001; Goto et al., 2005; Kanematsu et al., 2006). This task probably is taken over by other members of the MAP1-LC3 family in GABARAP knock-out mice.

The γ2 subunit has long been discussed as an important candidate for mediating synaptic targeting or anchoring (Alldred et al., 2005; Christie et al., 2006). This concept was obvious as γ2 and δ do not occur together in one receptor subtype and follow distinct assembly rules (Sieghart et al., 1999). As mentioned earlier, δ subunit containing receptors are well described as being localized mostly outside of the synaptic areas where they mediate tonic inhibition (Farrant and Nusser, 2005). The currently accepted model of receptor structure predicts the presence of 2α, 2β, and 1γ subunit in the pentameric ion channel (Barrera and Edwardson, 2008). The γ2 subunit is known to

exist in two splice variants (γ2L and γ2S, distinguished by the insertion of eight amino acids in the TM3–4 intracellular loop of γ2L containing a PKC phosphorylation site). This might be of some importance in the regulation of receptor trafficking and synaptic targeting (Meier and Grantyn, 2004). Staining of hippocampal pyramidal neurons in culture reveals a strong diffuse cell surface distribution of γ2 in addition to synaptic clusters, while staining for α2 reveals predominantly synaptic clusters and staining for α1 reveals both: synaptic clusters and smaller clusters or diffuse staining in extrasynaptic areas (Tretter and Moss unpublished results). Therefore, many γ2 containing receptors are extrasynaptic. This is not only true for hippocampal cell cultures but also for $GABA_A$ receptor in the brain (Kasugai et al., 2010).

Hippocampal pyramidal cells express a large repertoire of $GABA_AR$ subtypes containing the γ2 but different α and β subunits in different synapses (Klausberger et al., 2002). Although synaptic targeting and anchoring must be distinguished as two different processes, α subunits are more likely to dominate over the γ2 subunit in at least one of these processes. Furthermore the γ2-containing $GABA_AR$ subtype $\alpha_5\beta_x\gamma_2$, that has received considerable attention in context with a role in cognition has been found to be located mostly at extrasynaptic sites where it was found to be associated with the sub-membrane protein radixin (Kneussel, 2005; Loebrich et al., 2006; Serwanski et al., 2006). Therefore, it was assumed that γ2 subunits might not be involved in synaptic targeting, but possibly in the synaptic anchoring of $GABA_AR$.

Finally, in 2008 a direct interaction between some $GABA_AR$ α subunits and the P1 splice variant of gephyrin could be successfully proven (Tretter et al., 2008). We found that some $GABA_AR$ mutants were expressed on neuronal surfaces, but were clustering-deficient. An overlay assay protocol was adapted to specific conditions: following a Western blot, the GST fusion proteins of $GABA_AR$ intracellular domains were slowly renatured with a decreasing gradient of guanidinium hydrochloride and were incubated with *in vitro* translated gephyrin in the absence of detergent. This was a prerequisite for a positive and specific signal. The expectation that the large intracellular loop between TM3 and 4 contains the interaction site with gephyrin was thus verified. The first α subunit to be shown to interact with gephyrin was α2. The identified minimal core-sequence that is necessary for receptor clustering comprises amino acids 336–347 (**Figure 1C**), or amino acids 364–375, when numbering includes the signal sequence. In order to obtain a positive signal in yeast-two-hybrid screens an extended sequence of amino acids, 330–347 (358–375 including the signal sequence) was necessary (Saiepour et al., 2010). The motif is a highly hydrophobic stretch of amino acid residues in the center of the large intracellular loop. Subsequently, we also identified gephyrin interaction motifs in the homologous region of α1 and α3 (Mukherjee et al., 2011; Tretter et al., 2011). Interestingly these α subunits show little conservation of the amino acid sequence in this area (**Figure 1C**), which either implies different binding sites on gephyrin, or suggests an important role of a higher order structure that is conserved among these subunits.

BINDING SITE OF $GABA_A$ RECEPTOR α SUBUNITS ON GEPHYRIN

The binding site of the GlyR β subunit (GlyR β) on gephyrin was initially identified as a stretch of residues at the beginning of gephyrin's C-terminal E-domain (GephE, for domain structure see **Figure 3C**). In 2006, the GlyR β-loop was co-crystallized with the E-domain of gephyrin, showing that amino acids at the beginning and the end of the E-domain seem to be involved in GlyR β subunit binding (Schrader et al., 2004; Kim et al., 2006). Based on the structure of the GlyR β subunit-gephyrin complex and sequence similarities in the $GABA_AR$ α3 subunit we hypothesized, that binding of α3 to gephyrin very likely occurs in a similar three-dimensional binding pocket of the gephyrin E-domain. As a crystal structure is currently not available, we first roughly mapped the binding sites of $GABA_AR$ α2 and α3 subunits on gephyrin by using alanine mutagenesis in a yeast two-hybrid system (Saiepour et al., 2010; Tretter et al., 2011). The identified sequences for α2 (residues 325–343) and α3 (residues 325–334) overlap and include the critical aminoacids involved in GlyR β subunit binding (Asp327, Phe330) (**Figure 3C**). These results imply an evolutionarily conserved binding site for $GABA_AR$ α subunits and GlyR β subunit on gephyrin.

The key residues for the interaction were further characterized in a follow-up study (Maric et al., 2011). Although the identified binding sites on α1, α2, and α3 are only moderately conserved, $GABA_AR$ α1, α2, α3, and GlyR β subunits compete for the same binding site on the gephyrin molecule (Maric et al., 2011; and **Figure 3D**). Phe330 and Asp327 of GephE are the main critical residues mediating the GlyR β-gephyrin interaction (Kim et al., 2006). The crystal structure of the gephyrin E-domain itself and in complex with a bound GlyR β peptide reveals the formation of Geph-E dimers (indicated by the grey structure in **Figure 3A**), but the main interaction site seems to be located on one molecule (indicated by the colored structure in **Figure 3A**) (Kim et al., 2006). It can be predicted that Phe330 in the gephyrin E-domain also mediates hydrophobic interactions with conserved aromatic residues in the $GABA_AR$ α subunits. The motif "FSIV" in GlyR β (**Figures 3B,D**) fits into the three-dimensional binding pocket formed mainly by residues of the N-terminus of GephE (subdomain III in **Figure 3C**), but also a few residues near the C-terminus of GephE (subdomain IV in **Figures 3B,C** color-coded in light blue) (Kim et al., 2006). This sequence of GlyR β is highly conserved in $GABA_AR$ α3 ("FNIV") (**Figure 3D**). Another interesting conserved amino acid is a tyrosine in each of the $GABA_AR$ α subunit interaction domains (α1Y347, α2Y346, α3Y375) (**Figure 3D**). Mutation of the respective tyrosine to phenylalanine or alanine reduces gephyrin-binding significantly (Maric et al., 2011). A related phenylalanine (Glyβ F408) is also found in a similar position of the GlyR β subunit, yet it is shifted by three residues toward the C-terminus (**Figure 3D**).

Isothermal titration calorimetry is a frequently used thermodynamic method to quantify the strength and nature of protein-protein interactions. We used this method to additionally prove, that GlyR β and $GABA_AR$ α1, α2, α3 binding sites compete for the same site on GephE. The interaction of gephyrin E domain or full-length gephyrin with GlyR β residues 378–425 of the large

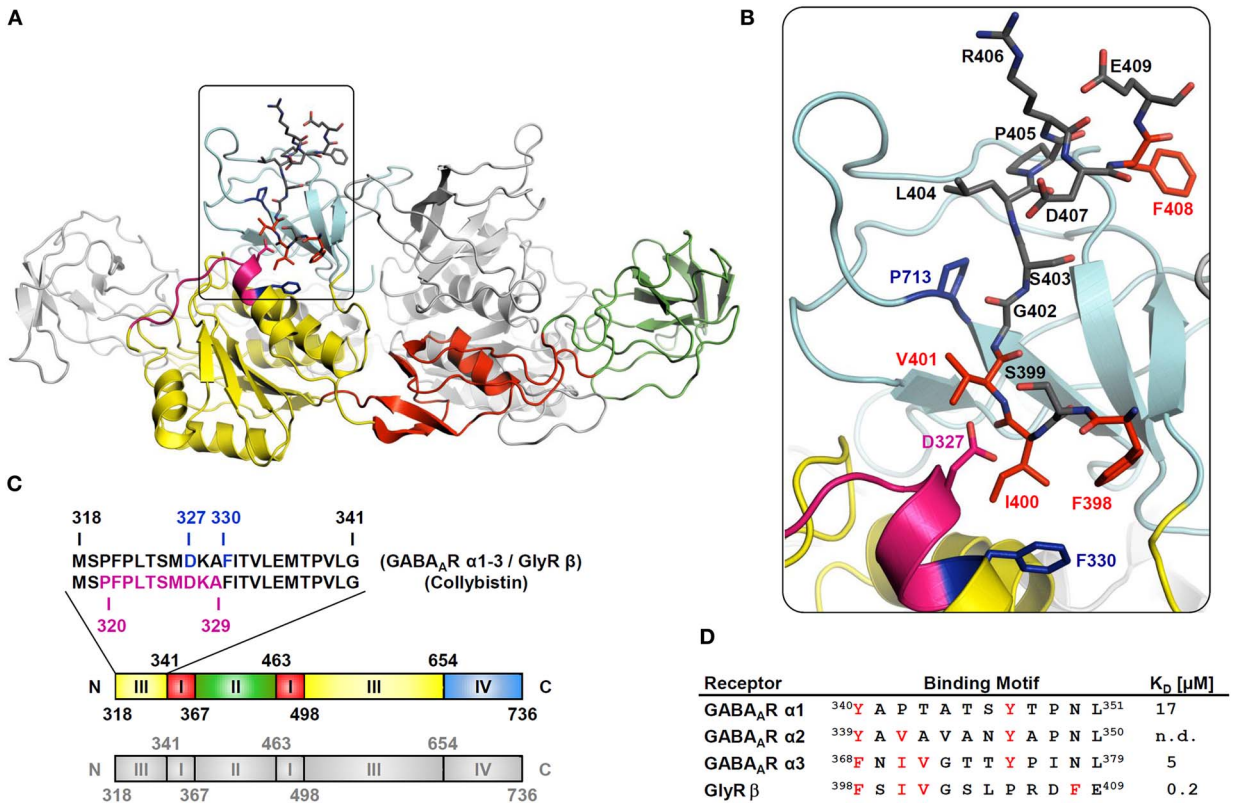

FIGURE 3 | Structural determinants of gephyrin, interacting with GABA$_A$Rs, GlyRs, and collybistin. (A) Crystal structure of the gephyrin E domain (GephE) complexed with GlyR β, modified for comparison with GABA$_A$R α subunits and collybistin. The ribbon diagram of GephE is colored to highlight its subdomain architecture. The GephE-domain is complemented by a second monomer shown in grey, representing the dimer that usually forms *in vitro*. Identified binding sites for collybistin on gephyrin are shown in magenta and overlapping binding sites for GlyR β and GABA$_A$R α subunits are shown in light blue. The GlyR β peptide as well as crucial interacting amino acids in GephE (D327, F330, P713) are shown in stick representation. **(B)** The major contributing residues to the interaction are conserved among GABA$_A$R α3 and GlyR β and are marked in red. **(C)** Subdomain-structure of GephE and amino acid sequence of GephE harboring the main binding site to GABA$_A$R α1, α2, and α3 subunits, GlyR β subunit and collybistin. Crucial residues for the interaction with receptors are highlighted in light blue (Asp327, Phe330), the overlapping collybistin binding site is shown in magenta. **(D)** The table compares homologous sequences crucial for gephyrin-binding in GlyR β and GABA$_A$R α subunits from rat. Residues critically contributing to the interaction and conserved among all subtypes are shown in red. The dissociation constant K_D indicates the binding strength as determined by isothermal titration calorimetry. (n.d. = not detectable).

cytoplasmic loop was analyzed intensively via ITC and based on the resulting biphasic binding curve a two-binding-sites model was used to describe the binding event. In particular, the high affinity binding site of the elongated GlyR β was described to be in the nanomolar range with values varying between 22–30 nM (Specht et al., 2011; Herweg and Schwarz, 2012) and 140–400 nM (Schrader et al., 2004; Kim et al., 2006) depending on the stoichiometry values which were estimated at 0.28–0.29 (Specht et al., 2011; Herweg and Schwarz, 2012) and 0.7–1.0 (Schrader et al., 2004; Kim et al., 2006), respectively. The low affinity site displayed affinities of 3–6 μM (Specht et al., 2011; Herweg and Schwarz, 2012) and 16–30 μM (Schrader et al., 2004; Kim et al., 2006) with a stoichiometry of 0.6 (Specht et al., 2011; Herweg and Schwarz, 2012) and 0.6–0.8 (Schrader et al., 2004; Kim et al., 2006). This deviation may be explained by lab-specific systematic differences in protein activity; depending on the purification protocol the discrepancy between active molecules and apparent concentration may vary. A low active fraction of macromolecules in the cell will yield overestimated affinities and underestimated stoichiometry values. In contrast, the discrepancy of a single binding site in the crystal structure versus two binding sites in ITC remains to be explained on the molecular level. Remarkably, both, alanine point mutations and phosphomimetic substitutions in the GlyR β binding motif, result in monophasic binding curves describing a single binding site with low micromolar affinity (Kim et al., 2006; Specht et al., 2011). We propose that structural rearrangements in either the receptor loop or gephyrin may explain the second apparent binding site. Interestingly, measurements of the affinity of the GABA$_A$R α subunits to gephyrin revealed monophasic binding curves with affinities in the low micromolar range for α3: $K_D = 5.3\,\mu M$ and α1: $K_D = 20\,\mu M$. The hydrophobic α2 peptide could not be synthesized in sufficiently high amounts to perform similar experiments (Maric et al., 2011). The affinities of the GABA$_A$R α subunits to gephyrin are, therefore, one to two orders of magnitude weaker and are comparable to those of PDZ domain-ligand interactions such

as PSD-95 with its binding partners at glutamatergic synapses (Jemth and Gianni, 2007). *In vivo,* the affinity may be influenced by various additional factors including the presence of full-length proteins, the contribution of other subunits in the pentamer, post-translational modifications and accessory proteins like collybistin (see below). GlyRs differ from $GABA_A$Rs also in the stoichiometry and assembly of subunits. The higher affinity of gephyrin to one GlyR β subunit and the presence of 2 or 3β subunits in one GlyR pentamer is a plausible explanation for the co-purification of gephyrin with GlyRs from detergent extracts (Sola et al., 2004).

The α subunits seem to be the lead subunits in gephyrin-dependent $GABA_A$R clustering. Deletion of the gephyrin-binding motifs in tagged recombinant α subunits prevented recombinant receptors from co-clustering with endogenous gephyrin after transfection into cultured hippocampal neurons, but did not prevent assembly with endogenous β and γ subunits and surface expression of receptors. *Vice versa*, it has been shown, that the insertion of the α2-binding site into the intracellular region of α6, or even into unrelated proteins like CD4, redirects these proteins to gephyrin clusters (Tretter et al., 2008). The sequence of events, however, is still not fully understood. Several studies have investigated $GABA_A$R assembly. As membrane proteins, $GABA_A$Rs pass the ER/Golgi, where individual subunits are assembled into pentamers with the help of chaperones like calnexin and binding immunoglobulin protein (BIP) (Connolly et al., 1996). N-terminal and some cytoplasmic sequences are essential for receptor assembly (Taylor et al., 1999, 2000; Klausberger et al., 2001, 2002; Sarto et al., 2002; Bollan et al., 2003; Ehya et al., 2003; Sarto-Jackson et al., 2006), however, it is still unknown, at which stage gephyrin associates with $GABA_A$Rs.

Although gephyrin-binding sites have now been identified on $GABA_A$R α subunits, there is considerable evidence, that other subunits are somehow involved in the interaction.

We assume that apart from the α subunits, γ2 also plays an active part in the clustering process explaining the earlier results from γ2 knock-out mice (Schweizer et al., 2003). However, the precise mechanism remains to be determined. Although speculative at this point, it is quite possible that an interface formed between the cytoplasmic loops of the α1, α2, or α3 subunits and the γ2 subunit might be crucial for, or at least strengthen gephyrin-binding, while the α subunit provides the major binding site and probably also mediates synapse-specificity. This interface might be critical: receptors without a γ2 or δ subunit not only appear as fully functional recombinant receptors at the surface of transfected heterologous cells, but have also been identified as an endogenous receptor population in neurons (Bencsits et al., 1999). In hippocampal neurons they are located extrasynaptically and contribute to tonic inhibition (Mortensen et al., 2010). Although the presence of such subunit combinations in synaptic areas is also possible, they probably are of minor quantitative importance and experimentally difficult to distinguish from γ2-containing receptors, especially if they occur in the same synapse.

We cannot make any predictions for $\alpha_{4/6}\beta_{2/3}\delta$ containing receptors yet, as α4 and α6 subunits have not been investigated in detail regarding a possible interaction with gephyrin. However, δ-containing receptors have long been considered to be responsible for providing significant tonic inhibition of neurons at extrasynaptic sites. These δ-containing receptors are rarely seen clustered, and are mostly distributed evenly on the neuronal surface (Nusser et al., 1998). The stoichiometry of these receptors might be similar to the α–β–γ receptors, except that δ replaces the γ subunit although some authors have observed a variable stoichiometry in recombinant δ-containing receptors depending on the availability of the δ subunit (Kaur et al., 2009; Baur et al., 2010; Wagoner and Czajkowski, 2010).

GEPHYRIN-INDEPENDENT $GABA_A$ RECEPTOR CLUSTERING

Immunohistochemical studies investigating the distribution of $GABA_A$R subunits and gephyrin in tissue sections from wild-type and mutant mice have indicated that $GABA_A$R subunit clusters are not always associated with gephyrin. This gephyrin-independent $GABA_A$R clustering is not fully understood at present. One alternative and completely independent form of clustering has been described and characterized: Radixin—a protein from the band 4.1 or ERM (ezrin-radixin-moesin) family—has been shown to cluster α5-containing receptors at extrasynaptic sites (Funayama et al., 1991; Loebrich et al., 2006). These receptors have gained considerable attention in cognition and α5-selective inverse agonists are under investigation as cognition enhancers in neurodegenerative disorders like Alzheimer's disease (AD) (Martin et al., 2009). Radixin connects α5-containing $GABA_A$Rs to the actin cytoskeleton. The N- and C-termini of radixin form intra-molecular associations that are disrupted upon activation. Membrane association is mediated upon PIP_2 binding by the radixin N-terminus, followed by phosphorylation of its C-terminus. The protein conformation changes to an open state, and the F-actin binding site becomes exposed. F-actin binding of radixin is essential for α5-containing $GABA_A$R clustering. It is certainly interesting, why extrasynaptic receptors need clustering at certain locations instead of being equally distributed on the neuronal surface. Loebrich et al. discuss this point and hypothesize about potential neuronal-glial contact points in analogy to observations with the location of NMDA receptors (Loebrich et al., 2006). Glia cells are currently emerging as active players in information processing in addition to their supportive role for neurons (Dityatev and Rusakov, 2011). Small amounts of radixin and α5-containing receptors are also observed in gephyrin-positive synaptic areas where they might contribute to synaptic plasticity (Christie and de Blas, 2002).

