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Development of GABAergic Synapses in Cultured Hippocampal Neurons

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Abstract

The formation and maturation of GABAergic synapses was studied in cultured hippocampal pyramidal neurons by both performing immunocytochemistry for GABAergic markers and recording miniature inhibitory postsynaptic currents (mIPSCs). Nascent GABAergic synapses appeared between 3–8 days *in vitro* (DIV), with GABA_A receptor subunit clusters appearing first, followed by GAD-65 puncta, and then functional synapses. The number of GABAergic synapses increased from 7–14 DIV, with a corresponding increase in frequency of mIPSCs. Moreover, these new GABAergic synapses formed on neuronal processes farther away from the soma, contributing to decreased mIPSC amplitude and slowed mIPSC 19–90% rise-time. The mIPSC decay quickened from 7–14 DIV with a parallel change in the distribution of the $\alpha 5$ subunit from diffuse expression at 7 DIV to clustered expression at 14 DIV. These $\alpha 5$ clusters were mostly extrasynaptic. The $\alpha 1$ subunit was expressed as clusters in none of the neurons at 7 DIV, in 20% at 14 DIV, and in 80% at 21 DIV. Most of these $\alpha 1$ clusters were expressed at GABAergic synapses. In addition, puncta of GABA transporter 1 (GAT-1) were localized to GABAergic synapses at 14 DIV, but were not expressed at 7 DIV. These studies demonstrate that mIPSCs appear after pre- and postsynaptic elements are in place. Furthermore, the process of maturation of GABAergic synapses involves increased synapse formation at distal processes, expression of new GABA_A receptor subunits, and GAT-1 expression at synapses; these changes are reflected in altered frequency, kinetics and drug sensitivity of mIPSCs.

Keywords

synaptogenesis; GABA_A receptor; GABA transporter; mIPSC

Introduction

Three basic stages of synaptogenesis have been described: 1) initial contact between presynaptic and postsynaptic elements, 2) maturation of the synapse, and 3) emergence of adult isoforms (Sanes et al., 2000). Most studies of synaptogenesis have concentrated on the neuromuscular junction and glutamatergic synapses (Sanes and Lichtman, 2001; Goda and Davis, 2003). Although the formation and organization of GABAergic synapses is thought

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to generally follow the same process (Moss and Smart, 2001; Meier, 2003), the development of GABAergic synapses has not been fully characterized.

Nascent GABAergic synapses should contain both presynaptic and postsynaptic elements, as well as produce synaptic transmission (Ahmari and Smith, 2002). Previous studies demonstrated that GABA_A receptors formed clusters before presynaptic terminals emerged (Scotti and Reuter, 2001), and this clustering occurred in the absence of scaffolding proteins and GABA release (Craig et al., 1994; Rao et al., 2000; Scotti and Reuter, 2001; Christie et al., 2002). Also, during maturation, GABA_A receptors became selectively clustered across from terminals that release the neurotransmitter GABA (Craig et al., 1994). However, these studies did not specify the relationship of GABAergic protein expression to the onset of GABAergic synaptic transmission.

Once GABAergic synapses are formed, they undergo profound maturational changes in both composition and function. Molecular changes may occur at the presynaptic terminal, within GABA_A receptor subunit composition or GABA transporter subtype. A well-studied locus of alteration is the composition of GABA_A receptor subunits (Killisch et al., 1991; Laurie et al., 1992; Brooks-Kayal et al., 1998). The GABA_A receptor channel is composed of five subunits derived from eight subfamilies of subunit genes. Each subunit gene family has multiple members (α has six, β has four, γ has three, ρ has three, and δ , ϵ , π , and θ each have one). There is a regionally distinct progression in cellular expression patterns of GABA_A receptor subunits during postnatal development. However, few studies correlate the maturation of synapses with functional changes in transmission.

Functionally, kinetics of GABA_A receptor-mediated synaptic currents alter during development. Changes in the frequency amplitude and decay of miniature inhibitory postsynaptic currents (mIPSCs) occur during postnatal development of various brain regions, including the cerebellum (Tia et al., 1996; Vicini et al., 2001), hippocampus (Cohen et al., 2000), thalamus (Okada et al., 2000), superior colliculus (Jüttner et al., 2001), and brainstem (Sanes et al., 1993). However, the temporal relationship of developmental changes in the kinetics of mIPSCs to the composition of GABAergic synapses has not been studied.

This study explored the relationship between the molecular and functional maturation of GABAergic synapses in cultured hippocampal neurons prepared using methods previously established (Fletcher et al., 1991; Rao et al., 2000; Mangan and Kapur, 2004) that provide the advantages of accessibility to and visibility of individual synapses.

Materials and methods

Cell Culture

Neuronal hippocampal/glial co-cultures were prepared from 18 day embryonic rats as previously described (Goslin et al., 1998). Glial cell cultures were prepared 10 days prior to co-culturing with hippocampal neurons when, in a laminar flow hood, neonatal Sprague-Dawley rat pups were decapitated after being placed on ice for 2-3 minutes. Hippocampal neuron cultures were prepared later when Sprague-Dawley rat fetuses were removed from the pregnant mother after she was anesthetized with halothane. Fetuses were then decapitated and brains removed. These methods were approved by the University of Virginia Animal Care and Use Committee and conform to NIH guidelines. Briefly, neurons were isolated by trypsin treatment, triturated and plated on poly-L-lysine-coated glass coverslips in minimum essential medium (MEM) with 15% horse serum at a density of 10,000-100,000 cells/35 mm². After attachment of cells, coverslips were transferred and neurons grew over a glial cell monolayer in serum-free MEM with N2 supplements. The

population of neurons in culture consisted primarily of pyramidal neurons which could be distinguished morphologically from GABAergic interneurons that composed approximately 6% of cells (Benson et al., 1994). Neurons were analyzed 1-21 days after plating. However, since expression of presynaptic and postsynaptic markers remained relatively constant from 14-21 DIV, only data from 14 DIV is presented.

Immunocytochemistry

Single-label and double-label immunocytochemistry for presynaptic and postsynaptic GABAergic markers were performed according to the methods previously described (Swanwick et al., 2004). The $\gamma 2$ and $\beta 2/3$ subunits of the GABA_A receptor were used as postsynaptic markers, as the $\gamma 2$ subunit is required for synaptic targeting of GABA_A receptors (Essrich et al., 1998), and $\beta 2/3$ is one of the most abundant subunits of GABA_A receptors in the brain (McKernan and Whiting, 1996). The 65 kDa isoform of glutamic acid decarboxylase (GAD-65), the synthetic enzyme for GABA, was used as a presynaptic marker. Two forms of GAD exist: GAD-65 and GAD-67 (Erlander et al., 1991). All GABAergic neurons make both isoforms but GAD-65 immunoreactivity is concentrated in synapses, whereas GAD-67 immunoreactivity is prominent in cell bodies (Kaufman et al., 1991; Dupuy and Houser, 1996). All measurements were performed in pyramidal neurons that were visually identified based on morphology (Benson et al., 1994).

Primary antibodies—All primary antibodies were diluted in 0.1 M PBS (pH 7.1) containing 2% normal goat serum. The specificity of each antibody was verified by the lack of staining after the omission of the primary antibody or serial dilution.

A mouse monoclonal antibody recognized amino acids 1-3 common to the $\beta 2$ and $\beta 3$ subunits of the GABA_A receptor (clone 62-3G1, 2 μ g/mL, Upstate, Lake Placid, NY, #05-474). This antibody visualizes two bands on a western blot: 55 kDa ($\beta 2$) and 57 kDa ($\beta 3$), and immunoreactivity to these bands was specifically blocked by the corresponding peptide (Li and De Blas, 1997). A rabbit antibody recognized the $\gamma 2$ subunit of the GABA_A receptor (amino acids 319-366, 2 μ g/mL). Immunoblotting using this antibody shows a band of 45-49 kDa (Mossier et al., 1994; Sun et al., 2004) and adsorption of the antibody blocked its detection (Togel et al., 1994). Immunocytochemical characterization of this antibody has been performed previously (Sperk et al., 1997; Nusser et al., 1998; Sun et al., 2004).

A mouse monoclonal antibody recognized GAD-65 (GAD-6 clone, 1 μ g/mL, Chemicon, Temecula, CA, #MAB351). This antibody binds to a single band of 65 kDa on a western blot and its specificity was verified through adsorption (Chang and Gottlieb, 1988). A rabbit antibody raised against GAD-65 was also used (GAD-6 clone, 1:1000, Chemicon, Temecula, CA, # AB5082). It also recognized a band of 65 kDa on a western blot whose detection was blocked by preadsorption with the peptide and has been immunocytochemically characterized (Mi et al., 2002).