Generally, gephyrin and radixin clustering are regarded as independent processes. Furthermore, those two proteins might still not tell the full story of $GABA_A$R clustering. Clusters of α1-containing receptors have also been observed in gephyrin knock-out mice. This phenomenon might reflect an unknown compensatory mechanism (e.g., other clustering factors or mechanisms), or a hypothetical $GABA_A$R-intrinsic property (like receptor-receptor interactions) (Ghai et al., 2011).

Furthermore, it should be mentioned, that $GABA_A$Rs frequently contain mixed α or β subunits (Benke et al., 2004). It is tempting to speculate that these "mixed" receptors have not only specific functional properties, but are also clustered in a special way. Mixed α1–α5 receptors might still be clustered by radixin.

Alternatively, these receptors could also be clustered by gephyrin, if the α1 subunit is in a favorable configuration, maybe positioned adjacent to the γ2 subunit. This could be an additional explanation for the low abundance, but not complete absence of α5-containing receptors at synaptic sites (Serwanski et al., 2006).

The characterization of gephyrin-binding sites on the α1, α2, and α3 subunits is only the starting point for understanding the $GABA_AR$-gephyrin interactions. One aspect can be an additional interaction of gephyrin with other subunits of the $GABA_AR$ pentamer. An association of gephyrin with extrasynaptic β3-containing receptors during development and synaptogenesis has been described (Danglot et al., 2003). The authors suggest that β3-containing receptors first appear in small clusters at the cell surface and only later on, when these receptors also contained γ2 subunits, they become associated with synaptic sites. A supporting observation for this hypothesis might also be the fact that homozygous γ2 knock-out mice are born alive and die only a few weeks after birth (Günther et al., 1995). Recently, a contribution of gephyrin to phasic as well as to tonic inhibition has been postulated (Marchionni et al., 2009).

GEPHYRIN STRUCTURE, POLYMORPHISMS, AND ENZYMATIC ACTIVITY

Gephyrin was initially recognized as a 93 kDa protein co-purifying with GlyR (Pfeiffer et al., 1982). The protein's first attributed function was a link of GlyR to microtubules (Langosch et al., 1992). Almost 400 papers have since been published on this 93 kDa protein including the identification of a continuously increasing number of gephyrin-interacting proteins, still leaving us with a fragmented picture of how inhibitory post-synapses are organized and dynamically regulated with gephyrin as a central platform. Gephyrin is a phylogenetically old protein with 29 exons in mouse, appearing in multiple splice variants. These isoforms vary between species, tissues and maybe even cell types (Ramming et al., 2000).

In bacteria the main function of gephyrin is molybdenum cofactor (Moco) biosynthesis, which is essential for the activity of various metabolically important enzymes, like aldehyde oxidase, xanthine oxidoreductase, and sulfite oxidase. This biosynthetic activity, which is dependent on the presence of cassettes C2 and C6 has been maintained in higher organisms, in addition to the role of gephyrin in inhibitory neurotransmitter receptor clustering (Stallmeyer et al., 1999). The essential role of gephyrin in Moco biosynthesis in higher organisms is reflected in the fact that Moco-deficiency (independent of gephyrin) leads to neurodegeneration and Moco-deficient mice die at postnatal day 11 (Kügler et al., 2007). On the other hand, gephyrin knock-out mice die immediately after birth (P0) with a stiff musculature that obviously results from an impaired GlyR function and absent motoneuron inhibition. The early postnatal death of gephyrin knock-out mice could not be prevented by restoring Moco biosynthesis in transgenic mice (Grosskreutz et al., 2003). Zebrafish contain duplicated gephyrin genes (*GPHNA* and *GPHNB*) that are differentially distributed in tissues and both mediate Moco biosynthesis and GlyR clustering. Alternative splicing creates a high degree of variability that generates isoforms with diverse functions and properties (Ogino et al., 2011).

The importance of an intact GlyR-gephyrin system is illustrated by severe illnesses like Startle disease (hyperekplexia) showing neonatal hypertonia and an exaggerated startle reflex in response to auditory or tactile stimuli. This phenotype is mainly caused by multiple mutations of proteins involved in glycinergic neurotransmission, like GlyR, gephyrin, collybistin, and glycine transporters that also affect the GABAergic system (Harvey et al., 2008).

The most frequently mentioned splice variant P1 of gephyrin, first described by Prior et al. (1992), is composed of a 20 kDa N-terminal domain (G-domain: residues 1–181) and a C-terminal E-domain (43 kDa, residues 318–736), which are homologous to the bacterial Moco-synthesizing enzymes MogA and MoeA, respectively (Schwarz et al., 2001). In higher organisms the two domains are connected by a linker (18–21 kDa), also referred to as C-domain (Feng et al., 1998). The G-domain has an intrinsic tendency to form trimers and, *in vitro*, the E-domains form dimers (Schwarz et al., 2001; Sola et al., 2001, 2004). As large gephyrin clusters appear at synapses and in light of the *in vitro* observations, an assumption of the presence of higher order structures ("super-structures") was postulated (Kirsch and Betz, 1995; Kneussel and Betz, 2000; Xiang et al., 2001). A long-standing hypothesis predicts a hexagonal lattice as a combination of the three-fold and two-fold symmetry elements (Kneussel and Betz, 2000). The authors present the hypothesis that gephyrin is initially present as trimers in the cytosol. The metastable characteristics of the E-domain prevents dimerization, but is stabilized upon GlyR binding and that the trimers are transported in vesicles to the synapse or are randomly inserted (Rosenberg et al., 2001; Hanus et al., 2004).

Some splice forms disrupt the formation of higher order structures like the C5 cassette in the G-domain, that prevents trimerization (Bedet et al., 2006). Gephyrin containing the C5 cassette can act as a dominant-negative variant that can still bind GlyRs, but favors diffusion of these receptors and removal from synapses. This model provides a hypothesis for a dynamic anchoring of receptors. A recent study provides more insight into biophysical properties of gephyrin (Herweg and Schwarz, 2012). The authors compared other isoforms (Gephyrin P1, Gephyrin-C3, Gephyrin-C4) in two expression systems: SF9 insect cells and human embryonic kidney-HEK cells. Geph-C3 is a non-neuronal form with the sole metabolic function of Moco biosynthesis. The C3 cassette is spliced by the neuronal factor Nova, which is absent in glia cells. Geph-C4 is a neuronal form that tends to accumulate at the plasma membrane, while Geph-C3 exhibits diffuse cytosolic staining in liver and glia cells. The choice of the expression system had an impact on which higher order structures could be identified by size exclusion chromatography: from *E. coli* trimeric gephyrin was isolated, while SF9 cells produced hexamers and even higher oligomeric complexes. The authors also looked at the dynamics of the subdomains. The G- and C-domain seem to stabilize each other, while the E-domain is more flexible remaining open for interactions with other proteins. Most gephyrin interaction partners bind close to or inside the E-domain.

It is well known, that gephyrin is a phosphoprotein. This post-translational modification influences gephyrin clustering and also receptor binding (Zita et al., 2007; Bausen et al., 2010; Charrier

et al., 2010). The comparison of the two expression systems allowed to further investigate the influence of different states of phosphorylation on gephyrin oligomerization. In addition to the previously characterized phosphorylation sites (Ser188, Ser194, Ser200, Ser270) Herweg and Schwarz added an additional 18 sites to the list, most of them located in the G-domain. These modifications can be expected to be celltype-specific and maybe also synapse-specific in higher organisms.

These studies mainly focus on GlyR-gephyrin interactions but are equally valuable for studying the interaction between gephyrin and $GABA_A$Rs. The gephyrin variants might differ from synapse to synapse, but a mechanistic understanding of this complex system will help to better understand the interactions of gephyrin with $GABA_A$Rs.

THE ROLE OF THE CYTOSKELETON IN $GABA_A$R TRANSPORT TO AND FROM THE NEURONAL SURFACE AND IN GEPHYRIN-MEDIATED CLUSTERING

Neurotransmitter receptors are assembled in the ER/Golgi compartments and transported to the cell surface in membrane vesicles budding from the trans-Golgi along "trails" of the cytoskeleton. Specific motor proteins are responsible for the anterograde or retrograde transport. Gephyrin has been demonstrated to interact with microtubules, actin filaments and the microfilament system (Kirsch et al., 1995). Exon 14 of the murine gene of gephyrin contains a region that is highly homologous to motifs in the microtubule-associated proteins MAP2 and tau, which are essential for tubulin polymerization and microtubule binding. This could be the site of the direct interaction of gephyrin with tubulin. An indirect connection of gephyrin to microtubules is also mediated by adaptor proteins like KIF5 and the dynein light chains (DYNLL1/2) (Fuhrmann et al., 2002; Maas et al., 2009). The dynein components DNLL1/2 bind to gephyrin and mediate the retrograde transport of gephyrin-GlyR complexes (Fuhrmann et al., 2002).

Recombinant gephyrin has also been shown to interact with "uncapped" actin via the adaptor Mena/VASP (Mammalian enabled/Vasodilator stimulated phosphoprotein) (Bausen et al., 2006). A complex of profilin and actin-monomers is bound by Mena/VASP and increases the efficiency of actin polymerization. Actin filament formation and bundling is a prerequisite for morphological changes and dynamic cellular processes (Dent et al., 2011). The regulation of synaptic strength is usually accompanied by a change of the synaptic structure (Izeddin et al., 2011). Dendritic spines change their size and shape in response to synaptic activity (Penzes and Cahill, 2012). GABAergic synapses are mostly found on shafts, but will also depend on the microfilament system (Luccardini et al., 2004). A contribution of actin microfilaments in gephyrin clusters has been demonstrated by the observation that cytochalasin D, an inhibitor of actin polymerization and disruptor of actin microfilaments, disrupts small gephyrin clusters in the early stages of maturation of hippocampal neurons in culture (Bausen et al., 2006). The actin-binding protein profilin is an interactor of gephyrin (Mammoto et al., 1998). Profilin also binds membrane lipids like phosphatidyl (4, 5) bisphosphate. It is assumed that through binding to membranes profilin is kept in an inactive form that can be released by phospholipase C. Gephyrin actually competes with G-actin and phospholipids for the same binding site on profilin (Giesemann et al., 2003). The interplay of these proteins and the dynamics of the microfilament system at synaptic sites might play a role in receptor density and possibly in synaptic plasticity.

There are several cytoskeletal regulatory proteins that have been found to interact with gephyrin: some of those proteins are GEFs or GAPs of the Rho family of small GTPases: the Cdc42-GEF "ARHGEF9" (collybistin) (see below), the Rho-GAP "SRGAP2" (SLIT-ROBO Rho GTPase-activating protein 2) and the Rac-GAP "WRP" (WAVE-associated Rac GAP). WRP affects gephyrin cluster size and plays a role in a human 3p-syndrome mental retardation and mutations of this protein are also linked to schizophrenia (Endris et al., 2002; Lewis et al., 2008; Addington and Rapoport, 2009; Okada et al., 2011).

COLLYBISTIN AND CELL ADHESION MOLECULES (CAMs) REGULATE GABAergic SYNAPSE FORMATION

Collybistin is a member of the Dbl family of guanine nucleotide exchange factors (GEFs) and interacts with gephyrin (Grosskreutz et al., 2001). It is a RhoGEF for the small GTPase Cdc42 and is especially important for $GABA_A$R-gephyrin clustering, as it seems to be a versatile modulator of this interaction (Kins et al., 2000). Small GTPases of the Rho family (Rho, Rac, Cdc42) are generally involved in establishing cellular polarity, transcriptional regulation, actin cytoskeleton rearrangements, intracellular trafficking, and endocytosis. They are activated by GEFs and inactivated by GAPs (GTPase activating proteins). The RhoGEF Kalirin has also been identified as an important signaling molecule at glutamatergic synapses and has been implicated in psychiatric diseases (Penzes et al., 2000; Penzes and Remmers, 2012). The involvement of RhoGEFs in cytoskeletal dynamics provides a compelling reason for their presence at dynamic synapses, as actin rearrangements will be involved in protein trafficking and synaptic stabilization. An interesting feature is the synapse-specificity of these proteins. It has been shown that collybistin exerts important functions in clustering of $GABA_A$Rs (Harvey et al., 2004).

Collybistin controls the sub-membrane accumulation and synaptic localization of gephyrin by translocating gephyrin from cytoplasmic aggregates to submembranous compartments. Collybistin knock-out mice reveal a region-specific loss of gephyrin and $GABA_A$R clusters in the hippocampus and amygdala, suggesting that gephyrin clustering might vary in different regions (Papadopoulos et al., 2007). Generally, these mice reveal a mild phenotype with no detectable impairment of gephyrin-GlyR clustering at glycinergic synapses. Collybistin occurs (species-dependently) in different alternatively spliced forms. The main variants are currently named CB 1, 2, and 3 and are distinguished by their C-terminus. Change of splice variants and mutations in the gene frequently play a role in pathological states (Harvey et al., 2008). The RhoGEF activity seems not to be important for gephyrin clustering of $GABA_A$Rs at synaptic sites. The only small GTPase, which is currently known to be associated with CB and gephyrin, Cdc42, is not vital for synapse formation and $GABA_A$R-gephyrin clustering (Reddy-Alla et al., 2010). CB2 and CB3 splice variants occur with or without a Src-homology-domain-3 (SH3):

CB(SH3+) and CB(SH3–). The presence of the SH3-domain keeps CB in an inactive conformation that might be useful during intracellular transport, while CB(SH3–) isoforms are constitutively active in clustering gephyrin without the presence of an activating protein. The cell adhesion protein neuroligin-2 (NL-2) and the $GABA_AR$ α2 subunit can activate CB(SH3+) isoforms by binding to the SH3-domain (Poulopoulos et al., 2009; Saiepour et al., 2010). An attractive hypothesis for the sequence of events that leads to the establishment of the GABAergic postsynapse is presented in the review by Papadopoulos and Soykan (2011) in Frontiers.

Interesting features include the competition of NL-2 and $GABA_AR$ α2 subunits for the collybistin SH3-domain and the competition of collybistin and $GABA_AR$ α2 for slightly shifted, yet still overlapping sites on gephyrin (Tretter et al., 2011 and **Figure 3C**). The binding sites of CB and $GABA_AR$ subunits α2 and α3 on gephyrin are positioned close to each other (see **Figure 3**). Saiepour et al. showed that the $GABA_AR$ α3 subunit directly interacts with gephyrin, while α2, gephyrin and CB form a ternary complex, where CB strengthens the otherwise weak α2-gephyrin interaction (Saiepour et al., 2010). Therefore, synapses containing $GABA_ARs$ with an α3 subunit do not need collybistin as a cofactor, while collybistin is mandatory for synapses with α2-containing $GABA_ARs$ (see **Figures 2A,B**). The theory of a selective association of CB with certain $GABA_AR$ subtypes has been rejected in the paper by Patrizi et al., yet the interaction of CB with the synaptic complexes seems to be only transient (Patrizi et al., 2011). Therefore, not all synaptic puncta are associated with CB immunoreactivity.

During synaptogenesis cell adhesion molecules like neurexin and neuroligin play very critical roles in stabilizing both excitatory and inhibitory synapses (Sudhof, 2008). Neurexin/Neuroligin knock-out mice exhibit significant behavioral phenotypes like memory deficits, hyperactive behavior or increased anxiety, revealing their importance in proper synaptogensis (Blundell et al., 2009, 2010). Other members of the CAM-family, the beta-integrins, have also been shown to be involved in gephyrin-mediated GlyR dynamics at spinal cord synapses as intercellular interactors and downstream signaling receptors (Charrier et al., 2010). Whether they play a role in GABAergic synapses remains to be investigated. At the axon initial segment (AIS) the CAM neurofascin has been shown shown to organize $GABA_AR$-gephyrin clusters in this area (Burkarth et al., 2007).

Recently, the cell adhesion molecule neuroplastin-65 of the immunoglobulin superfamily that is involved in hippocampal synaptic plasticity has also been shown to be associated with $GABA_ARs$ containing α1 or α2, but not α3 subunits at GABAergic synapses (Sarto-Jackson et al., 2012). Here, neuroplastin-65 is partially colocalized with gephyrin. In addition, neuroplastin-65 also colocalizes with $GABA_AR$ α5 subunits at extra-synaptic sites. Down-regulation of neuroplastin-65 by shRNA causes a loss of $GABA_AR$ α2 subunits at GABAergic synapses. These data indicated that neuroplastin-65 might contribute to anchoring and/or confining of a subset of $GABA_AR$ subtypes to particular synaptic or extrasynaptic sites, thus affecting receptor mobility and synaptic strength. A possible direct interaction of neuroplastin-65 with gephyrin, collybistin, or radixin has not been investigated (Sarto-Jackson et al., 2012). Given the many interaction partners of $GABA_ARs$, some of which have been identified only recently, a detailed understanding of the sequence of events during synaptogenesis including the role of different cell adhesion molecules is still missing.

Gephyrin has been shown to be bound to GlyRs already during the transport from the Golgi to the surface (Maas et al., 2006). Whether this is also the case with $GABA_ARs$ is not known. Recently the protein Muskelin has been identified and characterized as an intracellular transport protein for $GABA_AR$ (Heisler et al., 2011). An association of gephyrin with this complex has not yet been identified. In any case, the thoroughly documented constant dynamic synthesis, assembly, endocytosis and recycling process of $GABA_ARs$ provides a large pool of available "free" receptors, either in intracellular vesicles waiting to be delivered to the cell surface, or already present in the plasma membrane at extrasynaptic sites. Overexpression of collybistin recruits those receptors into even more numerous and especially much larger synaptic clusters (Chiou et al., 2011). This would also represent a hypothetical model for GABAergic synaptic plasticity.

A new feature of gephyrin clustering at GABAergic synapses is the role of the heat-shock protein 70 (Hsp 70). This protein has been repeatedly identified as co-purifying with $GABA_A$ receptors (Moss et al., 1995). A recent paper describes a role of Hsp70 in the regulation of gephyrin clustering (Machado et al., 2011). Hsp70 seems to increase the synaptic accumulation of gephyrin at inhibitory synapses as well as gephyrin polymerization independently of the interaction with associated inhibitory neurotransmitter receptors. This observation probably needs further investigations to assess its significance.

GEPHYRIN AS A HUB FOR SIGNAL TRANSDUCTION PATHWAYS TO THE NUCLEUS, INFLUENCING PROTEIN TRANSLATION

The mammalian target of Rapamycin (mTOR)-pathway is a central signaling pathway that integrates input from external signals (like growth factors and insulin) but also cellular, nutritional, energy, and redox state and is frequently disregulated in cancer. There are two known complexes: mTOR1 and mTOR2. An abundance of growth factors and nutrients leads to the initiation of protein synthesis by mTOR1 and the stimulation of RhoA, Rac, Cdc42, and PKC by mTOR2. One component, RAFT1 has been identified as a gephyrin-interacting protein (Sabatini et al., 1999). The binding site of RAFT1 for gephyrin has 45% sequence similarity to the GlyR β binding site. Therefore, the binding site on gephyrin might be roughly the same. RAFT1 (rapamycin and FKBP12 target-FRAP/mTOR) mediates drug actions of the immunosuppressant rapamycin and affects protein translation by phosphorylating the 70 kDa S6 kinase, which in turn phosphorylates 4E-BP1 (a repressor of protein translation initiation) and the ribosomal S6 protein. RAFT1 mutants that no longer interact with gephyrin do not signal to its downstream molecules. The functional consequences of this link between mTOR and gephyrin can be manifold and need further detailed investigations.

Gephyrin and collybistin have also been found as components of the eukaryotic translation initiation factor 3-complex-eIF3H (Sertie et al., 2010). This finding is rather surprising, but at the

same time very interesting, as it implies a role of collybistin and gephyrin as regulators of local synaptic protein synthesis. Gephyrin might be the link to study local protein synthesis at GABAergic synapses.

DYNAMICS AT GABAergic SYNAPSES: A MECHANISM OF PLASTICITY

Neurotransmitter receptors do not sit still at the cell surface; many are highly mobile as they can diffuse very rapidly within the lipid membrane due to Brownian motion (Choquet and Triller, 2003). However, transient interactions with their specific scaffold proteins significantly slow down their movement resulting in a reduced rate of diffusion. Using quantum dots and further developments in sophisticated technology allow real-time observation of moving and synaptically trapped receptors and can generate very insightful information about protein-protein interaction (Bannai et al., 2006, 2009; Alcor et al., 2009). Data derived from single particle tracking (SPT) experiments can be particularly useful to understand weaker interactions where standard biochemical methods are of limited value (Triller and Choquet, 2008). Indeed SPT data provided critical insights into the scaffolding action of gephyrin when previously published data on the mobility of GlyRs or γ2-containing $GABA_A$Rs were compared. The median diffusion coefficient (D) of endogenous γ2-containing $GABA_A$Rs (0.012 $\mu m^2/s$) turned out to be approximately 10-fold higher compared to the diffusion coefficient derived for endogenous GlyRs on gephyrin puncta (Meier et al., 2001; Ehrensperger et al., 2007; Lévi et al., 2008). These data can be explained by a different strength of interaction with the cytoskeleton and are consistent with our finding that gephyrin binds at least 10 times stronger to GlyRs than to $GABA_A$Rs. The higher mobility of $GABA_A$Rs might be a prerequisite for different forms of synaptic plasticity at GABAergic synapses in the brain facilitating processes like learning and memory.

GEPHYRIN MEDIATES TRANS-SYNAPTIC SIGNALING AND CROSSTALK WITH THE GLUTAMATERGIC SYSTEM

Previous observations from the glutamatergic synapse already implicate that the multiple interactions of scaffold proteins renders them indispensible for up- and downstream signaling and crosstalk with other neurotransmitter systems. PSD-95, the main scaffold protein of glutamatergic synapses, has been shown to balance excitation and inhibition in the brain (Keith and El-Husseini, 2008). Varley and co-workers used single chain antibody fragments to remove gephyrin from synapses. They observed a reduction in the probability of GABA release from the presynaptic neuron. Additionally the frequency of spontaneous and miniature glutamatergic events was significantly reduced. A reduction of NL-2 and the vesicle transporters for GABA (VGAT) and glutamate (VGLUT) was also observed (Varley et al., 2011). This implies the initiation of a postsynaptic as well as a presynaptic signaling cascade upon interaction between neurexin and neuroligin. NL-2, which as described above, was shown to be physically interacting with gephyrin and collybistin is a nucleation factor for synapse formation and regulates synapse stability. It has also been demonstrated that presynaptic neurexin physically interacts with α1-$GABA_A$Rs and can negatively modulate the inhibitory synaptic transmission in a neuroligin-independent pathway (Zhang et al., 2010). These data suggest that GABAergic synapse formation and maintenance are very complex processes and are regulated in multiple sophisticated ways. Regardless, our data demonstrate that the interaction between gephyrin and $GABA_A$Rs is very critical for the transient stability of mature synapses and dynamic modulation of this interaction can be very appropriate to tune the efficacy of inhibitory neurotransmission thus mediating synaptic plasticity. It is quite likely that mechanisms of gephyrin-mediated clustering of $GABA_A$Rs are not only dependent on the cell type but also on subcellular domains of neurons. Most investigations focus on dendritic clustering, however, $GABA_A$R-gephyrin clusters also occur in (peri)somatic and axo-axonic synapses (Poulopoulos et al., 2009; Panzanelli et al., 2011). GABAergic synaptogenesis in different areas of the neuron (dendritic, somatic, axonal domains) most likely will be differentially regulated. This extends to different cell adhesion molecules and the contribution of other cofactors like the dystrophin-glycoprotein complex. In somatic areas gephyrin clusters are frequently observed to be smaller and probably only transient (Panzanelli et al., 2011).

PHOSPHORYLATION CAN DYNAMICALLY REGULATE THE NUMBER OF POSTSYNAPTIC $GABA_A$Rs

The number of receptors present at postsynaptic sites is not constant but changes over time. We assume that, like in many other biological processes, there is a natural random mobility of receptors and that in addition, their number at the synapse can be regulated dynamically depending on the cells' or system's needs (Wenner, 2011). Currently documented ways of regulating protein activity include site-specific phosphorylation, mono- and polyubiquitination, sumoylation, acetylation, and proteolysis (Luscher et al., 2011). Neuronal activity can influence the confinement to anchoring proteins and the dwell time in microdomains of the synapse of the postsynaptic AMPA receptor, $GABA_A$Rs, and GlyRs (Groc et al., 2004; Lévi et al., 2008). In many cases, neuronal activity-dependent phosphorylation regulates the interaction between scaffolding proteins and the synaptic receptors and thereby tunes synaptic strength (Muir et al., 2010).

Several studies have already shown that gephyrin is phosphorylated by many different kinases on several serines and threonines mostly in the linker (Zita et al., 2007; Bausen et al., 2010; Tyagarajan et al., 2011; Herweg and Schwarz, 2012). Phosphorylation of gephyrin is critical for the postsynaptic clustering of $GABA_A$Rs. Tyagarajan et al. demonstrated that Ser270 of gephyrin is phosphorylated by GSK3β. GSK3β activity can negatively modulate the clustering of $GABA_A$Rs and therefore, their function. Thus, abolishing phosphorylation at this residue resulted in a higher density of gephyrin clusters and a higher frequency of mIPSCs, an effect that could be induced by the GSK3β inhibitor and mood-stabilizing drug lithium (Tyagarajan et al., 2011). It is, however, interesting to note that the GSK3β phosphorylation site is located outside of the gephyrin E-domain, the domain that primarily mediates interactions with GlyRs and $GABA_A$Rs. This modification apparently influences the folding and oligomerization of the gephyrin molecule.

Where kinases are active players, matching phosphatases are usually not far away. Protein phosphatases 1 and 2B (PP1, PP2B) have been shown to associate with the gephyrin-platform and dephosphorylate gephyrin and associated proteins (Bausen et al., 2010). The phosphatase inhibitors calyculin A and ocadaic acid reduce the size of postsynaptic gephyrin clusters.

Gephyrin also undergoes proline-directed phosphorylation (on Ser/Thr-Pro consensus sites), followed by the recruitment of Pin1 (peptidyl-prolyl isomerase NIMA interacting protein1) that interacts with gephyrin in a phosphorylation-dependent manner (Zita et al., 2007). The binding site of Pin1 comprises Ser188, Ser194, and Ser200 that were found to be phosphorylated upon expression in SF9 cells (Herweg and Schwarz, 2012). Binding of Pin1 induces a conformational change of gephyrin and enhances GlyR β binding. Whether this plays a role also in $GABA_A$R-gephyrin interactions remains to be determined.

There is strong evidence that phosphorylation of GlyRs and $GABA_A$Rs also regulates their interaction with gephyrin. A PKC phosphorylation site in the intracellular loop of GlyR β has been shown to negatively affect the binding affinity of gephyrin (Specht et al., 2011) (**Figure 1C**). A putative phosphorylation site (Thr375) has been identified in the gephyrin-binding site of the $GABA_A$R α1 subunit (Mukherjee et al., 2011) (**Figure 1C**). Here, the introduction of a charged residue (aspartate) as a phosphomimetic significantly reduces the binding affinity to gephyrin.

The binding sites of $GABA_A$ receptor α1, α2, and α3 to gephyrin contain additional amino acids that could be kinase targets (Tretter et al., unpublished results). It can be anticipated that multiple and diverse phosphorylation events are involved in the dynamics of glycinergic and GABAergic synapses, regulating inhibitory synaptic plasticity.

THE ROLE OF GEPHYRIN IN DISEASE

A compromised function of $GABA_A$Rs has been implicated in a range of neurological and psychiatric disorders, such as anxiety, insomnia, cognitive disorders, depression, epilepsy, and schizophrenia (Fritschy and Brunig, 2003). Generally spoken, epileptic seizures are believed to be conditions of imbalanced excitation and inhibition in certain brain areas that tend to progress with time involving pathologies like inflammation, necrosis, gliosis, etc. Early events are down-regulations of tonic GABA currents, loss of GABAergic interneurons and, therefore, alterations in neuronal circuit activities (Fritschy, 2008). The progression of the disease state can best be observed in animal models of temporal lobe epilepsy. Typical animal models are the kindling model and the pilocarpine injection. Fang et al. observed a reduction of immunostaining for gephyrin during both the acute and the latent period, followed by an increase during the chronic phase (Fang et al., 2011). As $GABA_A$Rs and gephyrin are functionally interdependent, the down-regulation of one of these proteins automatically also results in the loss of other partners or of other partner proteins at synapses. This might be the case during initial phases of the disease, where inhibition is disturbed either through functional over-excitation or a destruction of GABAergic interneurons and, therefore, lack of GABAergic input, and it might also be the case in a later upregulation, maybe as a neuronal attempt to increase the efficiency of inhibitory synapses (Andre et al., 2001).

Recently, an interesting observation was made concerning a possible specific role of gephyrin in epilepsy. Förstera et al. found a prevalence of alternatively spliced forms of gephyrin in brain tissue from drug-resistant mesial temporal lobe epilepsy patients (Forstera et al., 2010). Mesial temporal lobe epilepsy is a special form of focal epilepsy. The authors found four abnormally spliced gephyrin variants lacking several exons in the G-domain. As those exons are important in mediating gephyrin-gephyrin interaction, the oligomerization of the pathological forms of gephyrin is most likely impaired. Additionally they act in a dominant-negative way by interacting with regularly spliced gephyrin at synapses, thereby disrupting the normal gephyrin scaffold. Similar observations have been made experimentally in an earlier study (Calamai et al., 2009). Any fragment of gephyrin, that has the capability to assemble with endogenous gephyrin can act as a dominant-negative molecule interfering with the oligomerization process at the synapse. This affects cluster size and receptor mobility and finally synaptic strength. Mesial temporal lobe epilepsy frequently is a progressive illness. Patients usually have a history of risk factors like febrile seizures or other harmful triggers (Yang et al., 2010). As it is a multifactorial disease, the sequence of events is difficult to track down (Bozzi et al., 2011).

The role of collybistin as a cofactor in gephyrin clustering is emphasized by human disease states, where mutations of collybistin result in X-linked mental retardation associated with epilepsy due to impaired $GABA_A$R clustering (Shimojima et al., 2011). Mutations in the human CB gene (*ARHGEF9* on Xq11.1) were found in patients with diverse neurological abnormalities, including hyperekplexia, epilepsy, insomnia, aggressive behavior, and anxiety (Kalscheuer et al., 2009).

Although the sequence of molecular events in the course of developing AD is not yet resolved, one aspect might also be neuronal excitotoxicity contributing to synaptic degeneration and neuronal death. A reduced expression of gephyrin has been observed in all regions of brains from deceased AD patients (Agarwal et al., 2008). Loss of synaptic gephyrin also means a reduced neuronal inhibition and a higher susceptibility to excitotoxicity.

Loss of gephyrin is also the main problem in the autoimmune disease "Stiff man syndrome" where the body produces autoantibodies against gephyrin (Butler et al., 2000). The symptoms primarily reflect the dysfunction of GlyRs in the spinal cord.

CONCLUSION AND OUTLOOK

The strong interaction of gephyrin and GlyRs has provided an excellent model for the investigation of multiple aspects of the inhibitory postsynapse formation over the past 20 years. GlyRs and $GABA_A$Rs are structurally related, but have distinct functions in the nervous system. The weaker interaction of $GABA_A$Rs with gephyrin as compared to GlyRs confers a higher mobility of $GABA_A$Rs in synaptic areas, possibly requiring additional associated regulatory proteins. Knowledge on these associated proteins is slowly appearing. We are only beginning to understand, how GABAergic synapses are assembled. In addition, $GABA_A$Rs are

much more heterogeneous than GlyRs with regard to the number of subunits and the subunit composition. Future research will have to investigate the interaction of individual $GABA_AR$ subtypes with gephyrin and other interacting proteins, the involvement of cofactors and synapse-specificity.

Gephyrin as the central scaffold is a protein with many faces. Its extensive heterogeneity through alternative splicing, post-translational modifications and complex biophysical properties of its subdomains creates a multiplicity of structural scaffolds that can be used to accommodate and organize $GABA_AR$ subtypes and associated proteins. Whether certain gephyrin isoforms confer synaptic specificity or control the dynamics at the synapse, currently is not known at present. A variety of proteins have recently been identified to interact with gephyrin and/or with $GABA_ARs$. Structural analysis should give the most reliable information on directly interacting proteins, but is still a huge technical challenge for hetero-oligomeric receptors. The heterogeneity of $GABA_ARs$, gephyrin and cofactors provide a high flexibility for these synapses that for sure will play a role in synapse identity and synaptic plasticity.

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The role of collybistin in gephyrin clustering at inhibitory synapses: facts and open questions

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Collybistin (Cb) is a brain-specific GDP/GTP-exchange factor, which interacts with the inhibitory receptor anchoring protein gephyrin. Data from mice carrying an inactivated Cb gene indicate that Cb is required for the formation and maintenance of gephyrin and gephyrin-dependent $GABA_A$ receptor ($GABA_AR$) clusters at inhibitory postsynapses in selected regions of the mammalian forebrain. However, important aspects of how Cb's GDP/GTP-exchange activity, structure, and regulation contribute to gephyrin and $GABA_AR$ clustering, as well as its role in synaptic plasticity, remain poorly understood. Here we review the current state of knowledge about Cb's function and address open questions concerning its contribution to synapse formation, maintenance, plasticity, and adaptive changes in response to altered network activity.

Keywords: inhibitory synapse, GEF, GABA(A) receptor, GlyR, synaptogenesis, neuroligin, SH3-domain, PI3P

INTRODUCTION

Fast synaptic transmission in the nervous system is mediated by ligand-gated ion channels, which are highly concentrated at postsynaptic membrane specializations (**Figure 1**). At inhibitory synapses, the scaffolding protein gephyrin has been shown to be essential for the synaptic localization of glycine receptors (GlyRs) and major $GABA_A$ receptor ($GABA_AR$) subtypes (Kneussel and Betz, 2000; Moss and Smart, 2001). Ablation of gephyrin expression by either antisense depletion in cultured neurons or gene knockout (KO) in mice prevents the clustering of GlyRs (Kirsch and Betz, 1993; Feng et al., 1998) and α2- and γ2-subunit containing $GABA_ARs$ (Essrich et al., 1998; Kneussel et al., 1999; Luscher and Keller, 2004) at developing postsynaptic sites. In gephyrin KO animals, the loss of gephyrin from inhibitory synapses leads to death on postnatal day 0 (P0; Feng et al., 1998). The generalized stiffness of the musculature seen in the newborn gephyrin-deficient mice resembles the rigid, hyperextended phenotype resulting from poisoning with the GlyR antagonist strychnine and is consistent with a loss of motoneuron inhibition (Feng et al., 1998). Besides its role in GlyR and $GABA_AR$ clustering, gephyrin has an essential function in molybdenum cofactor (MoCo) biosynthesis (Feng et al., 1998; Stallmeyer et al., 1999). However, MoCo-deficient mice (Lee et al., 2002) survive for up to 11 days after birth. Furthermore, transgenic expression of the plant homolog Cnx1 partially rescues MoCo synthesis in gephyrin KO mice but does not prolong their survival (Grosskreutz et al., 2003). The early postnatal lethality of the gephyrin KO mice thus is likely due to the lack of postsynaptic inhibitory receptors and not MoCo-deficiency.

Gephyrin is a 93-kDa protein that contains three major domains: an amino-terminal G-domain, a carboxyterminal E-domain and a central linker region. The crystal structures of the G- and E-domains have been determined. The G-domain is homologous to the *Escherichia coli* protein MogA and forms a trimer (Liu et al., 2000; Sola et al., 2001), whereas the E-domain is homologous to the *E. coli* protein MoeA and forms a dimer (Sola et al., 2004; Kim et al., 2006). Based on the oligomerization properties of the E- and G-domains, full-length gephyrin was proposed to form a hexagonal lattice beneath the plasma membrane onto which inhibitory receptors are anchored (Kneussel and Betz, 2000; Sola et al., 2004). Consistent with this model, disruption of G-domain trimerization and E-domain dimerization through the introduction of charged residues at the oligomerization interfaces impaired postsynaptic gephyrin clustering (Saiyed et al., 2007).