A rabbit antibody recognized the $\alpha 1$ subunit of the GABA_A receptor (amino acids 1-16, 1.5 μ g/mL, Alomone Labs, Jerusalem, Israel, #AGA-001). This antibody reveals a single band of ~50 kDa on a western blot (Sun et al., 2004) that was blocked by preadsorption with the synthetic peptide. A rabbit antibody recognized the $\alpha 2$ subunit of the GABA_A receptor (C-terminal peptide, 1:2000, Abcam Inc, Cambridge, MA, #ab8342). On a western blot this antibody (1:2500) revealed a single band of 53 kDa (C. Sun and J. Kapur, unpublished observations) and its specificity has been characterized previously (Poulter et al., 1999; Brandon et al., 2000). A rabbit antibody recognized the $\alpha 4$ subunit of the GABA_A receptor (amino acids 1-14, 5 μ g/mL). A 67 kDa band is visible using this antibody for western blotting (Kern and Sieghart, 1994; Bencsits et al., 1999, Sun et al., 2004) and this band is not detectable after preadsorption with the synthetic peptide (Kern and Sieghart, 1994).

Moreover, this antibody has previously been characterized immunocytochemically (Sperk et al., 1997; Sun et al., 2004; Mangan et al., 2005). A rabbit antibody recognized the $\alpha 5$ subunit of the GABA_A receptor (amino acids 337-388, 5 μ g/mL). This antibody detects a single band of 47 kDa on a western blot (Sieghart et al., 1993; S.A. Trotter and J. Kapur, unpublished observations) and adsorption of the antibody blocked this detection (Sieghart et al., 1993). A rabbit antibody recognized the δ subunit of the GABA_A receptor (amino acids 1-44, 5 μ g/mL). A band of ~53-57 kDa is visible using western blotting (Sperk et al., 1997; Sun et al., 2004). Immunocytochemical characterization of this antibody has been performed previously (Sperk et al., 1997; Nusser et al., 1998; Sun et al., 2004; Mangan et al., 2005). Also, no specific labeling was evident in δ subunit-deficient mice (Peng et al., 2002).

A rabbit antibody recognized GABA transporter 1 (amino acids 588-599, 1:500, Chemicon, Temecula, CA #AB1570W). This antibody detects a single band of 67 kDa on a western blot (Vitellaro-Zuccarello et al., 2003) and this immunoreactivity was prevented by preadsorption with the synthetic peptide (Ribak et al., 1996). A rabbit antibody recognized GABA transporter 3 (amino acids 607-627, 1:500, Chemicon, Temecula, CA #AB1574). A single band of 70 kDa is visible using this antibody for western blotting (Vitellaro-Zuccarello et al., 2003), and this was also prevented by preadsorption with the synthetic peptide (Ribak et al., 1996).

Secondary Antibodies—Secondary antibodies included goat anti-mouse IgG or goat anti-rabbit IgG conjugated with Alexa 488 and Alexa 594 fluorochromes (4 μ g/mL, Molecular Probes, Eugene, OR). All secondary antibodies were diluted in 0.1 M PBS (pH 7.1) containing 2% normal goat serum.

Image Acquisition and Analysis

Pyramidal neurons were randomly selected for immunocytochemical analysis from ≥ 2 cultures. Fluorescent images of cells were captured on a CoolSNAPcTM CCD camera (Roper Scientific Photometrics, NJ) mounted on a Eclipse TE200 fluorescent microscope (Nikon, Japan) driven by Metamorph imaging software (Universal Imaging Corporation, Downingtown, PA). High resolution digital images of each fluorochrome were acquired using a 60X/1.4 NA lens. Brightness and contrast of fluorescent images were adjusted using Metamorph software so that only punctate fluorescence, but no weak diffuse background labeling, was visible.

Definition of puncta/clusters—For cluster count, size, and colocalization, thresholds were set to detect punctate fluorescence that was two times higher than diffuse background labeling. Number of clusters was then measured. Aggregations of ≥ 2 pixels were selected as clusters, which corresponded to ≥ 0.15 μ m diameter at 60X magnification, as determined by 1 μ m diameter fluorescent microspheres. Images were also visually inspected to eliminate the soma, fused puncta, or obvious debris from being selected for analysis. Controls lacking primary antibody showed nonspecific labeling that could appear as granular clusters < 7 pixels (< 0.54 μ m diameter), so if average cluster size was < 7 pixels, cluster number was recorded as 0. Due to fluorescent intensity of the soma, only clusters on processes were quantitated for all neurons. Number of clusters was quantified per field after a single neuron was centered in the visual field.

Analysis of colocalization—A binary image was created from each thresholded image. Binary images were then added together to display overlapping puncta. Number of colocalized puncta or clusters was then measured. Data of number, size and colocalization of puncta or clusters was analyzed using GraphPad Prism 4.0 (San Diego, CA). All values are reported as mean $\pm$ s.e.m. Values compared between 7 and 14 DIV were analyzed using a

two-tailed student's t-test with a significance value of $p < 0.05$. Values for the size of $\beta_2/3$ and γ_2 clusters compared between 3, 7, and 14 DIV were evaluated using a one-way ANOVA followed by Bonferroni's Multiple Comparison Test.

Quantification of 20X fields—To analyze the percentage of neurons expressing GAD-65 puncta from 3-8 DIV, images were obtained using a 20X objective. Random fields in the center, top, bottom, left, and right regions of the slide were chosen at each age from ≥ 2 cultures. The number of neurons expressing GAD-65 puncta was divided by the number of total neurons in the field to obtain the percentage of neurons with GAD-65 puncta.

Photomicrograph production—Images were saved as 8-bit TIFF files and opened in Adobe Photoshop 6.0 (San Jose, CA), where overall brightness was increased for final production.

mIPSC Recording

Synaptic currents mediated by the GABA_A receptor were recorded from visually identified hippocampal pyramidal neurons using the whole-cell patch clamp method as described in the past (Hamill et al., 1981; Mangan and Kapur, 2004). Membrane properties of these neurons at 14 DIV were previously characterized (Mangan and Kapur, 2004). mIPSCs were recorded by blocking both excitatory neurotransmission and action potentials: glutamate receptor-mediated synaptic currents were blocked using 50 μM D(-)-2-amino-5-phosphonovaleric acid (D-APV), and 20 μM 6,7-dinitroquinoxaline-2/3-dione (DNQX) in the external solution, and action potentials were blocked using 1 μM tetrodotoxin (TTX) in the external solution. Bath application of the GABA_A receptor antagonist bicuculline (5 μM) eliminated all currents observed, verifying that recorded currents were GABAergic mIPSCs.

Patch electrodes were filled with internal recording solution containing (in mM): CsCl 153.3, MgCl₂ 1.0, EGTA 5.0, and HEPES 10.0, with a pH of 7.40 and osmolarity of 290–300 mOsm. CsCl was used to block potassium currents. MgATP (4 mM) was included in the intracellular solution before recording. The external recording medium contained (in mM): NaCl 146.0, KCl 2.5, MgCl₂ 3.0, CaCl₂ 2.0, Glucose 10.0, and HEPES 10.0, with a pH of 7.4 and osmolarity of 315 – 330 mOsm. Internal and external solutions contained equimolar concentrations of chloride ions, and no GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) were evident at a clamped membrane potential of 0 mV. Recordings were made at a holding potential of -60 mV.

Neurons were studied on the stage of an inverted microscope at room temperature. Thick-walled (1.5 mm outer diameter, 0.86 mm inner diameter) borosilicate patch electrodes (World Precision Instruments, Sarasota, FL) were pulled on a P-97 Flaming–Brown horizontal puller (Sutter Instruments, CA) using a 2-stage pull to a final resistance of 2-5 M Ω . Currents were recorded with an Axopatch 200A amplifier and low-pass filtered at 3 kHz with an 8 pole Bessel filter prior to digitization, storage and display using the patch clamp technique (Hamill et al., 1981). Currents were recorded using axoscope software (Axon Instruments, CA) digitized at 400 Hz. Series resistance and capacitance were compensated for each neuron. After baseline and input resistance became stable, mIPSC recordings were made for 5 minute epochs during 30-60 minutes.

Electrophysiological Analysis

Pyramidal neurons were randomly selected for recording from ≥ 2 cultures. MiniAnalysis software (Synaptosoft, Decatur, GA) was used to analyze mIPSC frequency, amplitude, 10-90% rise-time, and decay. Mean frequency is reported for 7 and 14 DIV. Frequency at 7 and 14 DIV was compared using a Kolmogorov-Smirnov (K-S) test with a significance

value of $p < 0.05$. Medians were measured for mIPSC amplitude and 10-90% rise-time because in frequency histograms of each parameter the frequency distributions were largely skewed to the right, so the mean of medians is reported for amplitude and 10-90% rise-time at 7 and 14 DIV. Decay was analyzed by fitting with two-exponential curves and accepting the fit if $R^2 > 0.70$. Decays were analyzed until 20 values were obtained for each neuron. Because the range of fitted mIPSC decays is also not normally distributed, the mean of median values of the decay of each neuron is also reported. For mIPSC amplitude, 10-90% rise-time, and decay, values compared between two groups were analyzed using a two-tailed student's t-test with a significance value of $p < 0.05$. All values are reported $\pm$ s.e.m.