Gephyrin depends on both actin microfilaments and microtubules for synaptic targeting and submembranous scaffold formation (Kirsch and Betz, 1995; Allison et al., 2000; Bausen et al., 2006; Maas et al., 2006). These interactions are thought to be critical for the postsynaptic localization and intracellular transport of gephyrin (Kneussel, 2006; Fritschy et al., 2008; **Figure 1**). Furthermore, in dissociated hippocampal neurons small highly mobile, probably vesicle-associated gephyrin particles have been shown to be added to and removed from synaptic gephyrin clusters over a time scale of a few minutes (Maas et al., 2006). This, together with a direct interaction of gephyrin with the microtubule-associated motor proteins kinesin superfamily protein 5 (KIF5; Maas et al., 2009) and dynein light chains 1 and 2 (dlc1/2; Fuhrmann et al., 2002), provides evidence for an active intracellular transport of gephyrin. Gephyrin has also been shown to bind to mammalian enabled (Mena)/vasodilator stimulated phosphoprotein (VASP; Giesemann et al., 2003; Bausen et al., 2006). Furthermore, it has been shown that recombinant gephyrin colocalizes with "uncapped" actin and that this interaction requires ena/VASP as an adaptor (Bausen et al., 2006). In addition, cytochalasin D application induced a preferential loss of small gephyrin clusters from immature but not more differentiated hippocampal neurons, suggesting a transient and early

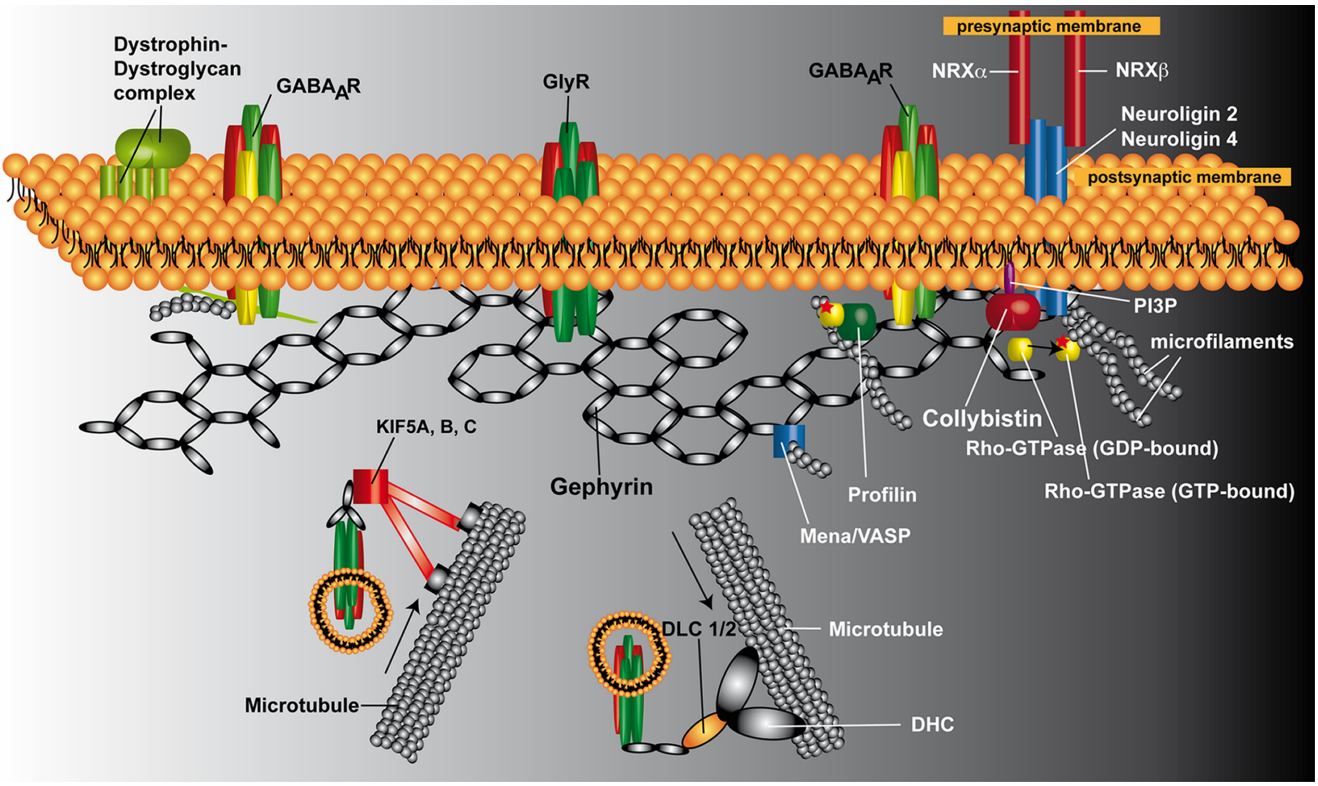

FIGURE 1 | Schematic representation of the postsynaptic protein scaffold at inhibitory synapses. Gephyrin is proposed to form a hexagonal lattice beneath the plasma membrane, onto which inhibitory receptors (GlyRs and GABA$_A$Rs) are anchored. Cb is required for the postsynaptic clustering of gephyrin at GABAergic synapses. Neuroligin 2 binds to Cb and functions as a specific activator of Cb, thus guiding membrane tethering of the gephyrin scaffold to inhibitory postsynaptic sites. However, GABA$_A$Rs containing the α2 subunit in the cerebellar cortex require the dystrophin–dystroglycal complex and not gephyrin for their targeting to inhibitory postsynapses, suggesting the existence of dystrophin-dependent and gephyrin-independent mechanism for the clustering of selected GABA$_A$R subtypes. At glycinergic synapses, gephyrin and GlyRs are targeted to the postsynapses independently of Cb. Newly synthesized GlyRs reach the plasma membrane through active transport along microtubules. Gephyrin interacts directly with KIF5 and GlyRs, and the gephyrin/KIF5/GlyR complex moves in anterograde directions toward the plus ends of microtubules. A GlyR/gephyrin/dynein transport complex mediates retrograde minus end-directed microtubule transport to intracellular compartments upon GlyR internalization. Of the known additional gephyrin-interacting factors, profilin and Mena/VASP are shown. Abbreviations: DHL, dynein heavy chain; DLC 1/2, dynein light chain 1/2; GABA$_A$R, GABA$_A$ receptor; GlyR, glycine receptor; KIF5A, B, C, kinesin family protein 5A, B, and C; Mena, microfilament adaptors of the mammalian enabled; NRXα, neurexin α; NRXβ, neurexin β; PI3P, phosphatidylinositol-3-phosphate; VASP, vasodilator stimulated phosphoprotein.

role of the actin cytoskeleton in gephyrin scaffold formation (Bausen et al., 2006). Gephyrin binds also to polymerized tubulin (Kirsch et al., 1991). Whether this interaction involves adaptor proteins like the tubulin- and gephyrin-binding protein KIF5 (Maas et al., 2009) has not been investigated. However, exon 14 of the murine gephyrin gene encodes a 14 amino acid sequence that is 80 and 60%, respectively, identical to imperfect repeat motifs of MAP2 and tau (Ramming et al., 2000). In these microtubule-associated proteins, three such repeat motifs are known to be required for tubulin polymerization and microtubule binding. Thus, gephyrin trimers might directly bind to microtubules via these motifs.

Furthermore, gephyrin binds the cytoplasmic loop of the GlyR β-subunit (Meyer et al., 1995; Sola et al., 2004), the brain-specific guanine nucleotide exchange factor (GEF) collybistin (Cb; Kins et al., 2000), the postsynaptic adhesion molecule neuroligin 2 (NL2; Poulopoulos et al., 2009) and the actin-binding protein profilin (Mammoto et al., 1998). Thus, gephyrin clearly is not just a simple scaffolding protein.

In the present review we focus on the GDP/GTP-exchange factor Cb and summarize the current state of knowledge concerning its role in inhibitory synapse formation. In addition, we discuss unmet challenges and open questions which should be addressed to deepen our understanding of the molecular mechanisms involved in Cb-dependent clustering of gephyrin and GABA$_A$Rs at inhibitory postsynapses.

STRUCTURE OF Cb

A yeast two-hybrid screen with gephyrin originally identified two Cb splice variants (Cb I and Cb II) which both harbor a dbl-homology (DH) and a pleckstrin-homology (PH) domain connected by a short linker sequence (**Figure 2A**; Kins et al., 2000). Such DH/PH tandem domains are found in all known dbl-like GEFs and are considered a signature of this family of GEF. The

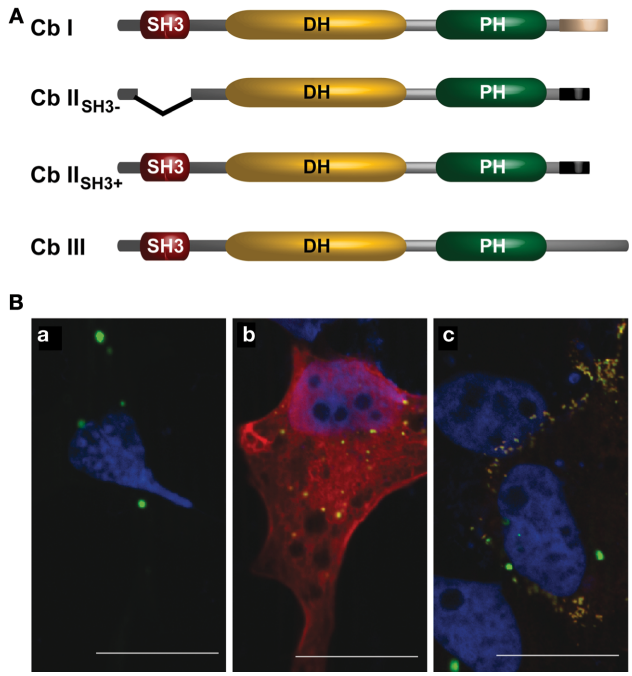

FIGURE 2 | (A) Schematic representation of the domain structures of known Cb isoforms (according to Kins et al., 2000; Harvey et al., 2004). **(B)** Co-expression of Cb isoforms differentially affects gephyrin distribution in COS7 cells. COS7 cells expressing either **(a)** GFP–gephyrin alone (green), or together with **(b)** Myc-tagged CbII$_{SH3+}$ (red), or **(c)** Myc-tagged CbII$_{SH3-}$ (red) are shown. Nuclei (blue) were visualized by DAPI-staining. Note that co-expression of Cb II$_{SH3-}$ and gephyrin induces the formation of numerous submembranous gephyrin clusters **(c)**. Scale bars, 20 μm.

DH-domain mediates the GDP/GTP-exchange activity of bdl-like oncoproteins, and the human homolog of Cb, hPEM-2, has been first shown to be a GEF specific for the small GTPase Cdc42 (Reid et al., 1999). The PH region is thought to regulate the attachment of GEFs to membranes by binding to phosphoinositides (Hyvonen et al., 1995), and the PH-domain of Cb has been shown to interact specifically with phosphatidylinositol-3-phosphate (PI3P; Kalscheuer et al., 2009).

Murine Cb I and Cb II differ in their C-terminal regions (Kins et al., 2000). The longer variant Cb I contains a segment predicted to form a coiled coil, a structure known to mediate protein interactions. In 2004, Harvey et al. identified a third splice variant, Cb III (**Figure 2A**), which contains another C-terminus that shares 59 out of 60 residues with the C-terminus of human Cb (hPEM-2; Reid et al., 1999; Harvey et al., 2004).

A second region of variation is the N-terminus of Cb. The N-terminal region of Cb I contains an additional Src homology 3 (SH3) domain, whereas for Cb II two splice variants were detected, one containing (Cb II$_{SH3+}$) and one lacking (Cb II$_{SH3-}$) the SH3-domain (**Figure 2A**; Harvey et al., 2004). Since Cb II$_{SH3-}$ catalyzes nucleotide exchange on Cdc42 more efficiently than Cb I (Xiang et al., 2006), it is thought that similar to what is known for the homologous GEFs Asef 1 and 2 (Hamann et al., 2007; Mitin et al., 2007), the SH3-domain acts as an auto-inhibitory domain retaining Cb I, Cb II$_{SH3+}$, and Cb III in an inactive conformation. To demonstrate an interaction of Cb with gephyrin in mammalian cells, Kins et al. (2000) used an assay previously employed to characterize the interaction of the GlyR β-subunit with gephyrin (Meyer et al., 1995). This assay was based on the observation that recombinant gephyrin forms large intracellular aggregates that "trap" hetero-oligomeric GlyRs (Kirsch and Betz, 1995) or green fluorescent protein (GFP) carrying a gephyrin-binding motif (Kneussel et al., 1999). These gephyrin aggregates have been shown to colocalize with the microtubule organizing center (MTOC) marker γ-tubulin (Maas et al., 2006), the actin uncapping protein ena/VASP as well as cotransfected γ-actin (Bausen et al., 2006), and thus appear to be linked to cytoskeletal structures. **Figure 2Ba** illustrates the large aggregates generated upon over-expression of GFP–gephyrin in mammalian cell lines. Notably, upon co-expression Cb II$_{SH3+}$ colocalized with gephyrin in these large cytoplasmic aggregates (**Figure 2Bb**), whereas coexpressed Cb II$_{SH3-}$ not only colocalized but caused a redistribution of gephyrin into small (diameter, 0.2–0.5 μm) microclusters beneath the plasma membrane (**Figure 2Bc**). This observation led to the hypothesis that Cb II$_{SH3-}$ induces submembranous gephyrin microaggregates, which resemble the GlyR clusters found at the initial steps of postsynaptic membrane differentiation (Kins et al., 2000).

It is noteworthy that intracellular aggregates of neuronal endogenous gephyrin were also observed *in vivo* both at early stages of neuronal maturation (Colin et al., 1996, 1998; Sassoe-Pognetto and Wassle, 1997; Papadopoulos et al., 2008) and after partial denervation of the goldfish Mauthner cell, an identified neuron in the teleost brainstem (Seitanidou et al., 1992). This suggests that gephyrin accumulation in aggregates or "blobs" is not just an artifact caused by over-expression of recombinant gephyrin in heterologous cell lines. However, whether endogenously formed gephyrin aggregates colocalize with γ-tubulin (Maas et al., 2006), Mena (the neuronal ena/VASP isoform) and/or γ-actin (Bausen et al., 2006) or collybistin (Kins et al., 2000) remains to be elucidated.

FUNCTION OF Cb: INSIGHTS FROM THE ANALYSIS OF KO MICE

In vivo evidence for the requirement of Cb in gephyrin clustering was provided by inactivating the Cb gene in mice (Papadopoulos et al., 2007). This revealed that in the absence of this neuronal GEF, gephyrin and gephyrin-dependent GABA$_A$R subtypes are lost from postsynaptic sites in selected regions of the mammalian forebrain (**Figure 3** and not shown). As a consequence, reduced GABAergic transmission, altered hippocampal synaptic plasticity, increased anxiety scores and impaired spatial learning were observed in the Cb KO animals (Papadopoulos et al., 2007; Jedlicka et al., 2009). Furthermore, using the Cre-loxP strategy to specifically inactivate the Cb gene at different developmental stages, it could be shown that Cb is required for both the initial formation and the maintenance of GABAergic postsynapses in the mouse hippocampus (Papadopoulos et al., 2008). However, in the brainstem and spinal cord of Cb KO mice, glycinergic transmission and gephyrin clustering were indistinguishable from wildtype (WT) animals (Papadopoulos et al., 2007). These studies on Cb

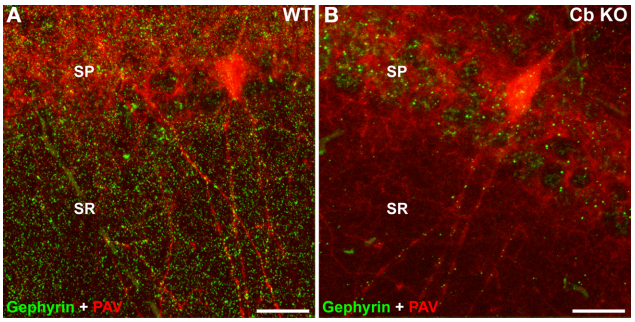

FIGURE 3 | Reduced density of gephyrin clusters in the Cb KO hippocampus. Brain sections through the hippocampus [CA1, stratum pyramidale (SP), and stratum radiatum (SR)] of WT **(A)** and Cb KO **(B)** mice were stained with the gephyrin-specific mAb 7a (green) and an antibody specific for parvalbumin (PAV; red) and processed for confocal microscopy. Note that most of the remaining gephyrin clusters in sections derived from Cb KO mice are localized on dendrites of PAV-positive interneurons. Scale bars, 20 μm.

KO mice extended the diversity of regulatory mechanisms underlying the formation and maintenance of inhibitory postsynaptic membrane specializations by disclosing two types of gephyrin-dependent clustering reactions for inhibitory neurotransmitter receptors, Cb-dependent and Cb-independent ones. In addition, the unimpaired clustering of $GABA_A$Rs containing the α2 and γ2 subunits in the cerebellum of the Cb KO mice, a region in which gephyrin cluster density was strongly affected, confirmed the existence of gephyrin-independent $GABA_A$R clustering mechanisms as previously proposed (Knuesel et al., 1999; Fischer et al., 2000; Sassoe-Pognetto and Fritschy, 2000; Kneussel et al., 2001; Levi et al., 2004). The reasons for the region-specific roles of Cb are currently unknown but might reflect compensation or interaction specificity. Interestingly, Mdx mice lacking dystrophin exhibit a marked reduction in the clustering of $GABA_A$Rs containing the α2 subunit but retain gephyrin clustering in the cerebellar cortex, suggesting a dystrophin-dependent and gephyrin-independent mechanism for the clustering of selected $GABA_A$R subtypes (Knuesel et al., 1999). In line with this view, a rather recently published study demonstrates that synArf-GEF, a member of the brefeldin A-resistant Arf-GEF/IQSEC family, activates Arf6 and localizes preferentially at inhibitory postsynaptic specializations by forming a complex with the dystrophin-associated glycoprotein complex (DGC) and the synaptic scaffolding molecule, S-SCAM (Fukaya et al., 2011). S-SCAM was previously shown to interact directly with two inhibitory postsynaptic components, β-dystroglycan and NL2 (Sumita et al., 2007), suggesting that the interaction of synArf-GEF with dystrophin and S-SCAM enables synARf-GEF to activate Arf6 in the proximity of the DGC and NL2 at inhibitory synapses. Thus, in addition to Cb, synArf-GEF is the second known regulator of GTPases that shows a preferential localization at inhibitory postsynaptic sites.

Similar to Cb-deficiency, deletion of NL2 in mice perturbs GABAergic transmission and leads to a loss of postsynaptic specializations (Poulopoulos et al., 2009). NLs have pivotal roles in organizing the assembly of different types of synapses (Varoqueaux et al., 2006) and ensuring proper synaptic connectivity (Shen and Scheiffele, 2010). NL2 binds to gephyrin through a conserved cytoplasmic motif and functions as a specific activator of Cb, thus guiding membrane tethering of the inhibitory postsynaptic scaffold (Poulopoulos et al., 2009). However, whereas Cb is required at both dendritic and perisomatic inhibitory synapses for proper clustering of gephyrin and gephyrin-dependent $GABA_A$Rs (Papadopoulos et al., 2007), NL2 critically functions at perisomatic synapses to drive postsynaptic differentiation, suggesting that other proteins can compensate the loss of NL2 at dendritic inhibitory postsynaptic sites (Poulopoulos et al., 2009; Jedlicka et al., 2011). In agreement with this hypothesis, NL4 was recently identified as a second postsynaptic adhesion protein capable of interacting with gephyrin and Cb, whose co-expression in heterologous cells results in the formation of NL4, gephyrin and Cb submembranous microaggregates (Hoon et al., 2011). In the retina of NL2 KO mice, the number of NL4 immunoreactive puncta was significantly upregulated as compared to WT, suggesting that NL4 can replace NL2 at a subset of inhibitory synapses, and that NL4 and NL2 are functionally related (Hoon et al., 2011).