Results

Emergence of Nascent Synapses

Comparisons of cultured hippocampal neurons prepared for immunocytochemistry at 3-7 days *in vitro* (DIV) revealed that GABA_A receptor clusters appeared before GABAergic terminals on pyramidal neurons. At 3 DIV, bright immunoreactive clusters of the $\gamma 2$ (Fig. 1A) and $\beta 2/3$ (Fig. 1D) GABA_A receptors subunits were evident in all pyramidal cells but immunoreactive puncta typical of presynaptic GAD-65 expression were not present (Fig. 1G). No mIPSCs could be recorded from any pyramidal neurons at 3 DIV (Fig. 1J), even when the cells were hyperpolarized to -80 mV to increase the Cl⁻ driving force (data not shown). However, at 7 DIV, $\gamma 2$ subunit clusters (Fig. 1B), $\beta 2/3$ subunit clusters (Fig. 1E), and GAD-65 puncta (Fig. 1H) were all visible and mIPSCs could be recorded (Fig. 1K). The average size of GAD-65 puncta was larger than the average size of $\gamma 2$ and $\beta 2/3$ clusters (Table 1) because unlike GAD-65, large clusters of $\gamma 2$ and $\beta 2/3$ are interspersed with very small clusters. The presence of postsynaptic markers together with presynaptic markers at 7 DIV but not 3 DIV suggested that nascent GABAergic synapses formed between 3-7 DIV. This process was studied in detail.

The appearance of synaptic activity lagged behind that of GABAergic markers. When the percentage of neurons containing GAD-65 puncta or mIPSCs were plotted as a function of DIV, mIPSCs lagged behind the appearance of GAD-65 cluster by a half day (Fig. 1M). GAD-65 puncta and mIPSCs were first observed at 5 DIV. At this time, images captured using a 20X objective (20X fields, each containing 3-12 neurons) showed that $42.5 \pm 4.2\%$ of neurons contained GAD-65 puncta ($n = 20$ fields at 20X), but mIPSCs were observed in only 30% of neurons tested ($n = 10$ neurons). Both of these percentages increased until at 7 DIV, when $92.9 \pm 1.4\%$ of neurons contained GAD-65 puncta ($n = 20$ fields at 20X) and mIPSCs were recorded from 83% of neurons analyzed ($n = 6$ neurons). By 8 DIV, GAD-65 puncta were present in all neurons examined ($n = 20$ fields at 20X), and mIPSCs were recorded from 94% of neurons tested ($n = 17$ neurons). (At 4 DIV, $n = 20$ fields at 20X for GAD-65 measurement and $n = 9$ neurons for mIPSC measurement; at 6 DIV, $n = 17$ fields at 20X for GAD-65 analysis and $n = 5$ neurons for mIPSC recordings).

Interestingly, the size of $\gamma 2$ and $\beta 2/3$ subunit clusters increased during the appearance of GAD-65 puncta. The size of $\gamma 2$ subunit clusters rose from 3 DIV (0.9 ± 0.05 μ m diameter, $n = 10$ neurons) to 7 DIV (Table 1, $p < 0.05$), and the size of $\beta 2/3$ subunit clusters increased from 3 DIV (1.1 ± 0.06 μ m, $n = 10$ neurons) to 7 DIV (Table 1, $p < 0.001$).

Developmental Increase in Synapse Number

GABAergic synapses proliferated during *in vitro* development. The number of presynaptic and postsynaptic markers per 60X field increased from 7-14 DIV (Table 1, $p < 0.001$ for $\gamma 2$ and $p < 0.0001$ for $\beta 2/3$ and GAD-65) due to increased neuronal outgrowth during this time, as previously reported (Swanwick et al., 2004). Presynaptically, the density of GAD-65

puncta also rose from 7 DIV (Fig. 1H) to 14 DIV (Fig. 1I, Table 1, $p < 0.0001$). On the postsynaptic membrane, there was only a slight increase in the density of $\gamma 2$ clusters (Fig. 1B,C) and $\beta 2/3$ clusters (Fig. 1E,F) from 7-14 DIV (Table 1). However, the synaptic localization of $\gamma 2$ and $\beta 2/3$ subunit clusters rose from 7-14 DIV, as demonstrated by the percentage of $\gamma 2$ and $\beta 2/3$ subunit clusters colocalized with GAD-65 puncta approximately doubling from 7 DIV (Fig. 1O, Q) to 14 DIV (Fig. 1P, R, Table 1, $p < 0.01$ for $\gamma 2$ and $p < 0.05$ for $\beta 2/3$). The size of $\beta 2/3$ clusters did not increase significantly from 7-14 DIV as they did from 3-7 DIV, in contrast to the size of $\gamma 2$ clusters from 7-14 DIV (Table 1, $p < 0.05$). However, the size of GAD-65 puncta approximately doubled from 7-14 DIV (Table 1, $p < 0.0001$).

The new GABAergic synapses formed were functional. One-minute traces from 7 DIV neurons showed few mIPSCs present (Fig. 1K) whereas those from 14 DIV neurons showed numerous mIPSCs (Fig. 1L). A cumulative probability plot demonstrated that a higher fraction of mIPSCs recorded from 14 DIV neurons had shorter inter-event intervals than mIPSCs recorded from 7 DIV neurons (Fig. 1N). Mean mIPSC frequency increased three-fold from 7 DIV to 14 DIV (Table 2, $p < 0.001$).

New Synapses were Located on Distal Dendrites

The increased number of GABAergic synapses per neuron may have resulted from either of two possibilities: 1) increased density of GABAergic synapses, or 2) constant density of GABAergic synapses, but increased area due to outgrowth of neuronal processes. The density of $\gamma 2$ and $\beta 2/3$ receptor clusters remained constant, but the number of neuronal processes increased from 7-14 DIV, as mentioned above, suggesting that the latter explanation is responsible for the increased density of GABAergic synapses per neuron. Moreover, whole-cell capacitance increased from 7.2 ± 0.9 pF at 7 DIV ($n = 11$ neurons) to 10.1 ± 1.3 pF at 14 DIV ($n = 16$ neurons), confirming that these neurons grow and their processes elongate during *in vitro* development.

In support, the mean distance of presynaptic GAD-65 puncta from the soma increased during *in vitro* development due to increased neuronal outgrowth. Images captured with a 20X objective show GAD-65 puncta distributed throughout neuronal processes at both 7 DIV (Fig. 2A) and 14 DIV (Fig. 2B), but an increased number and length of processes at 14 DIV. The distances of GAD-65 puncta from the soma at both days were calculated by drawing concentric regions around the soma in 25 μm diameter increments, so that puncta were classified into the following distances from the soma: 0-25 μm , 26-50 μm , 51-75 μm , 76-100 μm , 101-125 μm , 126-150 μm , 151-175 μm , and 176-200 μm . At 7 DIV, most dendrites were located within 150 μm of the soma. At 14 DIV, the dendritic arbor was more complex and several dendrites extended beyond the edges of the last concentric circle, which had a boundary of 200 μm from the soma. The number of synapses in each distance category was quantified. Whereas at 7 DIV the distribution of somatic distances were concentrated around smaller values and highest at 0-25 μm and 25-50 μm ($n = 5$ neurons, Fig. 2C), at 14 DIV the distribution of somatic distances shifted rightward towards larger values and the highest distances moved to 75-100 μm ($n = 5$ neurons, Fig. 2D). This shift is similar to one reported from Scholl's analysis by Benson and Cohen (1996). However, these calculations of physical distance actually underestimates the electrical distance of synapses from the soma, as outgrowth of neuronal processes does not always proceed linearly from the soma; some neurites may also curve back towards the soma as they grow and may therefore have been included in a category of smaller distance.

Synaptic currents generated by newly formed distal synapses are expected to be slowed and attenuated by increased electrical distance. In support, the 10-90% rise-time of mIPSC recorded from neurons slowed from 7-14 DIV (Table 2, $p < 0.01$). A frequency distribution

histogram of mIPSC rise-times showed a peak centered slightly above 1 msec at 7 DIV (Fig. 2H) whereas at 14 DIV (Fig. 2I), the distribution was skewed with a large number of 10-90% rise-times ranging from 2-3 msec. In addition, mIPSC amplitude decreased from 7-14 DIV (Table 2, $p < 0.01$). Average mIPSC traces recorded from pyramidal neurons at 14 DIV were much smaller than at 7 DIV (Fig. 2E). A frequency distribution histogram of mIPSC amplitudes recorded from a neurons at 7 DIV displays a wide distribution and many large values, including many amplitudes ranging from 200-400 pA (Fig. 2F), whereas in the histogram from a neuron 14 DIV, the number of amplitudes ranging from 0-200 pA was disproportionately higher (Fig. 2G). Therefore, despite maximal compensation for resistance and capacitance in pyramidal neurons, distal mIPSCs were filtered as they traveled to the somatic site of recording.