An interesting difference between the Cb KO and the NL2 KO mice emerged from analyzing the network activity in the dentate gyrus (DG) of Cb- and NL2-deficient mice after perforant-path (PP) stimulation *in vivo* (Jedlicka et al., 2009, 2011). Both in Cb KO and NL2 KO animals, an increase in granule cell (GC) excitability after PP stimulation *in vivo* was observed, as compared to WT. However, paired-pulse inhibition (PPI), which is a measure for somatic GABAergic network inhibition and depends on the activity of GABAergic interneurons in the dentate network (Sloviter, 1991; DiScenna and Teyler, 1994; Bronzino et al., 1997), was increased in Cb KO mice as compared to WT, consistent with enhanced GABAergic network inhibition in the DG of the Cb-deficient animals (Jedlicka et al., 2009). In contrast, in the DG of NL2-deficient mice PPI was severely impaired and associated with reduced perisomatic clustering of $GABA_A$Rs containing the γ2 subunit, and the $GABA_A$R-mediated miniature inhibitory postsynaptic currents recorded from NL2-deficient GCs were smaller (Jedlicka et al., 2011; **Figure 4**). As the reduction of gephyrin and $GABA_A$R clusters containing the α2 and γ2 subunits seen in the DG was more pronounced in the Cb KO than the NL2 KO mice (Jedlicka et al., 2011), the enhanced PPI in the Cb KO animals was unexpected (**Figure 4**). A possible explanation could be non-linear network effects (Kapfer et al., 2007): reduced dendritic inhibition should make GCs more excitable, and hence GCs may recruit GABAergic feedback inhibition, mediated by soma-targeting interneurons, more effectively (Miles et al., 1996; Jedlicka et al., 2009; **Figure 4B**). Additional factors might also contribute to the compartment-specific alteration of GABAergic inhibition, e.g., a loss of Cb- and gephyrin-dependent $GABA_A$Rs from soma-targeting interneurons (Simburger et al., 2001) leading to disinhibition. Furthermore, compartment-specific alterations of the $GABA_A$ reversal potential (E_{GABA}) may significantly modulate the PPI of GCs, as altered expression of proteins involved in the regulation of chloride homeostasis has previously been reported to modulate PPI in the DG (Kang et al., 2006; Kwak et al., 2006).

Electrophysiological recordings from the DG indicate that Cb KO mice display significantly higher field excitatory postsynaptic

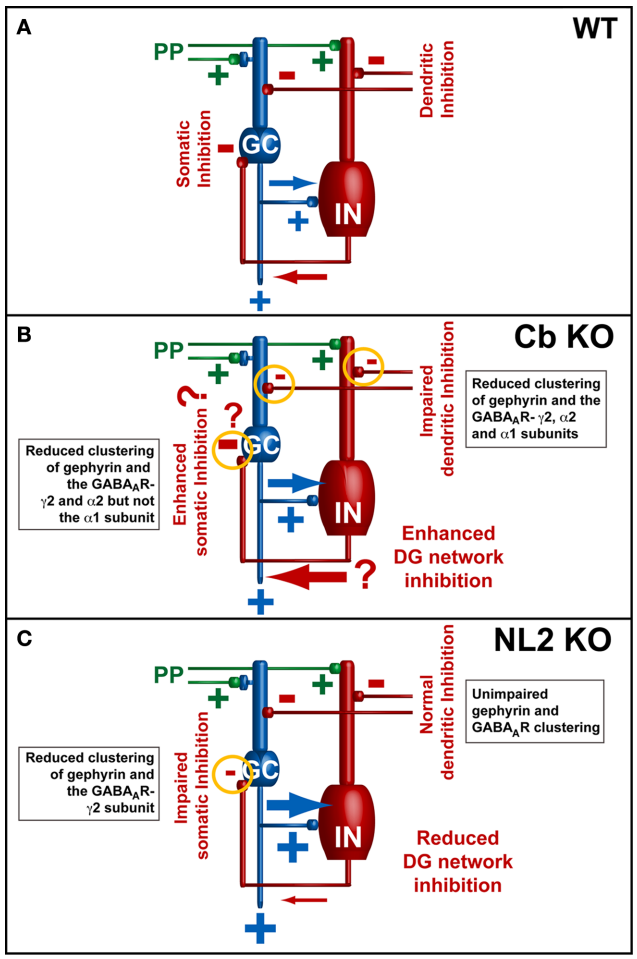

FIGURE 4 | Models of the neuronal network in the dentate gyrus (modified from Jedlicka et al., 2009; Jedlicka et al., 2011). Basic dentate gyrus circuitry: PP, perforant-path; GC, granule cells; IN, GABAergic interneurons. **(A)** PP-stimulation initiates feed-forward excitation of GCs (PP → GC) along with feed-forward (PP → IN → GC) and feedback inhibition (PP → GC → IN → GC) responsible for the paired-pulse inhibition (PPI) of GC spikes in wildtype animals. **(B,C)** Electrophysiological data from Cb KO and NL2 KO mice indicate that the reduction of GABAergic inhibition leads to an enhanced ability of GCs to fire evoked action potentials [indicated by the larger blue arrows in **(B,C)**]. A previously published computational model of $GABA_A$R-mediated PPI in the dentate gyrus indicated that PPI results mainly from a combination of perisomatic feed-forward and feedback inhibition of GCs by basket cells. Feed-forward inhibition mediated by basket cells appears to be the most significant source of PPI (Jedlicka et al., 2010). Thus, in the DG of NL2 KO mice **(B)**, the observed reduction in PPI might be a consequence of changes in perisomatic inhibition, which lead to an overall increase of the excitation/inhibition ratio in the network. However, in the DG of Cb KO mice **(A)** PPI is increased (Jedlicka et al., 2009). This might be a consequence of changes in dendritic inhibition that lead to enhanced recruitment of somatic GABAergic feedback inhibition (indicated by the large red arrow). In addition, other factors leading to enhanced somatic inhibition (indicated by question marks) and contributing to homeostatic adaptation of the inhibitory network in the DG of Cb KO mice might also be involved in the regulation of the excitation/inhibition balance.

potentials (fEPSPs) than WT littermates, suggesting a higher efficacy of excitatory synapses in the Cb-deficient hippocampus (Jedlicka et al., 2009). Previous experiments in acute hippocampal slices in the CA1 region had yielded similar results, revealing a trend to higher fEPSP slope values (Papadopoulos et al., 2007). Notably, in a few Cb KO mice, generalized tonic-clonic convulsions were observed during routine handling (Papadopoulos, unpublished observations). In order to establish the origin of these seizures, the expression of c-fos was monitored; the upregulation of this transcription factor upon neuronal activation by epileptic seizures has been widely documented (Morgan et al., 1987; **Figure 5**). Double-labeling with c-fos and glutamate decarboxylase 65 (GAD65) antibodies disclosed that c-fos was mostly upregulated in GAD65-labeled interneurons (**Figures 5D1–D0**). This suggests a preferential activation of GABAergic interneurons in the DG 2 h after a seizure. However, epileptic seizures in Cb KO mice are very infrequent events, and therefore have not been characterized more thoroughly. Notably, Tuff et al. (1983) found enhanced PPI of GC *in vivo* following kindling, an animal model for temporal lobe epilepsy (Tuff et al., 1983). The GCs of the DG, which function as a gateway and rate-limiting control unit for the flow of information through the hippocampal trisynaptic circuit (Winson and Abzug, 1978), would be an appropriate site of endogenous modulation. Further work is needed to understand whether the compartment-specific alterations of GABAergic inhibition observed in the Cb KO hippocampus, might reflect a carefully orchestrated response of the brain to repeated seizures, designed to stabilize GC excitability and to reduce the likelihood of hippocampal activation and subsequent seizures.

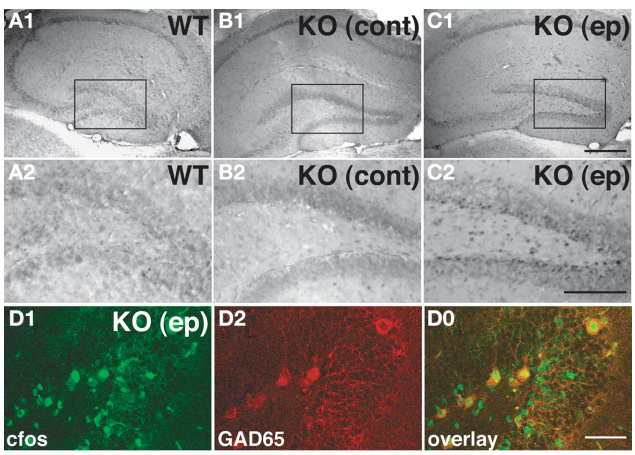

FIGURE 5 | Induction of c-fos in the hippocampus of a Cb KO mouse after an epileptic seizure episode. (A–C) Analysis of sections derived from brains of a WT mouse, a Cb KO mouse that had not shown seizures at least 2 h before decapitation (control) and a Cb KO mouse that had an epileptic seizure (ep) 2 h before decapitation disclosed differences between the patterns of c-fos expression in the hippocampus. Specifically, in sections of the Cb KO (ep) brain **(C1,C2)** cells at the border between the GC layer and the hilus exhibited strong c-fos immunoreactivity as compared to control littermates **(A1–B2)**. **(A2–C2)** Represent higher magnifications of the boxed areas indicated in **(A1–C1)**, respectively. Scale bars, 400 μm **(C1)**, 200 μm **(C2)**. **(D)** Coronal section from the dentate gyrus of a Cb KO (ep) mouse double-stained for c-fos **(D1)** and GAD65 **(D2)**. Note that GAD65-labeled cells show strong c-fos labeling in the overlay **(D0)**. Scale bar, 50 μm.

ROLES OF Cb DOMAINS IN GEPHYRIN CLUSTERING AT INHIBITORY POSTSYNAPSES

THE SH3-DOMAIN

As mentioned above, the SH3-domain of Cb is believed to act as an auto-inhibitory domain which retains Cb in an inactive conformation. Harvey et al. (2004) examined SH3 domain usage in the Cb II and Cb III splice variants and found that in postnatal rodent brain the "inactive" Cb II_{SH3+} and Cb III_{SH3+} isoforms are predominant (Harvey et al., 2004). Moreover, in transfected cortical neurons both the SH3-containing and SH3-lacking splice variants of Cb were found to similarly redistribute gephyrin into synaptic clusters (Harvey et al., 2004). This suggested that in specific neuronal subpopulations SH3-containing Cb isoforms are locally "activated" by an SH3-interacting protein. In agreement with this hypothesis, NL2 and the α2 subunit of $GABA_A$Rs were recently shown to interact with both gephyrin and the SH3-domain of Cb and to induce Cb-dependent gephyrin clustering by relieving SH3-mediated inhibition (Poulopoulos et al., 2009; Saiepour et al., 2010). This led to a model of postsynaptic membrane differentiation according to which the recruitment of NL2 under GABAergic presynaptic terminals is thought to create nucleation sites for gephyrin deposition and subsequent inhibitory receptor clustering by activating Cb through a mechanism involving NL2–Cb and NL2–gephyrin interactions (Poulopoulos et al., 2009).

Based on this model, the SH3 domain of Cb is now thought to function as trigger for a switch mechanism, which is specifically activated by NL2 (see **Figure 6**). Additionally, the inability of NL1 and NL3 to bind to and activate Cb (Poulopoulos et al., 2009) explains why these NL isoforms do not initiate inhibitory synaptic assembly. A more detailed structure-function analysis of the tripartite NL2–Cb–gephyrin complex is required for further validation and refinement of the signaling mechanism that underlies the assembly of the GABAergic inhibitory synapses. In addition, a more in-depth investigation of the NL2/NL4-dependent but Cb-independent clustering of GlyRs (Poulopoulos et al., 2009; Hoon et al., 2011) is needed in order to unravel how the formation of inhibitory synapses is triggered in brainstem and spinal cord, where glycinergic rather than GABAergic inhibition prevails.

THE DH-DOMAIN

Dbl-homology-domains are known to mediate the GDP/GTP-exchange activity of bdl-like oncoproteins (Hart et al., 1994). A hallmark of small GTPases is to undergo structural changes in response to alternate binding of GDP and GTP. The GDP-bound "off" state and the GTP-bound "on" state recognize different partner proteins, thereby allowing these G-proteins to function as molecular switches. The GTP-bound form interacts with effectors and activates pathways that affect cell morphology, trafficking, growth, differentiation and apoptosis. Inactivation of the GTP-bound form requires binding of the GTPase to another class of GTPase-interacting proteins, the GTPase activating proteins (GAPs; for review see Cherfils and Chardin, 1999). GEFs stimulate the dissociation of tightly bound GDP from small G-proteins in response to upstream signals. This reaction involves several stages: first, the GEF forms a low-affinity docking complex with the GDP-bound GTPase. GDP then dissociates from this initial complex, which results in a high-affinity binary GEF–G-protein complex. This intermediate does not accumulate in the cell because it is rapidly dissociated upon GTP binding (Cherfils and Chardin, 1999).

Although previous experiments have clearly shown that Cb's gephyrin clustering function is "activated" by NL2, the signaling mechanisms through which Cb activation induces gephyrin recruitment remain enigmatic. As Cdc42 is the only GTPase known to be activated by Cb (Reid et al., 1999; Xiang et al., 2006), it was previously believed that the interaction of Cb with Cdc42 and activated Cdc42 are required for gephyrin clustering at inhibitory postsynaptic sites (Kneussel and Betz, 2000). However, the analysis of Cb mutants deficient in catalyzing GDP/GTP-exchange on Cdc42 and of Cdc42 conditional KO mice clearly demonstrated that Cdc42 is not essential for inhibitory synapse formation (Reddy-Alla et al., 2010). Importantly, inactivation of the Cdc42 gene in the mouse forebrain at early developmental stages had no effect on gephyrin and $GABA_A$R cluster densities in the hippocampus (Reddy-Alla et al., 2010). These findings suggest that other, as yet unidentified Rho-like GTPases are activated by Cb at synaptic sites. Dbl-family proteins display varied selectivities, and the available analyses mainly focused on Cdc42, Rac1 and RhoA (Reid et al., 1999; Schmidt and Hall, 2002). Non-conserved residues that reside within the GTPase interaction sites of the DH-domains are likely candidates for determining specific coupling (Worthylake et al., 2000). A possible scenario thus could be that the DH-domain of Cb discriminates among different G-proteins, thereby regulating the spatio-temporal activation of more than one Rho–GTPase in response to distinct stimuli. Ultimately, different approaches will be needed to identify novel Cb cognate Rho–GTPases in the mouse brain and determine their importance for inhibitory synapse formation.

THE PH-DOMAIN

Deletion of the PH-domain of Cb has been previously shown to abolish PI3P binding without affecting the interaction of Cb with gephyrin (Kalscheuer et al., 2009). Furthermore, over-expression of a Cb II_{SH3-} deletion mutant lacking the PH-domain interfered with gephyrin clustering at synaptic sites (Harvey et al., 2004). However, based on these results it was not clear whether the effects of PH-domain deletion are indeed due to impaired phosphoinositide binding or due to more global effects on protein structure. Reddy-Alla et al. (2010) analyzed the interaction of the Cb PH-domain with PI3P in more detail. In this study, a Cb II_{SH3-} mutant was generated in which the arginine residues R303 and R304 were both replaced by asparagines. These residues reside within the β3–β4 loop of the Cb PH-domain, and their positively charged side chains are surface-exposed. The positively charged surfaces of the β1–β2 and β3–β4 loops of several other PH-domains are known to be involved in membrane lipid anchoring (Hyvonen et al., 1995; Yu et al., 2004; DiNitto and Lambright, 2006). Using immobilized PI3P and purified glutathione-*S*-transferase (GST)-tagged Cb II_{SH3-} and GST–Cb II_{SH3-}-RR303-304NN in a lipid overlay assay, the RR303-304 residues were confirmed to be essential for Cb II_{SH3-} binding to this phosphoinositide. Co-expression of the Cb II_{SH3-}-RR303-304NN mutant with GFP–gephyrin in NIH-3T3

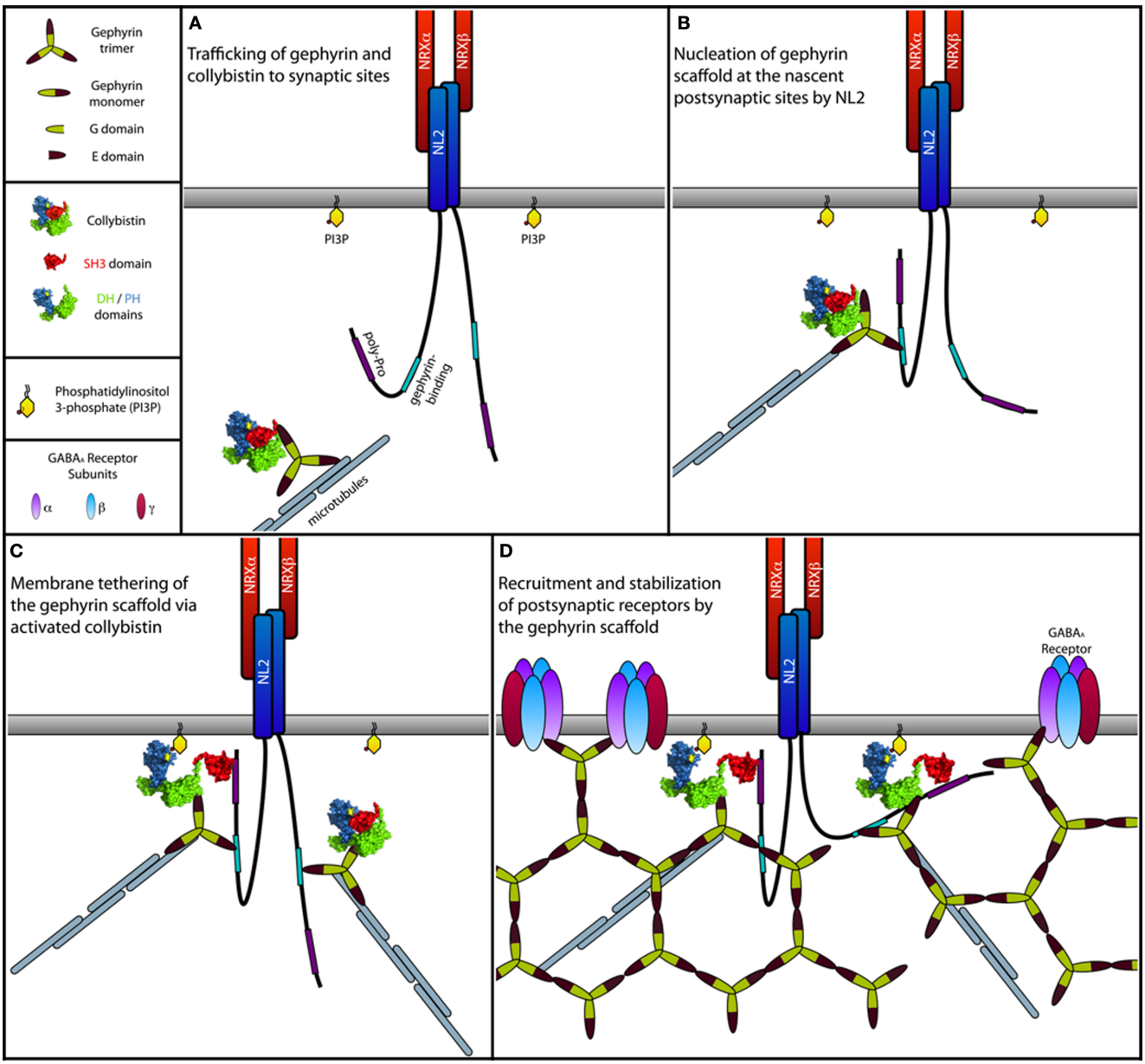

FIGURE 6 | Model of the NL2-induced activation of Cb at inhibitory postsynapses. (A) Gephyrin trimers associated with Cb are transported to subsynaptic sites along the microtubules by forming complexes with motor proteins. For simplicity, motor proteins are not indicated. During transport, Cb adopts a closed conformation, in which the SH3 domain folds back onto the tandem DH/PH-domains, similar to what is known for the autoinhibited form of Asef, the closest homolog of Cb (Kawasaki et al., 2007; Mitin et al., 2007). In this closed inactive conformation, both the catalytic activity of the DH-domain and binding of the PH-domain to membrane phosphoinositides (see text below) are suppressed due to steric hindrance imposed by the SH3 domain. **(B)** At postsynaptic sites, NL2 molecules clustered by presynaptically expressed Neurexins (NRXs) interact transiently with the cytoplasmic gephyrin–Cb complex. This interaction brings the proline-rich cytoplasmic domain of NL2 into close vicinity of the SH3 domain of Cb. **(C)** A typical SH3 domain–ligand interaction mediated by a PXXP motif in the cytoplasmic domain of NL2 leads to a structural rearrangement in Cb, resulting in a more open conformation of this GEF. Similar to the APC mediated activation of Asef (Mitin et al., 2007), binding of NL2 is thought to interfere with the intramolecular interactions between the SH3 and the DH/PH-domains. This transition toward an open state now allows the PH-domain to bind PI3P-rich domains within the plasma membrane, which might be generated postsynaptically as a product of a yet unknown signaling mechanism (see text below) induced by either NL2, Cb, or other inhibitory synaptic molecules. Together with Cb, gephyrin is co-recruited to the PI3P-rich membrane domains where it assembles into the postsynaptic gephyrin scaffold. **(D)** $GABA_A$Rs are then recruited to the growing postsynaptic scaffold.