Another possible explanation for decreased mIPSC amplitude could have been a reduced number of synaptic GABA_A receptors. However, as mentioned above, the size of $\gamma 2$ and $\beta 2/3$ subunit clusters, which should approximately correspond to number of receptors present, did not diminish from 7-14 DIV (Table 1). Alternatively, mIPSC amplitude could have declined due to a decrease in single channel conductance (Sigworth, 1981; Johnston and Wu, 1995; Cohen et al., 2000). However, when single channel conductance was estimated using non-stationary analysis, it had not significantly changed from 7-14 DIV.

Expression of GABA_A Receptor Subunits

In addition to increased frequency, diminished amplitude, and slowed 10-90% rise-time, the decay of mIPSCs quickened from 7-14 DIV. Decays of mIPSCs were best fit with a biexponential curve, where τ_1 represented the fast component of decay and τ_2 represented the slow component of decay. mIPSCs at 7 DIV returned to baseline current slower than mIPSCs at 14 DIV (Fig. 2H). Average mIPSC traces at 14 DIV were smoother than at 7 DIV because frequency increased during development. When mIPSC decay was quantified, τ_1 shortened from 7-14 DIV (Table 2, $p < 0.05$), whereas τ_2 remained relatively constant from 7-14 DIV (Table 2).

The mIPSC kinetics may be shaped by GABA_A receptor subunit composition (McKernan and Whiting, 1996). Therefore the distributions of the $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 5$ and δ subunits of the GABA_A receptor were examined at 7 and 14 DIV (Fig. 3). The $\alpha 2$ subunit immunoreactivity was clustered at 7 DIV (Fig. 3A) and 14 DIV (Fig. 3B), and the density and size of these clusters remained relatively constant during this time (Table 1).

The $\alpha 4$ subunit immunoreactivity was diffusely distributed at 7 and 14 DIV (Fig. 3C,D). The δ subunit, which is commonly co-assembled with the $\alpha 4$ subunit (McKernan and Whiting, 1996), was also expressed diffusely from 7-14 DIV, although it showed some tiny puncta no larger than nonspecific labeling (Fig. 3E,F). The absence of $\alpha 4$ and δ clusters on pyramidal neurons suggests that these subunits were not synaptic at either time point.

However, distribution of the $\alpha 5$ subunit immunoreactivity changed from 7-14 DIV. At 7 DIV, cell bodies of pyramidal neurons were diffusely stained for $\alpha 5$, with nonspecific granular labeling spread throughout the processes (Fig. 3G). However, at 14 DIV, the cell body was still intensely labeled but large clusters of $\alpha 5$ immunoreactivity had emerged in the neuronal processes (Fig. 3H). These $\alpha 5$ immunoreactive clusters were relatively dense and fairly small (Table 1). Only a small fraction of $\alpha 5$ clusters at 14 DIV were apposed to GABAergic presynaptic terminals (Fig. 3I, Table 1). Interestingly, clusters of $\alpha 5$ were no longer present at 21 DIV (data not shown).

Clusters of $\alpha 1$ subunit immunoreactivity only fully emerged in pyramidal neurons by 21 DIV (Fig. 4). In pyramidal neurons at 7 DIV, $\alpha 1$ subunit staining (Fig. 4A) was diffuse,

although large, distinct $\alpha 1$ subunit clusters were found on interneurons in the same cultures (data not shown). At 14 DIV (Fig. 4B), although approximately 20% of pyramidal neurons contained larger $\alpha 1$ subunit clusters similar to ones found on interneurons, the majority of pyramidal neurons displayed a mostly diffuse pattern of staining for this subunit. However, at 21 DIV, approximately 80% of pyramidal neurons contained abundant large, intense clusters of $\alpha 1$ immunoreactivity scattered throughout the neuronal processes (Fig. 4C). The density of $\alpha 1$ clusters per $10 \mu\text{m}^2$ at this time was 2.7 ± 0.4 ($n = 13$ neurons) and the diameter of the clusters was $3.4 \pm 0.2 \mu\text{m}$ ($n = 33$ neurons). Many $\alpha 1$ clusters ($55.5 \pm 5\%$, $n = 11$ neurons, Fig. 4D) overlapped with GAD-65 puncta, indicating that they were apposed to GABAergic presynaptic terminals.

To confirm this immunocytochemical data regarding $\alpha 1$ expression, mIPSCs were recorded in pyramidal neurons at 14 and 21 DIV before and after bath application of zolpidem (30 nM), a drug that potentiates GABA_A receptors containing the $\alpha 1$ subunit. Average mIPSC traces from a neuron at 21 DIV in the presence of zolpidem had more prolonged decay than those before drug application (Fig. 4F). The second component of mIPSC decay, τ_2 was significantly prolonged from 101.4 ± 12.2 msec before treatment ($n = 5$ neurons) to 148.8 ± 22.6 msec after zolpidem ($n = 5$ neurons, $p < 0.05$) but τ_1 remained unchanged, from 24.0 ± 1.8 msec to 23.2 ± 1.8 msec. In contrast, at 14 DIV, average mIPSC traces from the same pyramidal neuron before and after zolpidem treatment looked similar (Fig. 4E) and neither component of the decay was significantly affected, (τ_1 : 24.4 ± 1.7 msec vs. 23.8 ± 1.2 msec, $n = 5$ neurons; τ_2 : 91.8 ± 8.1 msec vs. 93.9 ± 10.0 msec, $n = 5$ neurons).

GABA Transporter Expression

There are four identified GABA transporters (GATs) (Guastella et al. 1990; Borden et al. 1992; Liu et al. 1993). GAT-2 and GAT-4 (also known as BGT-1) are expressed in both the peripheral and central nervous systems, but in the brain GAT-2 is limited to the meninges (Ikegaki et al. 1994) and GAT-4 is most concentrated in the anterior hypothalamus and septal area (Borden 1996). However, the expression of GABA transporters GAT-1 and GAT-3 is developmentally regulated in the hippocampus and cortex (Vitellaro-Zuccarello et al., 2003; Sipilä et al., 2004), and so the expression of these GABA transporter subtypes was examined during *in vitro* development.

GAT-1 distribution altered from 7-14 DIV. At 7 DIV, GAT-1 immunoreactivity was mostly diffuse (Fig. 5A) with small puncta present in some neurons. However, at 14 DIV, GAT-1 expression appeared strong and punctate (Fig. 5B, Table 1). Many GAT-1 puncta overlapped with GAD-65 puncta at 14 DIV, suggesting that these GAT-1 puncta at 14 DIV were synaptic (Table 1).

In contrast, small GAT-3 puncta were constantly present from 7-14 DIV. GAT-3 was densely distributed as small, granular puncta at both 7 DIV (Fig. 5D) and 14 DIV (Fig. 5E, Table 1). Few GAT-3 puncta overlapped with GAD-65 puncta (Fig. 5F, Table 1), implying that most GAT-3 puncta were extrasynaptic.

Discussion

This study provides to the best of our knowledge the first description of the relationship between functional maturation of GABAergic synapses with the development of various pre- and postsynaptic elements. The primary conclusions are that during *in vitro* development of GABAergic synapses 1) the onset of mIPSCs lags behind the formation of receptor clusters and GAD-65 puncta, 2) functional GABAergic synapses increase in number distally due to neuronal outgrowth, resulting in more frequent mIPSCs with slower rise times and smaller amplitudes, 3) decay of mIPSCs quickens, and 4) clusters of the $\alpha 5$

subunit of the GABA_A receptor and puncta of GAT-1 emerge. A summary of molecular and functional alterations during GABAergic synaptogenesis is illustrated in Figure 6.

Emergence of GABAergic Synapses

Clusters of GABA_A receptors were present early and increased in number during *in vitro* development. Previous studies also report that GABA_A receptor clusters are present in the dendrites, soma, and axon initial segment of pyramidal neurons as early as 3-4 DIV (Christie et al., 2002; Christie and De Blas, 2003; Elmariah et al., 2004; Elmariah et al., 2005), and some even describe the emergence of GABA receptor clusters 6 hours after plating (Scotti and Reuter, 2001). The emergence of presynaptic GABAergic terminals lagged behind that of GABA_A receptor clusters but also grew in number during *in vitro* development. Another marker of presynaptic GABAergic terminals, vesicular inhibitory amino acid transporter, rarely appeared as puncta at 4 DIV but was expressed as numerous puncta at 7 and 10 DIV (Elmariah et al., 2005). In the absence of GABAergic innervation, the GABA_A receptor clusters are mismatched with presynaptic glutamatergic terminals, but upon GABAergic innervation, the GABA_A receptor clusters become aligned with presynaptic GABAergic terminals (Rao et al., 2000; Christie et al., 2002; Christie and De Blas, 2003). Measurements of synaptic localization equivalent to the present study were obtained in a similar neuron-glia co-culture system, in which approximately one-third of GABA_A receptor clusters were synaptically localized by the end of the first week *in vitro*, and two-thirds were synaptically localized by the end of the second week *in vitro* (Elmariah et al., 2004; Elmariah et al., 2005).

Pre-existing GABA_A receptor clusters became larger during the emergence of presynaptic terminals, similar to previous reports (Christie et al., 2002; Christie and De Blas, 2003). The increased cluster size corresponded with increased synaptic localization, suggesting that extrasynaptic GABA_A receptors are being recruited into synapses. Indeed, when GABA_A receptor clusters became larger, surrounding smaller GABA_A receptor clusters disappeared, similar to observations made by Christie et al. (2002). The formation of larger clusters may be mediated by either the assembly of newly synthesized GABA_A receptor clusters or by the aggregation of smaller clusters. This aggregation could occur through lateral membrane diffusion or through internalization and reinsertion in the cell membrane. Signals that might mediate either of these processes are unknown.

An important question is whether clusters of GABA_A receptors alone possess functional capability. Whereas neurons containing clusters of GABA_A receptors alone did not exhibit mIPSCs, the present study did not analyze the onset of action potential-dependent spontaneous IPSCs (sIPSCs). Recent studies demonstrated paracrine release of GABA before synapse formation (Demarque et al., 2002) and that 50% of cortical neurons contained sIPSCs at birth (Owens et al., 1999). It is possible that mIPSCs and sIPSCs depend on different cellular release mechanisms. In support, Sara and colleagues (2005) recently showed that activity-dependent and activity-independent currents involve the release of vesicles from different pools. This suggests that the hippocampal neurons used in the present study may possess release machinery necessary for sIPSCs but lacked the appropriate signals to induce mIPSCs before they were joined together with nascent presynaptic terminals.

Maturation of GABAergic Synapses

There were striking molecular and functional differences between nascent GABAergic synapses and GABAergic synapses in relatively mature pyramidal neurons. The mIPSC rise time, amplitude and decay recorded from immature neurons were vastly different from those in more developed neurons. Similar alterations in mIPSC kinetics have also been reported in

CA1 pyramidal neurons during postnatal development (Cohen et al., 2000). Only $\alpha 2$, $\beta 2/3$, and $\gamma 2$ subunit-containing receptor clusters were present in nascent synapses whereas $\alpha 1$, $\alpha 5$, $\alpha 2$, $\beta 2,3$ and $\gamma 2$ subunit-containing receptors were expressed at GABAergic synapses in developing pyramidal neurons.

Clusters of $\alpha 5$ subunit-containing GABA_A receptors were only present in relatively mature pyramidal neurons. The presence of $\alpha 5$ subunits at GABAergic synapses may have contributed to altered mIPSC decay, as the $\alpha 5$ subunit is thought to be critical in determining dominant kinetics of GABA_A receptors (Serafini et al., 1998). Previous studies have also observed clusters of $\alpha 5$ in relatively mature neurons (Christie and De Blas, 2002; Brunig et al., 2002), and the larger clusters may be synaptic (Christie and De Blas, 2002). Overall $\alpha 5$ subunit mRNA and protein levels generally decline during postnatal development (Laurie et al., 1992; Poulter et al., 1992; Hutcheon et al., 2004), implying that the reduced amount of $\alpha 5$ subunit protein is redistributed into clusters. In confirmation, Ramos et al. (2004) showed a developmental change in $\alpha 5$ subunit distribution in hippocampal CA1 slices, in which $\alpha 5$ immunoreactivity was expressed in cell bodies early and descended into dendrites later during postnatal development. However, single-cell mRNA amplification revealed that $\alpha 5$ mRNA increased transiently in hippocampal neurons from approximately 7-14 DIV but then declined (Brooks-Kayal et al., 1998), introducing the alternative explanation that emergence of $\alpha 5$ clusters at 14 DIV correlates with this transient $\alpha 5$ mRNA increase. In support, at ~ 21 DIV, $\alpha 5$ clusters were either weakly stained (Brunig et al., 2002), or not present.

Previous studies have suggested that a developmental decrease in mIPSC decay may be due to a switch in expression from $\alpha 2$ subunit-containing GABA_A receptors to $\alpha 1$ subunit-containing GABA_A receptors (Lavoie et al., 1997; Okada et al., 2000; Jüttner et al., 2001; Vicini et al., 2001; Bosman et al., 2002; Goldstein et al., 2002). This study revealed that the distributions of $\alpha 2$ and $\alpha 1$ subunit immunoreactivity were unaltered between 7 and 14 DIV, although the clustering of $\alpha 1$ subunit-containing GABA_A receptors is just beginning at 14 DIV. This suggests that changes in expression of $\alpha 2$ and $\alpha 1$ subunits do not occur during this period, although quantitative methods such as western blotting would be needed for verification. Moreover, synaptic $\alpha 1$ subunit clusters only fully emerged by 21 DIV, suggesting that $\alpha 1$ subunit clusters may influence mIPSC kinetics later during *in vitro* development. Similar to our results, previous studies on mature cultured hippocampal neurons have reported that both $\alpha 1$ and $\alpha 2$ clusters are synaptic (Brunig et al., 2002; Christie et al., 2002; Mangan et al., 2005).

Another possible explanation for prolonged mIPSC decay at 7 DIV could have been the presence of $\alpha 4$ and δ subunits exclusively at this time. The $\alpha 4$ subunit preferentially combines with the δ subunit (McKernan and Whiting, 1996) and the δ subunit prolongs desensitization of whole-cell currents (Saxena and Macdonald, 1994) and preferentially desensitizes with slow and ultraslow phases of desensitization of recombinant receptors (Bianchi and Macdonald, 2002). However, the expression of $\alpha 4$ and δ subunits was not clustered at 7 DIV; immunostaining for these subunits was diffusely distributed at both 7 and 14 DIV.

It should be noted that developmental alterations in the nature of GABAergic synaptic transmission have been well established. In the neonatal brain, GABA depolarizes and excites neuronal membranes (Ben-Ari, 2002). However, starting from the end of the first postnatal week of life, GABA becomes inhibitory by a delayed expression of a Cl^- exporter, leading to a negative shift in the reversal potential for chloride ions (Rivera et al., 1999; Ben-Ari, 2002). However, the present study recorded mIPSCs using equimolar concentrations of chloride ions in the pipette and external solutions, so this change in Cl^-

reversal potential could not have been responsible for the alterations in mIPSC kinetics that were observed.

Maturation of GABA Transporters

Whereas GAT-3 was constantly expressed as small puncta during *in vitro* development, GAT-1 was only present in more mature pyramidal neurons. Furthermore, because many GAT-1 puncta were synaptic, unlike GAT-3, it is tempting to speculate that this localization may cause increased reuptake of synaptic GABA, thereby affecting the mIPSC kinetics observed in this study. However, the contribution of GABA transporters to mIPSC kinetics is thought to be limited. Whereas GABA transporter blockers prolonged evoked IPSCs (Dingledine and Korn, 1985; Roepstorff and Lambert, 1992, 1994; Thompson and Gahwiler, 1992; Isaacson et al., 1993; Draguhn and Heinemann, 1996), this effect was not evident in smaller evoked currents, sIPSCs, or mIPSCs, suggesting that transporters promote GABA clearance only when a large number of release sites are activated (Thompson and Gahwiler, 1992; Isaacson et al., 1993; Roepstorff and Lambert, 1994; Nusser and Mody, 2002; Jensen et al., 2003; Overstreet and Westbrook, 2003).

In summary, this study showed that during GABAergic synaptogenesis in cultured hippocampal neurons, the expression of GABAergic synaptic proteins correlated with alterations in synaptic function. mIPSCs appeared after both presynaptic and postsynaptic elements were present and functional GABAergic synapses increased in number distally during *in vitro* development. In addition, kinetics of GABAergic synaptic currents altered during maturation of GABAergic synapses, corresponding to their composition.

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Literature Cited

- Ahmari SE, Smith SJ. Knowing a nascent synapse when you see it. *Neuron*. 2002; 34:333–336. [PubMed: 11988164]
- Ben Ari Y. Excitatory actions of GABA during development: the nature of the nurture. *Nat Rev Neurosci*. 2002; 3:728–739. [PubMed: 12209121]
- Bencsits E, Ebert V, Tretter V, Sieghart W. A significant part of native gamma-aminobutyric Acid A receptors containing $\alpha 4$ subunits do not contain γ or δ subunits. *J Biol Chem*. 1999; 274:19613–19616. [PubMed: 10391897]
- Benson DL, Watkins FH, Steward O, Banker G. Characterization of GABAergic neurons in hippocampal cell cultures. *J Neurocytol*. 1994; 23:279–295. [PubMed: 8089704]
- Benson DL, Cohen PA. Activity-independent segregation of excitatory and inhibitory synaptic terminals in cultured hippocampal neurons. *J Neurosci*. 1996; 16:6424–6432. [PubMed: 8815921]
- Bianchi MT, Macdonald RL. Slow phases of GABA_A receptor desensitization: structural determinants and possible relevance for synaptic function. *J Physiol*. 2002; 544:3–18. [PubMed: 12356876]
- Borden LA, Smith KE, Hartig PR, Branchek TA, Weinshank RL. Molecular heterogeneity of the gamma-aminobutyric acid (GABA) transport system. Cloning of two novel high affinity GABA transporters from rat brain. *J Biol Chem*. 1992; 267:21098–21104. [PubMed: 1400419]
- Borden LA. GABA transporter heterogeneity: pharmacology and cellular localization. *Neurochem Int*. 1996; 29:335–356. [PubMed: 8939442]