cells led to the formation of large intracellular GFP–gephyrin aggregates, and its over-expression in cultured hippocampal neurons caused a strong reduction of synaptic GFP–gephyrin clusters colocalizing with epitope-tagged Cb (Reddy-Alla et al., 2010). Thus, Cb binding to PI3P appears to be essential for its efficient recruitment to postsynaptic sites together with gephyrin.

PI3P was considered for a long time as a lipid constitutively present on endosomes, mainly produced by class III phosphatidylinositol 3-kinase (PI3K; Gillooly et al., 2000; Vicinanza et al., 2008), and without any dynamic roles in signaling. However, during the past few years it has become increasingly evident that pools of PI3P can be specifically generated upon cellular stimulation, and PI3P has emerged as a critical intracellular second messenger involved in different pathways (for review see Falasca and Maffucci, 2006). Notably, PI3P is known to promote plasma membrane translocation of the glucose transporter 4 (Maffucci et al., 2003). Whether Cb can bind to gephyrin associated with PI3P-containing intracellular vesicles, and whether this interaction might be crucial for the translocation of gephyrin and Cb to the plasma membrane remains to be explored. More recently, Falasca et al. (2007) showed that activation of a member of the class II PI3Ks, PI3K–C2α, but not activation of the class III PI3K, hVps34, generates PI3P in the plasma membrane (Falasca et al., 2007). In addition, previous studies on the differential sensitivities to PI3K inhibitors of the endosomal versus the plasma membrane pool of PI3P strongly suggest that multiple enzymes are involved in the generation of PI3P (Maffucci et al., 2003, 2005). The general consensus is that class III PI3Ks are responsible for the production of the constitutive endosomal pool of PI3P (Schu et al., 1993). Several lines of evidence suggest a potential role of class II PI3K isoforms in agonist-mediated regulations of cellular functions. This includes activation of PI3K–C2α by insulin, TNFα and leptin (Brown et al., 1999; Ktori et al., 2003), and of PI3K–C2β by insulin and LPA (Brown and Shepherd, 2001; Maffucci et al., 2005). It appears feasible that inhibitory synapse function involves a yet unknown PI3K enzyme that generates PI3P at specific sites of the neuronal plasma membrane, and thereby regulates Cb-mediated gephyrin deposition at synaptic sites. Clearly further work is needed to fully understand how Cb regulates gephyrin deposition at inhibitory postsynapses.

PERSPECTIVES

Unraveling the molecular mechanisms of the Cb-dependent transport, clustering, and maintenance of gephyrin and $GABA_A$Rs at inhibitory synapses will be a key step toward a better understanding of GABAergic transmission in health and disease. This will require detailed knowledge of Cb's specificity for Rho–GTPases expressed in the mammalian forebrain. Once novel candidate GTPases have been identified, defining how their Cb-dependent activation affects gephyrin postsynaptic clustering would be the next step toward understanding the role of this GEF in inhibitory synapse formation. Previous work indicates that different actin-associated proteins (reviewed in Kneussel and Betz, 2000; Moss and Smart, 2001), including Mena, the neuronal ena/VASP isoform (Giesemann et al., 2003), interact with gephyrin. However, whether Cb can regulate actin cytoskeleton dynamics by activating a Rho–GTPase other than Cdc42 and whether a Rho–GTPase activation is required for gephyrin postsynaptic clustering is currently not known.

Despite the recent progress, the mechanism of PI3P-mediated anchoring of Cb at the postsynaptic membrane remains poorly understood. Whether PI3P is interacting with Cb directly at the plasma membrane upon stimulated accumulation of this phosphoinositide, or whether Cb can bind to gephyrin associated with PI3P at intracellular vesicles remains to be further investigated.

Furthermore, structural analysis of the tripartite NL2–gephyrin–Cb complex would be required for complete understanding how the interaction of NL2 with Cb's SH3-domain mediates Cb activation and subsequent gephyrin postsynaptic clustering.

In addition, a detailed spatio-temporal analysis on the expression of individual Cb isoforms in different regions of the mammalian brain, as well as an electron microscopic demonstration of the subcellular localization of the different isoforms during development will help to understand how the activation of Cb contributes to gephyrin postsynaptic clustering. Lastly, anatomical and electrophysiological studies with Cb-deficient mice will be required to identify the brain regions and neuron subclasses that contain Cb-dependent inhibitory postsynapses and to unravel the roles of Cb in inhibitory synaptic plasticity and adaptive responses to altered network activity.

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Selective changes of $GABA_A$ channel subunit mRNAs in the hippocampus and orbitofrontal cortex but not in prefrontal cortex of human alcoholics

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Alcohol dependence is a common chronic relapsing disorder. The development of alcohol dependence has been associated with changes in brain $GABA_A$ channel-mediated neurotransmission and plasticity. We have examined mRNA expression of the $GABA_A$ channel subunit genes in three brain regions in individuals with or without alcohol dependence using quantitative real-time PCR assay. The levels of selective $GABA_A$ channel subunit mRNAs were altered in specific brain regions in alcoholic subjects. Significant increase in the α1, α4, α5, β1, and γ1 subunit mRNAs in the hippocampal dentate gyrus region, and decrease in the β2 and δ subunit mRNAs in the orbitofrontal cortex were identified whereas no changes in the dorsolateral prefrontal cortex were detected. The data increase our understanding of the role of $GABA_A$ channels in the development of alcohol dependence.

Keywords: alcohol dependence, brain, $GABA_A$ channel, post-mortem

INTRODUCTION

Beverages containing alcohol are commonly consumed in today's societies and often abused. The brain is one of the main targets of alcohol. Long-term excessive consumption of alcohol can change the brain and lead to a variety of behavioral changes such as addiction and cognitive dysfunction (Harper, 1998). Magnetic resonance imaging studies have showed reduced hippocampal and prefrontal cortex volume of individuals suffering from alcohol dependence that may contribute to the cognitive deficit associated with chronic alcohol exposure (Jernigan et al., 1991; Sullivan et al., 1995). These aversive effects may be associated with direct and indirect actions of alcohol on various neurotransmitter and neuropeptide systems within the central nervous system (CNS; Harris et al., 2008; Vengeliene et al., 2008; Spanagel, 2009). Among those neurotransmitter receptors, a special focus has been on the association of alcohol action and alcoholism with γ-aminobutyric acid type A ($GABA_A$) ion channels during the last 30 years. Many $GABA_A$ channel subunit genes have been suggested to be associated with human alcoholism (Korpi and Sinkkonen, 2006), but detailed mechanisms remain poorly known and are inconsistent between studies.

The $GABA_A$ channels are GABA-gated anion channels that predominantly mediate inhibitory neurotransmission within CNS. Each $GABA_A$ channel complex is formed by five homologous protein subunits and to date 19 $GABA_A$ channel subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3) have been identified in mammals (Olsen and Sieghart, 2008). The various combinations of different subunits and associated proteins account for the diverse pharmacological and biophysical properties of the $GABA_A$ channel complex in the plasma membrane (Birnir and Korpi, 2007; Uusi-Oukari and Korpi, 2010). $GABA_A$ channels in neurons are present at synapses and extrasynaptic sites and mediate fast phasic inhibition and persistent tonic inhibition, respectively (Mody and Pearce, 2004; Semyanov et al., 2004; Lindquist and Birnir, 2006; Jin et al., 2011). Although many previous studies have shown that alcohol potentiates $GABA_A$ channels, how alcohol directly acts on $GABA_A$ channels remains unclear (Korpi et al., 2007). Alcohol action on $GABA_A$ channels depends on the concentration of alcohol and the channel subunits composition (Olsen et al., 2007). Some reports have demonstrated that low concentrations of alcohol (3–30 mM) can enhance the tonic inhibition mediated by extrasynaptic α4/6βδ $GABA_A$ channels but not the phasic inhibition mediated by γ2-containing synaptic $GABA_A$ channels (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Borghese et al., 2006; Korpi et al., 2007; Baur et al., 2009). In contrast, high concentration of alcohol (>60 mM) directly modulates $GABA_A$ channels associated with phasic inhibition and the putative alcohol-binding site was identified in-between transmembrane domains 2 and 3 (Mihic et al., 1997). In addition to the acute alcohol effects on $GABA_A$ channels, chronic exposure to alcohol can affect $GABA_A$ channel functions via alterations in subunit expression, post-translational modification, localization, intracellular signaling, and neurosteroid response (Kumar et al., 2009). In rodent models, chronic alcohol administration differentially changes the expression levels of $GABA_A$ channel subunit mRNAs and proteins across various brain regions (Grobin et al., 2000; Liang et al., 2006; Sarviharju et al., 2006). However, alcohol-dependent disorders in humans are not fully mimicked

by rodent models. Therefore, studies conducted on samples from post-mortem brains of individuals suffering from alcohol dependence may add important information and aid in understanding the mechanisms underlying human alcohol-dependent disorders.

In the present study, we performed quantitative real-time PCR (RT-qPCR) to investigate the expression of $GABA_A$ channel subunit mRNAs in the post-mortem hippocampal dentate gyrus, orbitofrontal, and dorsolateral prefrontal cortex (DL-PFC) of individuals suffering from alcohol dependence and have compared the results to brain samples from individuals without alcohol dependence.

MATERIALS AND METHODS

HUMAN SAMPLES

Twenty-one human controls and 19 individuals suffering from chronic alcohol dependence were included in the study. All individuals were Caucasian males. The individuals suffering from alcoholism consumed ≥80 g alcohol per day during the majority of their adult lives, met the criteria for Diagnostic and Statistical Manual for Mental Disorders, fourth edition and National Health and Medical Research Council/World Health Organization and did not have liver cirrhosis, Wernicke–Korsakoff's syndrome, or multi-drug abuse history. Individuals in the control group had either abstained from alcohol completely or were social drinkers who consumed ≤20 g of alcohol per day on average. Individuals in the control group were matched to individuals suffering from alcoholism by age and post-mortem interval (PMI). Post-mortem brain samples from hippocampal dentate gyrus (including both granule and molecular layer), orbitofrontal cortex (OFC; Brodmann's area 47), and DL-PFC (Brodmann's area 9) were collected at the New South Wales Tissue Resource Center (TRC), University of Sydney, Australia (http://sydney.edu.au/medicine/pathology/trc/index.php). The samples from all three brain regions were collected from the same donor in seven controls and ten individuals suffering from alcoholism. All samples were collected by qualified pathologists under full ethical clearance and with informed, written consent from the next of kin. The detailed demographic data for all subjects are given in **Table A1** in Appendix.

TOTAL RNA ISOLATION

Total RNA was isolated by using RNeasy Lipid Tissue Mini Kit (QIAGEN, MD, USA) or GenElute total RNA Miniprep (Sigma) and quantified with Nanodrop (Nanodrop Technologies, Inc.). The quality of RNA was evaluated by measuring RNA Quality Indicator (RQI) using Bio-Rad Experion (Bio-Rad Laboratories, Hercules, CA, USA) with Eukaryote Total RNA StdSens assay following the manufacturer's manual. RQI is equivalent to RNA integrity number (RIN) from Agilent (Denisov et al., 2008). RNA samples with RQI values greater than 5 are generally considered as suitable for RT-qPCR (Fleige and Pfaffl, 2006; Fleige et al., 2006). In this

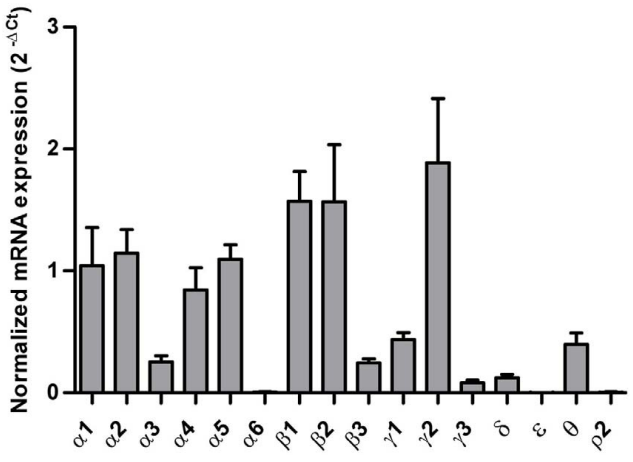

FIGURE 1 | Expression of $GABA_A$ channel subunit mRNAs in the hippocampal dentate gyrus region from control subjects ($n = 15$). The mRNA level of each subunit was normalized to reference genes *ACTB* and *UBC* and presented as mean ± SE.

Table 1 | Sample demographic information.

Characteristics	Hippocampal dentate gyrus			Dorsolateral prefrontal cortex			Orbitofrontral cortex		
	Controls	Alcoholics	*p* Value	Controls	Alcoholics	*p* Values	Controls	Alcoholics	*p* Value
Number	15	13		15	14		14	11	
Age (years)	57 ± 3	56 ± 4	0.909	59 ± 4	59 ± 4	0.981	59 ± 4	58 ± 5	0.774
PMI (h)	30 ± 4.8	30 ± 4.9	0.908	27 ± 4.2	29 ± 4.2	0.777	27 ± 4.6	29 ± 4.7	0.763
Brain pH	6.5 ± 0.05	6.5 ± 0.05	0.599	6.5 ± 0.06	6.5 ± 0.07	0.458	6.6 ± 0.06	6.5 ± 0.13	0.714
RNA quality indicator	6.9 ± 0.23	6.6 ± 0.14	0.23	7.3 ± 0.31	7.8 ± 0.31	0.206	7.7 ± 0.99	7.6 ± 0.96	0.936
Smoking history*	8(67%) S, 4(33%) NS	9(82%) S, 2(12%) NS	0.64	10 (77%) S, 3 (23%) NS	9(82%) S, 2(18%) NS	1.0	10(77%) S, 3(23%) NS	7(78%) S, 2(22%) NS	1.0

PMI, post-mortem interval; S, smoker; NS, non-smoker.

Age, PMI, brain pH, and RNA quality indicator are shown as mean ± SE, and the difference between controls and alcoholics was tested with Student's t-test or Mann–Whitney U-test.

**Smoking histories are not available for all subjects. The proportion of smokers and non-smokers between controls and alcoholics was tested with Fisher's exact test.*

study, samples with RQI less than 5 were not used for experiments. Average RQI of the samples was 7.29 ± 0.12 (mean ± SEM; 83% samples have RQI greater than 6) indicating high quality of isolated total RNA.