- Bosman LW, Rosahl TW, Brussaard AB. Neonatal development of the rat visual cortex: synaptic function of GABA_A receptor alpha subunits. *J Physiol.* 2002; 545:169–181. [PubMed: 12433958]
- Brandon NJ, Delmas P, Kittler JT, McDonald BJ, Sieghart W, Brown DA, Smart TG, Moss SJ. GABA_A receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. *J Biol Chem.* 2000; 275:38856–38862. [PubMed: 10978327]
- Brooks-Kayal AR, Jin H, Price M, Dichter MA. Developmental expression of GABA_A receptor subunit mRNAs in individual hippocampal neurons in vitro and in vivo. *J Neurochem.* 1998; 70:1017–1028. [PubMed: 9489721]
- Brunig I, Scotti E, Sidler C, Fritschy JM. Intact sorting, targeting, and clustering of gamma-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. *J Comp Neurol.* 2002; 443:43–55. [PubMed: 11793346]
- Chang YC, Gottlieb DI. Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J Neurosci.* 1988; 8:2123–2130. [PubMed: 3385490]
- Christie SB, Miralles CP, De Blas AL. GABAergic innervation organizes synaptic and extrasynaptic GABA_A receptor clustering in cultured hippocampal neurons. *J Neurosci.* 2002; 22:684–697. [PubMed: 11826098]
- Christie SB, De Blas AL. $\alpha 5$ subunit-containing GABA(A) receptors form clusters at GABAergic synapses in hippocampal cultures. *Neuroreport.* 2002; 13:2355–2358. [PubMed: 12488826]
- Christie SB, De Blas AL. GABAergic and glutamatergic axons innervate the axon initial segment and organize GABA_A receptor clusters of cultured hippocampal pyramidal cells. *J Comp Neurol.* 2003; 456:361–374. [PubMed: 12532408]
- Cohen AS, Lin DD, Coulter DA. Protracted postnatal development of inhibitory synaptic transmission in rat hippocampal area CA1 neurons. *J Neurophysiol.* 2000; 84:2465–2476. [PubMed: 11067989]
- Craig AM, Blackstone CD, Haganir RL, Banker G. Selective clustering of glutamate and gamma-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. *Proc Natl Acad Sci U S A.* 1994; 91:12373–12377. [PubMed: 7809044]
- Demarque M, Represa A, Becq H, Khalilov I, Ben Ari Y, Aniksztejn L. Paracrine intercellular communication by a Ca²⁺- and SNARE-independent release of GABA and glutamate prior to synapse formation. *Neuron.* 2002; 36:1051–1061. [PubMed: 12495621]
- Dingledine R, Korn SJ. Gamma-aminobutyric acid uptake and the termination of inhibitory synaptic potentials in the rat hippocampal slice. *J Physiol.* 1985; 366:387–409. [PubMed: 2414435]
- Draguhn A, Heinemann U. Different mechanisms regulate IPSC kinetics in early postnatal and juvenile hippocampal granule cells. *J Neurophysiol.* 1996; 76:3983–3993. [PubMed: 8985894]
- Dupuy ST, Houser CR. Prominent expression of two forms of glutamate decarboxylase in the embryonic and early postnatal rat hippocampal formation. *J Neurosci.* 1996; 16:6919–6932. [PubMed: 8824330]
- Elmariash SB, Crumling MA, Parsons TD, Balice-Gordon RJ. Postsynaptic TrkB-mediated signaling modulates excitatory and inhibitory neurotransmitter receptor clustering at hippocampal synapses. *J Neurosci.* 2004; 24:2380–2393. [PubMed: 15014113]
- Elmariash SB, Oh EJ, Hughes EG, Balice-Gordon RJ. Astrocytes regulate inhibitory synapse formation via Trk-mediated modulation of postsynaptic GABA_A receptors. *J Neurosci.* 2005; 25:3638–3650. [PubMed: 15814795]
- Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ. Two genes encode distinct glutamate decarboxylases. *Neuron.* 1991; 7:91–100. [PubMed: 2069816]
- Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B. Postsynaptic clustering of major GABA_A receptor subtypes requires the $\gamma 2$ subunit and gephyrin. *Nat Neurosci.* 1998; 1:563–571. [PubMed: 10196563]
- Fletcher TL, Cameron P, De Camilli P, Banker G. The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *J Neurosci.* 1991; 11:1617–1626. [PubMed: 1904480]
- Goda Y, Davis GW. Mechanisms of synapse assembly and disassembly. *Neuron.* 2003; 40:243–264. [PubMed: 14556707]
- Goldstein PA, Elsen FP, Ying SW, Ferguson C, Homanics GE, Harrison NL. Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the GABA_A receptor $\alpha 1$ subunit. *J Neurophysiol.* 2002; 88:3208–3217. [PubMed: 12466441]

- Goslin, K.; Asmussen, H.; Banker, G. *Culturing Nerve Cells*. Cambridge, MA: MIT Press; 1998. Rat hippocampal neurons in low-density culture; p. 339-370.
- Guastella J, Nelson N, Nelson H, Czyzyk L, Keynan S, Miedel MC, Davidson N, Lester HA, Kanner BI. Cloning and expression of a rat brain GABA transporter. *Science*. 1990; 249:1303–1306. [PubMed: 1975955]
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch*. 1981; 391:85–100. [PubMed: 6270629]
- Hutcheon B, Fritschy JM, Poulter MO. Organization of GABA receptor α -subunit clustering in the developing rat neocortex and hippocampus. *Eur J Neurosci*. 2004; 19:2475–2487. [PubMed: 15128401]
- Ikegaki N, Saito N, Hashima M, Tanaka C. Production of specific antibodies against GABA transporter subtypes (GAT1, GAT2, GAT3) and their application to immunocytochemistry. *Brain Res Mol Brain Res*. 1994; 26:47–54. [PubMed: 7854065]
- Isaacson JS, Solis JM, Nicoll RA. Local and diffuse synaptic actions of GABA in the hippocampus. *Neuron*. 1993; 10:165–175. [PubMed: 7679913]
- Jensen K, Chiu CS, Sokolova I, Lester HA, Mody I. GABA transporter-1 (GAT1)-deficient mice: differential tonic activation of GABA_A versus GABA_B receptors in the hippocampus. *J Neurophysiol*. 2003; 90:2690–2701. [PubMed: 12815026]
- Johnston, D.; Wu, SMS. *Foundations of Cellular Neurophysiology*. Cambridge: MIT; 1995.
- Jüttner R, Meier J, Grantyn R. Slow IPSC kinetics, low levels of $\alpha 1$ subunit expression and paired-pulse depression are distinct properties of neonatal inhibitory GABAergic synaptic connections in the mouse superior colliculus. *Eur J Neurosci*. 2001; 13:2088–2098. [PubMed: 11422449]
- Kaufman DL, Houser CR, Tobin AJ. Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *J Neurochem*. 1991; 56:720–723. [PubMed: 1988566]
- Kern W, Sieghart W. Polyclonal antibodies directed against an epitope specific for the $\alpha 4$ -subunit of GABA_A receptors identify a 67-kDa protein in rat brain membranes. *J Neurochem*. 1994; 62:764–769. [PubMed: 7507515]
- Killisch I, Dotti CG, Laurie DJ, Luddens H, Seeburg PH. Expression patterns of GABA_A receptor subtypes in developing hippocampal neurons. *Neuron*. 1991; 7:927–936. [PubMed: 1662520]
- Laurie DJ, Wisden W, Seeburg PH. The distribution of thirteen GABA_A receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci*. 1992; 12:4151–4172. [PubMed: 1331359]
- Lavoie AM, Tingey JJ, Harrison NL, Pritchett DB, Twyman RE. Activation and deactivation rates of recombinant GABA_A receptor channels are dependent on α -subunit isoform. *Biophys J*. 1997; 73:2518–2526. [PubMed: 9370445]
- Li M, De Blas AL. Coexistence of two β subunit isoforms in the same gamma-aminobutyric acid type A receptor. *J Biol Chem*. 1997; 272:16564–16569. [PubMed: 9195967]
- Liu QR, Lopez-Corcuera B, Mandiyan S, Nelson H, Nelson N. Molecular characterization of four pharmacologically distinct gamma-aminobutyric acid transporters in mouse brain [corrected]. *J Biol Chem*. 1993; 268:2106–2112. [PubMed: 8420981]
- Mangan PS, Kapur J. Factors underlying bursting behavior in a network of cultured hippocampal neurons exposed to zero magnesium. *J Neurophysiol*. 2004; 91:946–957. [PubMed: 14534286]
- Mangan PS, Sun C, Carpenter M, Goodkin HP, Sieghart W, Kapur J. Cultured hippocampal pyramidal neurons express two kinds of GABA_A receptors. *Mol Pharmacol*. 2005; 67:775–788. [PubMed: 15613639]
- McKernan RM, Whiting PJ. Which GABA_A-receptor subtypes really occur in the brain? *Trends Neurosci*. 1996; 19:139–143. [PubMed: 8658597]
- Meier J. The enigma of transmitter-selective receptor accumulation at developing inhibitory synapses. *Cell Tissue Res*. 2003; 311:271–276. [PubMed: 12658435]
- Mi R, Tang X, Sutter R, Xu D, Worley P, O'Brien RJ. Differing mechanisms for glutamate receptor aggregation on dendritic spines and shafts in cultured hippocampal neurons. *J Neurosci*. 2002; 22:7606–7616. [PubMed: 12196584]

- Moss SJ, Smart TG. Constructing inhibitory synapses. *Nat Rev Neurosci.* 2001; 2:240–250. [PubMed: 11283747]
- Mossier B, Togel M, Fuchs K, Sieghart W. Immunoaffinity purification of gamma-aminobutyric acidA (GABA_A) receptors containing $\gamma 1$ -subunits. Evidence for the presence of a single type of γ subunit in GABA_A receptors. *J Biol Chem.* 1994; 269:25777–25782. [PubMed: 7929282]
- Nusser Z, Sieghart W, Somogyi P. Segregation of different GABA_A receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci.* 1998; 18:1693–1703. [PubMed: 9464994]
- Nusser Z, Mody I. Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol.* 2002; 87:2624–2628. [PubMed: 11976398]
- Okada M, Onodera K, Van Renterghem C, Sieghart W, Takahashi T. Functional correlation of GABA_A receptor alpha subunits expression with the properties of IPSCs in the developing thalamus. *J Neurosci.* 2000; 20:2202–2208. [PubMed: 10704495]
- Overstreet LS, Westbrook GL. Synapse density regulates independence at unitary inhibitory synapses. *J Neurosci.* 2003; 23:2618–2626. [PubMed: 12684447]
- Owens DF, Liu X, Kriegstein AR. Changing properties of GABA_A receptor-mediated signaling during early neocortical development. *J Neurophysiol.* 1999; 82:570–583. [PubMed: 10444657]
- Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW, Houser CR. GABA_A receptor changes in δ subunit-deficient mice: altered expression of $\alpha 4$ and $\gamma 2$ subunits in the forebrain. *J Comp Neurol.* 2002; 446:179–197. [PubMed: 11932935]
- Poulter MO, Barker JL, O'Carroll AM, Lolait SJ, Mahan LC. Differential and transient expression of GABA_A receptor α subunit mRNAs in the developing rat CNS. *J Neurosci.* 1992; 12:2888–2900. [PubMed: 1322978]
- Poulter MO, Brown LA, Tynan S, Willick G, William R, McIntyre DC. Differential expression of $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ GABA_A receptor subunits in seizure-prone and seizure-resistant rat models of temporal lobe epilepsy. *J Neurosci.* 1999; 19:4654–4661. [PubMed: 10341263]
- Ramos B, Lopez-Tellez JF, Vela J, Baglietto-Vargas D, del Rio JC, Ruano D, Gutierrez A, Vitorica J. Expression of $\alpha 5$ GABA_A receptor subunit in developing rat hippocampus. *Brain Res Dev Brain Res.* 2004; 151:87–98.
- Rao A, Cha EM, Craig AM. Mismatched appositions of presynaptic and postsynaptic components in isolated hippocampal neurons. *J Neurosci.* 2000; 20:8344–8353. [PubMed: 11069941]
- Ribak CE, Tong WM, Brecha NC. GABA plasma membrane transporters, GAT-1 and GAT-3, display different distributions in the rat hippocampus. *J Comp Neurol.* 1996; 367:595–606. [PubMed: 8731228]
- Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K. The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature.* 1999; 397:251–255. [PubMed: 9930699]
- Roepstorff A, Lambert JD. Comparison of the effect of the GABA uptake blockers, tiagabine and nipecotic acid, on inhibitory synaptic efficacy in hippocampal CA1 neurones. *Neurosci Lett.* 1992; 146:131–134. [PubMed: 1337191]
- Roepstorff A, Lambert JD. Factors contributing to the decay of the stimulus-evoked IPSC in rat hippocampal CA1 neurons. *J Neurophysiol.* 1994; 72:2911–2926. [PubMed: 7897499]
- Sanes DH. The development of synaptic function and integration in the central auditory system. *J Neurosci.* 1993; 13:2627–2637. [PubMed: 8501528]
- Sanes, DH.; Reh, TA.; Harris, WA. *Development of the Nervous System.* New York: Academic Press; 2000.
- Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci.* 2001; 2:791–805. [PubMed: 11715056]
- Sara Y, Virmani T, Deak F, Liu X, Kavalali ET. An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. *Neuron.* 2005; 45:563–573. [PubMed: 15721242]
- Saxena NC, Macdonald RL. Assembly of GABA_A receptor subunits: role of the delta subunit. *J Neurosci.* 1994; 14:7077–7086. [PubMed: 7525894]

- Scotti AL, Reuter H. Synaptic and extrasynaptic gamma -aminobutyric acid type A receptor clusters in rat hippocampal cultures during development. *Proc Natl Acad Sci U S A*. 2001; 98:3489–3494. [PubMed: 11248105]
- Serafini R, Ma W, Maric D, Maric I, Lahjouji F, Sieghart W, Barker JL. Initially expressed early rat embryonic GABA_A receptor Cl⁻ ion channels exhibit heterogeneous channel properties. *Eur J Neurosci*. 1998; 10:1771–1783. [PubMed: 9751149]
- Sieghart W, Item C, Buchstaller A, Fuchs K, Hoger H, Adamiker D. Evidence for the existence of differential O-glycosylated alpha 5-subunits of the gamma-aminobutyric acid A receptor in the rat brain. *J Neurochem*. 1993; 60:93–98. [PubMed: 8380199]
- Sieghart W, Sperk G. Subunit composition, distribution and function of GABA_A receptor subtypes. *Curr Top Med Chem*. 2002; 2:795–816. [PubMed: 12171572]
- Sigworth FJ. Covariance of nonstationary sodium current fluctuations at the node of Ranvier. *Biophys J*. 1981; 34:111–133. [PubMed: 6260261]
- Sipila S, Huttu K, Voipio J, Kaila K. GABA uptake via GABA transporter-1 modulates GABAergic transmission in the immature hippocampus. *J Neurosci*. 2004; 24:5877–5880. [PubMed: 15229234]
- Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W. GABA_A receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. *Neuroscience*. 1997; 80:987–1000. [PubMed: 9284055]
- Sun C, Sieghart W, Kapur J. Distribution of $\alpha 1$, $\alpha 4$, $\gamma 2$, and δ subunits of GABA_A receptors in hippocampal granule cells. *Brain Res*. 2004; 1029:207–216. [PubMed: 15542076]
- Swanwick CC, Harrison MB, Kapur J. Synaptic and extrasynaptic localization of brain-derived neurotrophic factor and the tyrosine kinase B receptor in cultured hippocampal neurons. *J Comp Neurol*. 2004; 478:405–417. [PubMed: 15384067]
- Thompson SM, Gahwiler BH. Effects of the GABA uptake inhibitor tiagabine on inhibitory synaptic potentials in rat hippocampal slice cultures. *J Neurophysiol*. 1992; 67:1698–1701. [PubMed: 1629773]
- Tia S, Wang JF, Kotchabhakdi N, Vicini S. Developmental changes of inhibitory synaptic currents in cerebellar granule neurons: role of GABA_A receptor $\alpha 6$ subunit. *J Neurosci*. 1996; 16:3630–3640. [PubMed: 8642407]
- Togel M, Mossier B, Fuchs K, Sieghart W. γ -aminobutyric acid A receptors displaying association of $\gamma 3$ subunits with $\beta 2/3$ and different α subunits exhibit unique pharmacological properties. *J Biol Chem*. 1994; 269:12993–12998. [PubMed: 8175718]
- Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, Homanics GE. GABA_A receptor $\alpha 1$ subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. *J Neurosci*. 2001; 21:3009–3016. [PubMed: 11312285]
- Vitellaro-Zuccarello L, Calvaresi N, De Biasi S. Expression of GABA transporters, GAT-1 and GAT-3, in the cerebral cortex and thalamus of the rat during postnatal development. *Cell Tissue Res*. 2003; 313:245–257. [PubMed: 12898208]

Figure 1. Emergence and proliferation of GABAergic synapses

Clusters of GABA_A receptors were present before emergence of GABAergic presynaptic terminals and the number of functional GABAergic synapses increased from 7-14 DIV. By 3 DIV, $\gamma 2$ (A) and $\beta 2/3$ (D) clusters had appeared, but GAD-65 puncta were not present (G). Arrows mark examples of clusters. However, at 7 DIV, clusters of $\gamma 2$ (B), $\beta 2/3$ (E), and GAD-65 (H) were all present, and increased in number at 14 DIV (C, F, I).