QUANTITATIVE REAL-TIME RT-PCR

Total RNA (250 ng) was reverse transcribed into cDNA in a 20 µl reaction mixture using Superscript III reverse transcriptase (Invitrogen). RT negative control was performed by omitting reverse transcriptase in the reaction in order to confirm no genomic DNA contamination in the isolated RNA. Real-time PCRs were performed in a 10 µl reaction mixture containing 4 µl cDNA (1 ng), 1 × PCR reaction buffer, 3 mM $MgCl_2$, 0.3 mM dNTP, 1 × ROX reference dye, 0.8 U JumpStart *Taq* DNA polymerase (Sigma-Aldrich), 5 × SYBR Green I (Invitrogen), and 0.4 µM each of forward and reverse primers. The gene-specific primer pairs (primer sequences shown in **Table A2** in Appendix) were designed using Primer Express Software version 3.0 (Applied Biosystems), synthesized by Invitrogen and further validated using BioBank cDNA from human brain (PrimerDesign). Amplification was performed in 384-well optical plates using the ABI PRISM 7900HT Sequence Detection System (Applied

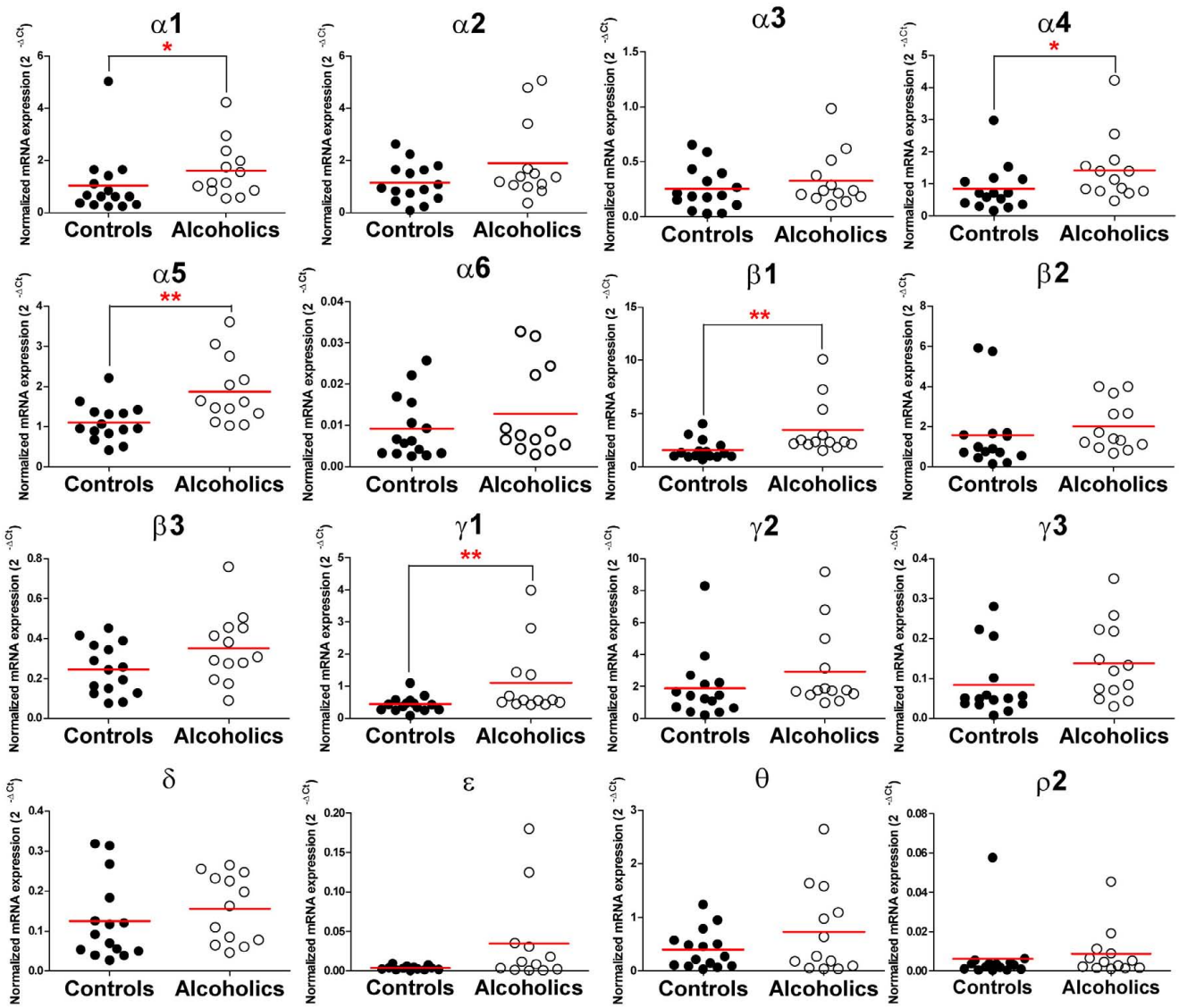

FIGURE 2 | Expression of $GABA_A$ channel subunit mRNAs in the hippocampal dentate gyrus region of controls (●, *n* = 15) and alcoholics (○, *n* = 13). Horizontal lines represented mean levels. Kruskal–Wallis ANOVA on ranks with Dunn's *post hoc* test, α1, $H(1, 28) = 4.39$, $p = 0.036$; α2, $H(1, 28) = 1.72$, $p = 0.19$; α3, $H(1, 28) = 0.73$, $p = 0.39$; α4, $H(1, 28) = 5.41$, $p = 0.02$; α 5, $H(1, 28) = 8.83$, $p = 0.003$; α6, $H(1, 28) = 1.17$, $p = 0.28$; β1, $H(1, 28) = 9.38$, $p = 0.002$; β2, $H(1, 28) = 2.68$, $p = 0.10$; γ1, $H(1, 28) = 7.02$, $p = 0.008$; γ2, $H(1, 28) = 2.83$, $p = 0.09$; γ3, $H(1, 28) = 3.31$, $p = 0.07$; δ, $H(1, 28) = 1.07$, $p = 0.3$; ρ2, $H(1, 28) = 1.97$, $p = 0.16$; θ, $H(1, 28) = 0.38$, $p = 0.53$; ε, $H(1, 28) = 2.32$, $p = 0.13$. One way ANOVA with Bonferroni *post hoc* test, β3, df = 23, $p = 0.06$.

Biosystems) with an initial denaturation of 5 min at 95°C, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. A melting curve was determined at the end of cycling to ensure the amplification of a single PCR product. Cycle threshold values (Ct) were determined with the SDS 2.3 and RQ Manager 1.2 softwares supplied with the instrument. The expression of each target gene relative to a normalization factor (geometric mean of two reference genes) was calculated with DataAssist v2.0 using the $2^{-\Delta Ct}$ method as previously described (Schmittgen and Livak, 2008). Reference genes beta actin (*ACTB*) and ubiquitin C (*UBC*) for hippocampal dentate gyrus (average expression stability value $M = 0.25$), ribosomal large P0 (*RPLP0*) and *ACTB* for prefrontal cortex (average expression stability value $M = 0.22$), and phosphoglycerate kinase 1 (*PGK1*) and peptidylprolyl isomerase A (*PPIA*) for OFC (average expression stability value $M = 0.125$) were selected for normalization according to previously developed approach for analysis of reference genes (Johansson et al., 2007; Kuzmin et al., 2009; Bazov et al., 2011). As the expression of reference genes may vary between different brain regions of human alcoholics, it is of great importance to use validated stable reference genes for normalization.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SigmaPlot and SigmaStat (Systat Software Inc., USA). Normality of data distribution was analyzed using Shapiro–Wilk normality test (see **Table A3** in Appendix). The differences between groups were assessed by one-way ANOVA with Bonferroni *post hoc* test (normally distributed data) or non-parametric Kruskal–Wallis ANOVA on ranks with Dunn's *post hoc* test (not normally distributed data). A general stepwise linear regression model was used to identify covariates (e.g., age and PMI). Variables with a significant association with group (controls and alcoholics) were included in the final statistical model as covariates. A significant level was set to $p < 0.05$.

RESULTS

The demographic characteristics of individuals in this study are shown in **Table 1** and **Table A1** in Appendix. There was no significant difference in age, PMI, brain pH, RNA quality indicator, and proportions of smokers and non-smokers between individuals with or without alcohol dependence (**Table 1**).

Expression of the 19 $GABA_A$ channel subunit mRNAs (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3) was quantified by RT-qPCR in the samples collected from the hippocampal dentate gyrus region (HP-DG), the orbitofronral cortex (OFC, Brodmann area 47), and the dorsolateral prefrontal cortex (DL-PFC, Brodmann area 9). Subunit genes that were not detected in any of the three brain regions were the π, ρ1, and ρ3 subunits.

INCREASED LEVELS OF mRNAS FOR $GABA_A$ CHANNEL SUBUNITS α1, α4, α5, β1, AND γ1 IN THE HIPPOCAMPAL DENTATE GYRUS REGION OF ALCOHOLICS

In the hippocampal dentate gyrus region of individuals without alcohol dependence, high expression of α1, α2, α4, α5, β1, β2, and γ2, modest expression of α3, β3, γ1, γ3, δ, and θ, low expression of α6, ε, and ρ2 subunit mRNAs were detected (**Figure 1**).

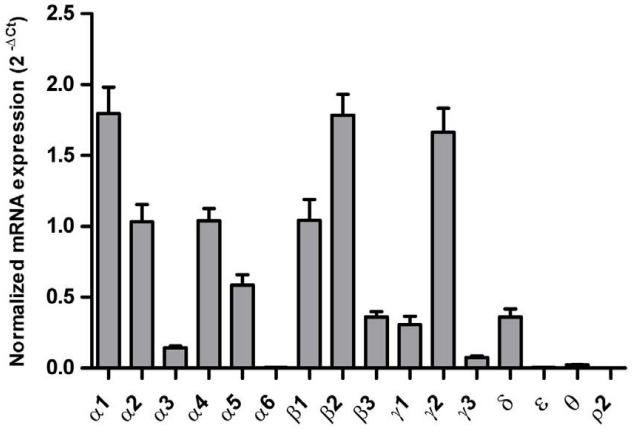

FIGURE 3 | Expression of $GABA_A$ channel subunit mRNAs in the orbitolfrontal cortex of controls ($n = 14$). The mRNA level of each subunit was normalized to reference genes *PPIA* and *PGK1* and presented as mean ± SE.

Interestingly, the mRNA levels of α1, α4, α5, β1, and γ1 subunits were significantly higher in the HP-DG of individuals suffering from alcohol dependence than in the controls. There was a 1.5-fold (α1), 1.6-fold (α4), 1.7-fold (α5), 2.1-fold (β1), and 2.3-fold (γ1) increase of mRNA in the hippocampal dentate gyrus region of individuals suffering from alcoholism as compared to those without alcohol dependence. Stepwise linear regression identified age, PMI, and brain pH as covariates for the α5 expression level. However, inclusion of these covariates in linear regression model did not affect the significant difference in α5 expression level between the two groups. The mRNA levels of other $GABA_A$ channel subunits did not differ between two groups (**Figure 2**).

DECREASED EXPRESSION OF $GABA_A$ CHANNEL SUBUNIT β2 AND δ mRNAS IN THE ORBITOFRONTAL CORTEX OF ALCOHOLICS

In the OFC of individuals without alcohol dependence, high expression of α1, α2, α4, β1, β2, and γ2, modest expression of α3, α5, β3, γ1, γ3, and δ, low expression of α6, ε, θ, and ρ2 subunit mRNAs were detected (**Figure 3**). Of particular interest is the result that the mRNA levels of the β2 and δ subunits were 26 and 47% lower, respectively, in individuals suffering from alcohol dependence as compared to those without alcohol dependence, while the mRNA expression of other subunits did not differ between the two groups (**Figure 4**). Stepwise linear regression identified age as a covariate for the β2 and δ expression level. However, inclusion of age as a covariate in linear regression model failed to affect the significant difference in the β2 and δ expression levels between the two groups.

UNALTERED EXPRESSION OF $GABA_A$ CHANNEL SUBUNIT mRNAS IN THE DORSOLATERAL PREFRONTAL CORTEX OF ALCOHOLICS

The mRNA expression profile of $GABA_A$ channel subunits in the DL-PFC in individuals without alcohol dependence closely resembled that observed in the OFC (**Figures 3** and **5**). Furthermore, no significant differences in the mRNA expression were observed for

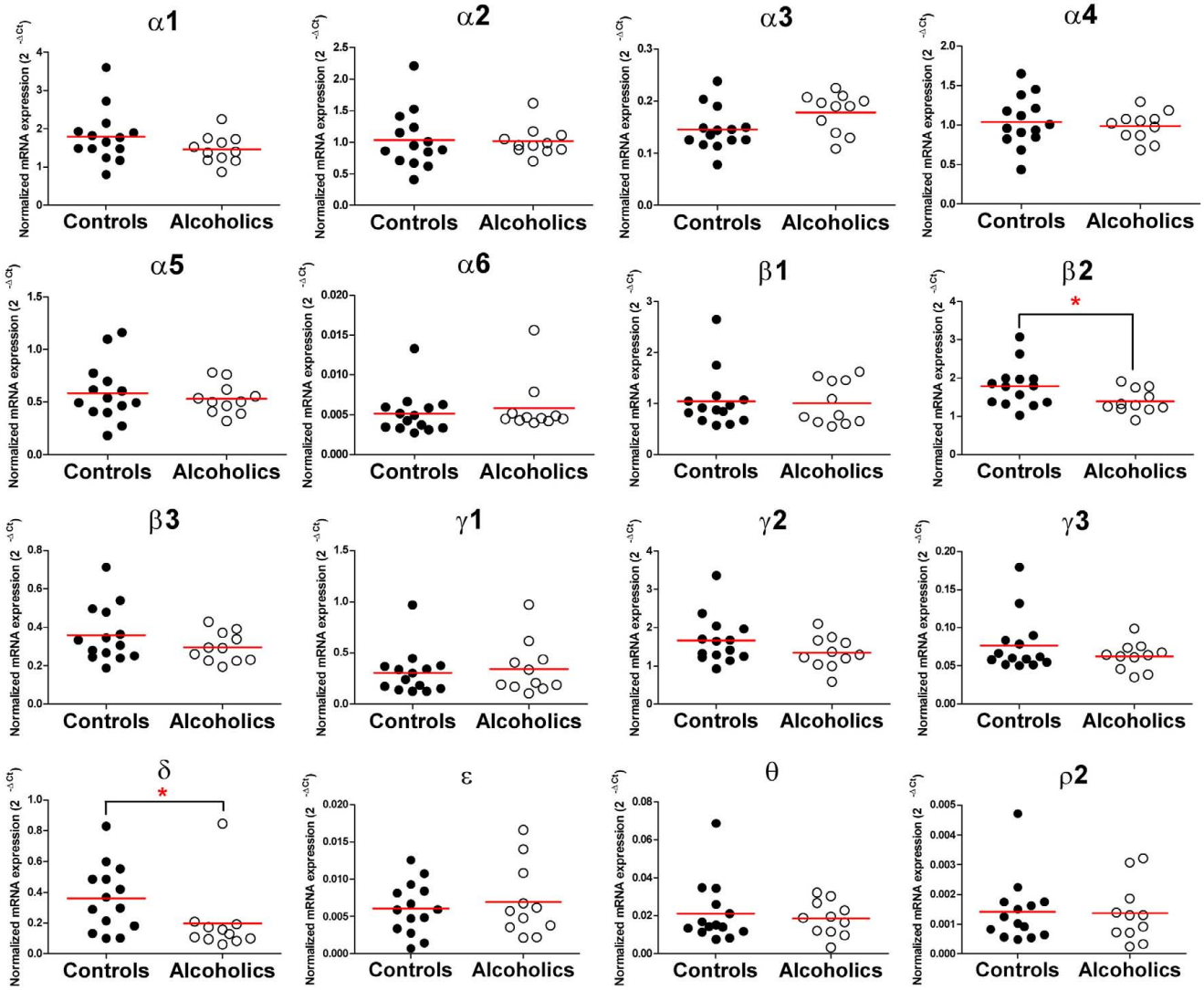

FIGURE 4 | Expression of $GABA_A$ channel subunits mRNA in the orbitofrontal cortex of controls (●, $n = 14$) and alcoholics (○, $n = 11$). Horizontal lines represented mean levels. One way ANOVA with Bonferroni *post hoc* test, α3, df = 23, $p = 0.052$; α4, df = 23, $p = 0.61$; α 5, df = 23, $p = 0.55$; β2, df = 23, $p = 0.045$; ε, df = 23, $p = 0.06$. Kruskal–Wallis ANOVA on ranks with Dunn's *post hoc* test, α1, $H(1, 25) = 2.03$, $p = 0.15$; α2, $H(1, 25) = 0.3$, $p = 0.58$; α6, $H(1, 25) = 0.51$, $p = 0.48$; β1, $H(1, 25) = 0.05$, $p = 0.83$; β3, $H(1, 25) = 1.09$, $p = 0.3$; γ1, $H(1, 25) = 0.15$, $p = 0.70$; γ2, $H(1, 25) = 1.59$, $p = 0.21$; γ3, $H(1, 25) = 0.15$, $p = 0.70$; θ, $H(1, 25) = 0.003$, $p = 0.96$; ρ2, $H(1, 25) = 0.003$, $p = 0.96$; δ, $H(1, 25) = 5.8$, $p = 0.016$.

any of the subunits between alcoholics and non-alcoholic controls (**Figure 6**).

DISCUSSION

The expression of specific $GABA_A$ channel subunit mRNAs was altered in specific brain regions in individuals suffering from alcoholism. In particular, there was a significant increase of the α1, α4, α5, β1, and γ1 subunit mRNAs in the HP-DG; a decrease of the β2 and δ in the OFC, but no change of any subunit expression in the DL-PFC. These data complement previous expression studies in individuals suffering from alcohol dependence providing further evidence for long-term changes in the $GABA_A$ channels in the CNS induced by long-term alcohol consumption (Lewohl et al., 1997, 2001; Mitsyama et al., 1998; Thomas et al., 1998; Buckley and Dodd, 2004).

Gene expression profiling studies by, e.g., quantitative PCR using human autopsy brain tissue can be affected by many pre- and post-mortem factors such as age, gender, ethnicity, and PMI. In this study we have tried to minimize the differences between the two groups of individuals we have studied. All individuals included in the study were Caucasian males and the two groups were matched for parameters such as age, PMI, brain pH, RNA quality indicator value, and proportions of smokers and non-smokers. In addition, the sample size in each group was between 11 and 15 that falls within the group–size range providing rather reliable statistical estimation (Hynd et al., 2003).

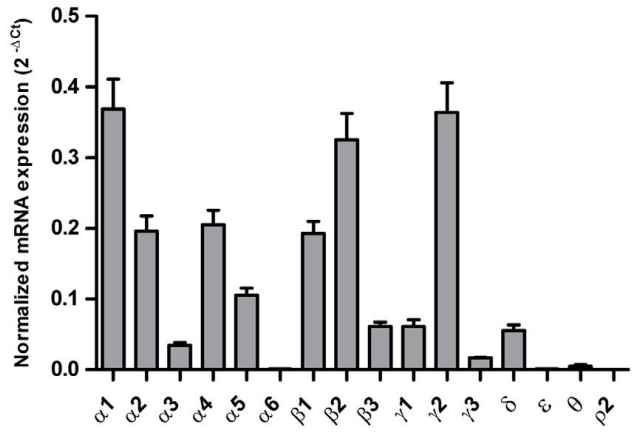

FIGURE 5 | Expression of $GABA_A$ channel subunit mRNAs in the prefrontal cortex of controls ($n = 15$). The mRNA level of each subunit was normalized to reference genes *ACTB* and *RPLP0* and presented as mean ± SE.

$GABA_A$ channels are pentameric GABA-gated chloride channels. A change of subunit composition in the $GABA_A$ channel complex can directly affect its cellular and sub-cellular location as well as physiological and pharmacological properties of the channel, including ethanol sensitivity (Birnir and Korpi, 2007). Several studies have indicated that the expression of $GABA_A$ channel subunits differs at both the mRNA and protein levels between post-mortem brains from non-alcoholic individuals and alcoholics, although the results remain somewhat ambiguous (Lewohl et al., 1997, 2001; Dodd and Lewohl, 1998; Buckley et al., 2000, 2006; Buckley and Dodd, 2004). It has been shown that the expression of the $GABA_A$ channel subunit α1 mRNA but not protein was elevated in the superior frontal cortex of alcoholics (Lewohl et al., 1997; Dodd and Lewohl, 1998). Similarly, in our study α1 mRNA level was increased in the hippocampal dentate gyrus of alcoholic individuals. However, chronic alcohol administration to rodents decreased or did not change the α1 mRNA and protein levels in the cerebral cortex, cerebellum, or hippocampus (Uusi-Oukari and Korpi, 2010). This discrepancy in results between humans and rodents can potentially be attributed to the difference in, e.g., in metabolism or length of alcohol exposure, transcriptional regulation and animal models of alcohol dependence, in addition to the species difference.