Correspondingly, no mIPSCs were observed at 3 DIV (J), but some mIPSCs were recorded at 7 DIV (K), and mIPSC frequency increased at 14 DIV (L). One-minute traces from 3 separate neurons are shown at 3 and 7 DIV, and one-minute traces from 2 separate neurons are shown at 14 DIV. The percentages of neurons containing GAD-65 puncta or mIPSCs from 3-8 DIV were each best fit with a sigmoidal dose-response curve (M). These curves were tightly correlated, but the curve representing percentage of neurons with mIPSCs lagged approximately half a day behind the curve illustrating percentage of neurons with GAD-65 puncta. Increased frequency of mIPSCs is shown with a cumulative frequency plot (N) for one neuron each at 7 and 14 DIV. The rates of colocalization of GAD-65 and $\gamma 2$ (O,P) and GAD-65 and $\beta 2/3$ (Q,R) also increased from 7-14 DIV. Images were captured at 60X and scale bar = 10 μm .

Figure 2. Distal formation of GABAergic synapses from 7-14 DIV

Distance of GAD-65 puncta from the soma increased from 7 DIV (A,C) to 14 DIV (B,D) due to increased outgrowth of neuronal processes. Images were captured at 20X and scale bar = 20 μ m. Amplitude of mIPSCs decreased from 7-14 DIV (E-G). Average mIPSC trace at 7 DIV is larger than at 14 DIV (E). A frequency histogram of mIPSC amplitudes at 7 DIV (F) shifts leftward at 14 DIV (G). 10-90% rise-time of mIPSCs slowed from 7-14 DIV (H-I). A frequency histogram of mIPSC 10-90% rise-time at 7 DIV (H) shifts rightward at 14 DIV (I). mIPSC decay also quickened from 7-14 DIV, as shown by average mIPSC traces normalized for amplitude (J).

Figure 3. Alterations in GABA_A receptor subunit composition from 7-14 DIV

The $\alpha 2$ subunit remained clustered increased from 7-14 DIV (A,B). The $\alpha 4$ (C,D) and δ (E,F) subunits were mostly diffusely distributed from 7-14 DIV. The $\alpha 5$ subunit was mostly diffusely distributed at 7 DIV (G) but became clustered at 14 DIV (H). Most $\alpha 5$ clusters at 14 DIV were not colocalized with GAD-65 puncta (I). Images were captured at 60X and scale bar = 10 μ m.

Figure 4. Expression of the $\alpha 1$ subunit of the GABA_A receptor from 7-21 DIV

Distribution of $\alpha 1$ subunits was mostly diffuse at 7 DIV (A). Approximately 20% of pyramidal neurons contained some small $\alpha 1$ clusters at 14 DIV (B), but $\alpha 1$ clusters fully emerged by 21 DIV (C). Most $\alpha 1$ clusters at 21 DIV were colocalized with GAD-65 puncta (D). Images were captured at 60X and scale bar = 10 μ m. In confirmation, treatment with 30 nM zolpidem, an $\alpha 1$ subunit selective agonist, had no effect on mIPSCs at 14 DIV (E) but prolonged decay at 21 DIV (F). Average mIPSC traces shown were normalized for amplitude.

Figure 5. Expression of GAT-1 and GAT-3 from 7-14 DIV

GAT-1 puncta emerged by 14 DIV (A,B). Most GAT-1 puncta at 14 DIV were colocalized with GAD-65 puncta (C). Small GAT-3 puncta were constantly present from 7-14 DIV (D,E). Most of these small GAT-3 puncta were not colocalized with GAD-65 puncta (F). Images were captured at 60X and scale bar = 10 μ m.

Figure 6. Summary of GABAergic synaptogenesis in cultured hippocampal neurons

Postsynaptic specializations were present by 3 DIV, but presynaptic terminals only emerged at 5 DIV. mIPSC onset coincided with first appearance of presynaptic terminals. Most neurons displayed mIPSCs by 7 DIV. Synapses increased in number distally from the soma from 7-14 DIV, mostly due to elongation of neuronal processes, causing decreased mIPSC amplitude and slowed mIPSC 10-90% rise-time between these time points. Whereas the $\alpha 4$ and δ subunits of the GABA_A receptor remained diffuse at 7 and 14 DIV, the $\gamma 2$ and $\beta 2/3$ subunits became clustered by 3 DIV, the $\alpha 2$ subunit were clustered at 7 and 14 DIV, the $\alpha 5$ subunit became clustered by 14 DIV but was not clustered at 21 DIV, and the $\alpha 1$ subunit became clustered by 21 DIV. GAT-1 puncta emerged by 14 DIV whereas GAT-3 appeared at 7 and 14 DIV. Most $\gamma 2$, $\beta 2/3$, $\alpha 2$, $\alpha 1$, and GAT-1 clusters were synaptic, whereas most $\alpha 5$ and GAT-3 clusters were extrasynaptic. Black circles indicate GABAergic synapses.

Figure 7.

Table 1
Measurements of GABAergic presynaptic and postsynaptic markers

	7 DIV	14 DIV
<i>(n = neurons)</i>		
<i>Number (per neuron in 60X field)</i>		
$\gamma 2$	106.5 $\pm$ 8.2 (n = 33)	164.6 $\pm$ 13.6 (n = 37) $p < 0.001$
$\beta 2/3$	109.0 $\pm$ 6.1 (n = 55)	181.3 $\pm$ 13.4 (n = 43) $p < 0.0001$ *
GAD-65	76.2 $\pm$ 6.6 (n = 37)	151.9 $\pm$ 11.2 (n = 32) $p < 0.0001$
<i>Density (per 10 μm^2)</i>		
$\gamma 2$	2.6 $\pm$ 0.5 (n = 16)	2.8 $\pm$ 0.3 (n = 16)
$\beta 2/3$	2.5 $\pm$ 0.3 (n = 25)	3.2 $\pm$ 0.3 (n = 30)
GAD-65	1.2 $\pm$ 0.1 (n = 25)	2.5 $\pm$ 0.2 (n = 30) $p < 0.0001$
$\alpha 2$	3.1 $\pm$ 0.5 (n = 12)	3.3 $\pm$ 0.5 (n = 12)
$\alpha 5$	N/A	2.8 $\pm$ 0.3 (n = 12)
GAT-1	N/A	2.3 $\pm$ 0.3 (n = 21)
GAT-3	4.4 $\pm$ 0.6 (n = 12)	4.2 $\pm$ 0.3 (n = 11)
<i>Size (μm diameter)</i>		
$\gamma 2$	1.3 $\pm$ 0.06 (n = 43)	1.6 $\pm$ 0.09 (n = 35) $p < 0.05$
$\beta 2/3$	1.8 $\pm$ 0.07 (n = 51)	1.8 $\pm$ 0.07 (n = 57)
GAD-65	3.3 $\pm$ 0.3 (n = 51)	7.6 $\pm$ 0.5 (n = 44) $p < 0.0001$
$\alpha 2$	2.2 $\pm$ 0.2 (n = 23)	2.1 $\pm$ 0.1 (n = 19)
$\alpha 5$	N/A	1.5 $\pm$ 0.2 (n = 12)
GAT-1	N/A	1.7 $\pm$ 0.4 (n = 21)
GAT-3	0.9 $\pm$ 0.1 (n = 12)	1.0 $\pm$ 0.1 (n = 11)
<i>Synaptic Localization (%)</i>		
$\gamma 2$	37.2 $\pm$ 3.0 (n = 10)	57.1 $\pm$ 5.4 (n = 11) $p < 0.01$
$\beta 2/3$	31.7 $\pm$ 4.0 (n = 10)	46.4 $\pm$ 4.8 (n = 10) $p < 0.05$
$\alpha 5$	N/A	17.0 $\pm$ 2.3 (n = 12)
GAT-1	N/A	49.3 $\pm$ 7.2 (n = 11)
GAT-3	13.8 $\pm$ 3.0 (n = 12)	13.8 $\pm$ 2.3 (n = 10)

* P values are based on ANOVA and posthoc Bonferroni's multiple comparison test when $\beta 2/3$ and $\gamma 2$ subunit size were compared between DIV 3, 7 and 14 and unpaired t-test for other parameters, which were compared only between DIV 7-14.

Table 2
Measurements of mIPSC kinetics

	7 DIV	14 DIV
<i>(n = neurons)</i>		
Frequency (Hz)	0.8 ± 0.2 (n = 11)	2.4 ± 0.7 (n = 17) $p < 0.001$
Amplitude (pA)	86.9 ± 11.0 (n = 11)	56.1 ± 3.9 (n = 17) $p < 0.01$
10-90% Rise-time (msec)	1.3 ± 0.1 (n = 11)	1.8 ± 0.1 (n = 17) $p < 0.01$
τ_1 (msec)	30.5 ± 3.1 (n = 5)	23.5 ± 1.5 (n = 8) $p < 0.05$
τ_2 (msec)	98.5 ± 9.4 (n = 5)	81.6 ± 11.2 (n = 8)

P values according unpaired t-test.