$GABA_A$ channels containing the α4, α5, or the δ subunit are of particular interest. These subunits are parts of extrasynaptic $GABA_A$ channels that participate in generating tonic neuronal inhibition that decreases action potential frequency in neurons (Pavlov et al., 2009; Jin et al., 2011). Activation of $GABA_A$ channels containing these subunits is thought to have implications for cognitive function. In a rodent model for excessive alcohol consumption, repeated ethanol withdrawals or longer ethanol exposure increased the α4 subunit protein expression in the hippocampus (Matthews et al., 1998; Cagetti et al., 2003). This is in accordance with our data where the α4 mRNA expression was elevated in alcoholic individuals as compared to the non-alcoholic subjects. In the human hippocampus, the α5 subunit is abundant in the dentate gyrus molecular layer as well as in mid-CA1 regions (Howell et al., 2000; Wainwright et al., 2000; Rissman et al., 2003), whereas in the rodent hippocampus, α5-containing $GABA_A$ channels are only highly expressed in CA1 pyramidal neurons (Sperk et al., 1997). Genetic or pharmacological manipulation of α5-containing $GABA_A$ channels in mice modulates hippocampus-dependent learning (Crestani et al., 2002; Caraiscos et al., 2004; Martin et al., 2010; Prut et al., 2010). It is possible that $GABA_A$ α4 or α5 subunit-selective compounds may potentially be used for the treatment of alcohol-induced cognitive deficit.

Ethanol can induce the release of endogenous GABAergic neurosteroids that further enhance the GABA signaling system in neurons (Biggio et al., 2007). The sensitivity to neurosteroids is higher in γ1 subunit-containing $GABA_A$ channels than in γ2 subunit-containing $GABA_A$ channels (Puia et al., 1993). Chronic ethanol administration in rodents significantly increases the mRNA expression of γ1 subunit in the cerebral cortex (Devaud et al., 1995) and in the hippocampus (Cagetti et al., 2003), and similarly in our study, up-regulation of the γ1 subunit was observed in the HP-DG from alcoholic individuals. Therefore, increased sensitivity to neurosteroids of $GABA_A$ channels may be associated with the alcohol dependence.

Some of the genes encoding the human $GABA_A$ channel subunits are organized into clusters on chromosomes. Chromosome 4 contains four $GABA_A$ channel genes: *GABRA2* (α2), *GABRA4* (α4), *GABRB1* (β1), and *GABRG1* (γ1) (Reich et al., 1998). As the change in the gene regulation of one $GABA_A$ channel subunit may affect the transcription levels of other $GABA_A$ subunit genes in the same cluster (Uusi-Oukari et al., 2000; Steiger and Russek, 2004), it is not surprising to see the up-regulation of three of them (α4, β1, and γ1), in HP-DG of alcoholic individuals in our study. It will be worth using genetic mapping approach to study whether these $GABA_A$ subunit expressions are associated with specific gene polymorphisms and whether the regulation of transcription is similar for these subunit genes (Joyce, 2007). Since the α1, α4, α5, and β1 $GABA_A$ subunits are abundant in the hippocampal dentate gyrus, the increase in the expression of these subunits may have significant functional consequence in alcohol-induced cognitive impairment. Further studies are needed to determine the protein level of these altered subunits and assess their putative functional impact in human alcoholism.

Chronic alcohol consumption in humans has been shown to cause impairment of executive and cognitive functions which require normal prefrontal cortical function (Goldstein et al., 2004; Crego et al., 2010). Here we have examined the expression of $GABA_A$ channel subunits in the sub-regions of cortex, DL-PFC, and OFC from alcoholics. In the OFC of alcoholic individuals, the β2 and δ $GABA_A$ subunits were significantly decreased. Whether this decrease contributes to the impaired GABAergic function in the OFC reported in studies involving alcoholics remains to be determined (Volkow et al., 1993, 1997). In contrast, none of the $GABA_A$ subunits were changed in the DL-OFC of individuals suffering from alcohol dependence as compared to non-alcoholic individuals. This is in agreement with two microarray studies showing no change of any $GABA_A$ subunit mRNAs in the frontal

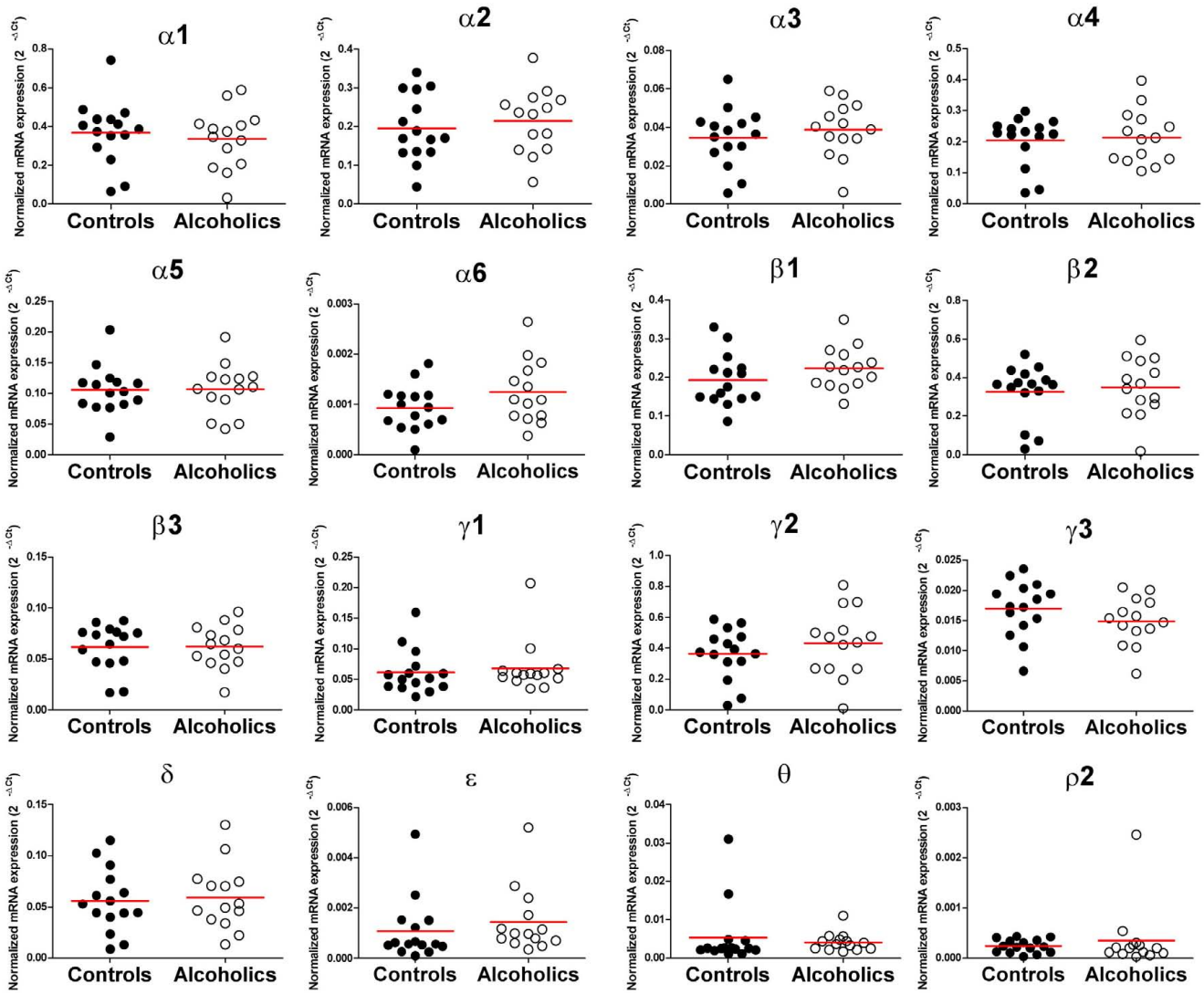

FIGURE 6 | Expression of $GABA_A$ channel subunit mRNAs in the prefrontal cortex of controls (●, $n = 15$) and alcoholics (○, $n = 14$). Horizontal lines represented mean levels. One way ANOVA with Bonferroni *post hoc* test, α1, df = 27, $p = 0.58$; α2, df = 27, $p = 0.55$; α3, df = 27, $p = 0.45$; α4, df = 27, $p = 0.76$; α5, df = 27, $p = 0.93$; α6, df = 27, $p = 0.13$; β1, df = 27, $p = 0.19$; β2, df = 27, $p = 0.66$; β3, df = 27, $p = 0.97$; γ2, df = 27, $p = 0.36$; γ3, df = 27, $p = 0.19$; δ, df = 27, $p = 0.76$; Kruskal–Wallis ANOVA on ranks with Dunn's *post hoc* test, γ1, $H(1, 29) = 0.69$, $p = 0.41$; ε, $H(1, 29) = 0.13$; θ, $H(1, 29) = 1.29$, $p = 0.26$; ρ^2, $H(1, 29) = 0.62$, $p = 0.43$.

cortex of alcoholic subjects (Mayfield et al., 2002; Flatscher-Bader et al., 2005).

In conclusion, we report brain area-specific selective changes in the mRNA expression of $GABA_A$ channel subunits in individuals suffering from alcohol dependence compared to control cases. It is of particular interest that several of the subunits that change with chronic alcohol consumption (e.g., α4, α5, and δ) are present in many extrasynaptic $GABA_A$ channels mediating tonic inhibition. As tonic inhibition has a significant role in determining baseline excitability of neurons this is perhaps not surprising but highlights the importance of $GABA_A$ channels located outside of synapses for drug effects.

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APPENDIX

Table A1 | Demographic data of controls and alcoholics.

Subject no.	Age (years)	PMI (hours)	Brain pH	Brain regions	Smoking history	Cause of death
CONTROLS						
1	34	20.5	6.73	DL-PFC, OFC	Yes	Acute exacerbation of asthma
2	78	6.5	6.2	DL-PFC, OFC	No	Adenocarcinoma
3	63	72	6.9	DL-PFC, OFC, HP-DG	Yes	Coronary artery atherosclerosis
4	82	23.5	6.4	DL-PFC, OFC	NA	Sepsis
5	38	13.5	6.26	DL-PFC, OFC, HP-DG	Yes	Atherosclerotic cardiovascular disease
6	69	16	6.6	DL-PFC, OFC, HP-DG	Yes	Atherosclerotic cardiovascular disease
7	56	24	6.53	DL-PFC, OFC, HP-DG	Yes	Coronary artery atheroma
8	59	20	6.56	DL-PFC, OFC, HP-DG	Yes	Coronary thrombosis
9	56	25	6.1	DL-PFC	NA	Ischemic heart disease
10	56	37	6.76	DL-PFC, OFC, HP-DG	Yes	Left ventricular scarring, hypertension, cardiomegaly
11	82	36	6.24	DL-PFC, OFC, HP-DG	No	Myocardial infarction
12	44	50	6.6	DL-PFC, OFC	Yes	Ischemic heart disease
13	66	22	6.31	HP-DG	NA	Respiratory failure
14	56	48	6.49	HP-DG	Yes	Cardiac failure
15	43	66	6.2	HP-DG	No	Aspiration pneumonia
16	58	12	6.46	HP-DG	Yes	Ischemic heart disease
17	37	24	6.37	HP-DG	NA	Electrocution
18	46	25	6.65	HP-DG	NA	Mitral valve prolapse
19	58	15	6.71	HP-DG	No	Perforated gastric ulcer
20	68	22	6.59	HP-DG	No	Asphyxia
21	53	16	6.84	DL-PFC, OFC	No	Dilated cardiomyopathy
ALCOHOLICS						
1	70	62	6.82	HP-DG	Yes	Cardiomyopathy
2	38	22	6.78	HP-DG	Yes	Ischemic heart disease
3	34	8.5	6.61	DL-PFC, OFC, HP-DG	Yes	Hanging
4	77	20	6.34	DL-PFC, OFC, HP-DG	Yes	Bronchopneumonia
5	65	32	5.66	DL-PFC	NA	Complications of chronic alcoholism
6	50	17	6.3	HP-DG	No	Ischemic heart disease
7	79	48	6.34	DL-PFC, OFC, HP-DG	Yes	Ischemic heart disease
8	39	24	6.56	DL-PFC, OFC, HP-DG	Yes	Aortic stenosis
9	56	22	6.52	DL-PFC, OFC, HP-DG	Yes	Gastro-intestinal hemorrhage
10	59	24	6.57	DL-PFC, OFC	No	Cardiomyopathy
11	56	15	6.66	DL-PFC, OFC, HP-DG	NA	Ischemic heart disease and emphysema
12	56	45	6.51	DL-PFC, OFC, HP-DG	NA	Bleeding esophageal varices
13	44	15	6.48	DL-PFC, OFC, HP-DG	No	Ischemic heart disease
14	81	36	6.44	DL-PFC, OFC, HP-DG	Yes	Sepsis
15	62	49	6.49	DL-PFC	Yes	Ischemic heart disease
16	66	11.5	6.4	DL-PFC	Yes	Pneumonia
17	53	57	6.75	DL-PFC, OFC, HP-DG	Yes	Chronic airflow limitation
18	61	24	6.52	DL-PFC, OFC	Yes	Ischemic heart disease
19	57	18	6.6	DL-PFC, OFC	Yes	Ischemic heart disease

PMI, post-mortem interval; DL-PFC, dorsolateral prefrontal cortex; OFC, orbitofrontal cortex; HP-DG, hippocampal dendate gyrus; NA, not available.

Table A2 | Human primers list for quantitative real-time RT-PCR.

Gene	Primer	Product size (bp)	Reference number
α1 (*GABRA1*)	F: GGATTGGGAGAGCGTGTAACC	66	NM_000806
	R: TGAAACGGGTCCGAAACTG		
α2 (*GABRA2*)	F: GTTCAAGCTGAATGCCCAAT	160	NM_000807
	R: ACCTAGAGCCATCAGGAGCA		
α3 (*GABRA3*)	F: CAACTTGTTTCAGTTCATTCATCCTT	102	NM_000808
	R: CTTGTTTGTGTGATTATCATCTTCTTAGG		
α4 (*GABRA4*)	F: TTGGGGGTCCTGTTACAGAAG	105	NM_000809
	R: TCTGCCTGAAGAACACATCCA		
α5 (*GABRA5*)	F: ACGGTGGGCACTGAGAACAT	64	NM_000810
	R: GGAAGTGAGCTGTCATGATTGTG		
α6 (*GABRA6*)	F: ACCCACAGTGACAATATCAAAAGC	67	NM_000811
	R: GGAGTCAGGATGCAAAACAATCT		
β1(GABRB1)	F: GTACAAAATCGAGAGAGTCTGGG	144	NM_000812
	R: GCG AATGTCATATCCTTTGAGCA		
β2(*GABRB2*)	F: GCAGAGTGTCAATGACCCTAGT	137	NM_021911
	R: TGGCAATGTCAATGTTCATCCC		
β3(*GABRB3*)	F: CAAGCTGTTGAAAGGCTACGA	108	NM_000814
	R: ACTTCGGAAACCATGTCGATG		
γ1(*GABRG1*)	F: CCTTTTCTTCTGCGGAGTCAA	91	NM_173536
	R: CATCTGCCTTATCAACACAGTTTCC		
γ2(*GABRG2*)	F: CACAGAAAATGACGGTGTGG	136	NM_000816
	R: TCACCCTCAGGAACTTTTGG		
γ3(*GABRG3*)	F: AACCAACCACCACGAAGAAGA	113	NM_033223
	R: CCTCATGTCCAGGAGGGAAT		
δ (*GABRD*)	F: ACCACGGAGCTGATGAACTT	109	NM_000815
	R: AGGGCATGTAGGATTGGATG		
ε (*GABRE*)	F: TGGATTCTCACTCTTGCCCTCTA	107	NM_004961
	R: GGAGTTCTTCTCATTGATTTCAAGCT		
θ (*GABRQ*)	F: CCAGGGTGACAATTGGCTTAA	63	NM_018558
	R: CCCGCAGATGTGAGTCGAT		
π (*GABRP*)	F: GGCCTTGCTAGAATATGCAGTTG	76	NM_014211
	R: CTTTGTTGTCCCCCTATCTTTGG		
ρ1(*GABRR1*)	Hs00266687_m1 from applied biosystem	94	NM_002042
ρ2 (*GABRR2*)	F: CCTAGAAGAGGGCATAGACATCG	99	NM_002043
	R: TCCAGTAGCTGCTGCATTGTTTG		
ρ3 (*GABRR3*)	F: TGATGCTTTCATGGGTTTCA	111	NM_001105580
	R: CGCTCACAGCAGTGATGATT		
β-actin (*ACTB*)	F: CCTGGCACCCAGCACAAT	144	NM_001101
	R: GGGCCGGACTCGTCATACT		
RPLP0	F: CCTCATATCCGGGGGAATGTG	95	NM_001002
	R: GCAGCAGCTGGCACCTTATTG		
PPIA	F: CCCACCGTGTTCTTCGACAT	116	NM_021130
	R: CCAGTGCTCAGAGCACGAAA		
PKG1	F: AGGGAAAAGATGCTTCTGGG	71	NM_000291
	R: AAGTGAAGCTCGGAAAGCTTCTAT		
UBC	F: CGGTGAACGCCGATGATTAT	124	NM_021009
	R: ATCTGCATTGTCAAGTGACGA		

Table A3 | Analysis of normality of RT-qPCR data distribution by Shapiro–Wilk normality test (*p* values are shown below).

	HP-DG	OFC	DL-PFC
α1	$p < 0.05$	$p < 0.05$	$p = 0.239$
α2	$p < 0.05$	$p < 0.05$	$p = 0.878$
α3	$p < 0.05$	$p = 0.977$	$p = 0.741$
α4	$p < 0.05$	$p = 0.994$	$p = 0.522$
α5	$p < 0.05$	$p = 0.114$	$p = 0.256$
α6	$p < 0.05$	$p < 0.05$	$p = 0.493$
β1	$p < 0.05$	$p < 0.05$	$p = 0.214$
β2	$p < 0.05$	$p = 0.75$	$p = 0.111$
β3	$p = 0.429$	$p < 0.05$	$p = 0.073$
γ1	$p < 0.05$	$p < 0.05$	$p < 0.05$
γ2	$p < 0.05$	$p < 0.05$	$p = 0.821$
γ3	$p < 0.05$	$p < 0.05$	$p = 0.349$
δ	$p < 0.05$	$p < 0.05$	$p = 0.281$
ε	$p < 0.05$	$p = 0.135$	$p < 0.05$
θ	$p < 0.05$	$p < 0.05$	$p < 0.05$
ρ2	$p < 0.05$	$p < 0.05$	$p < 0.05$

$p < 0.05$ indicates the data are not normally distributed. HP-DG, hippocampal dendate gyrus; OFC, orbitofrontal cortex; DL-PFC, dorsolateral prefrontal cortex.