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Differential dependence of GABAergic and glutamatergic neurons on glia for the establishment of synaptic transmission

Authors:

Paul Turko[1,2], Keenan Groberman[1], Ferdinand Browa[1], Stuart Cobb[2,3] and Imre Vida[1]

Address and contact information:

1. Institute for Integrative Neuroanatomy, NeuroCure Cluster of Excellence, Charité - Universitätsmedizin Berlin, 10115, Berlin, Germany.
2. Institute of Neuroscience and Psychology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK
3. Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, EH8 9XD

E-mail: *paul.turko@charite.de* or *imre.vida@charite.de*

Telephone: +49 (0)30-450-528-414

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Abstract (188 Words)

In the mammalian cortex, GABAergic and glutamatergic neurons represent two major neuronal classes, which establish inhibitory and excitatory synapses, respectively. Despite differences in their anatomy, physiology and developmental origin, both cell types require support from glial cells, particularly astrocytes, for their growth and survival. Recent experiments indicate that glutamatergic neurons also depend on astrocytes for synapse formation. However, it is not clear if the same holds true for GABAergic neurons. By studying highly pure GABAergic cell cultures, established through fluorescent activated cell (FAC) sorting, we find that, although purified GABAergic neurons are smaller and have reduced survival when compared to those in conventional primary cultures, they establish robust synaptic transmission in the absence of glia. While support from glial cells reverses morphological and survival deficits, it does little to alter synaptic transmission. In contrast, in cultures of purified glutamatergic neurons, morphological development, survival and synaptic transmission are collectively dependent on glial support. Our results demonstrate a fundamental difference in the way GABAergic and glutamatergic neurons depend on glia for the establishment of synaptic transmission, a finding that has important implications for our understanding of how neuronal networks develop.

Introduction (395 Words)

Neurons depend on glia, in particular astrocytes, for a number of vital functions related to their development and maintenance. Not only do they provide mechanical support and play a critical role in maintaining homeostasis, by regulating ion and transmitter levels in the interstitial space (Simard and Nedergaard 2004; Witcher et al. 2007), they also promote the growth and survival of neurons by supplying trophic factors, nutrients and valuable energy substrates (Lindsay 1979; Banker 1980; Le Roux and Reh 1994; Magistretti 2006; Bélanger et al. 2011). Thus, support provided by glial cells is widely considered to be essential for neuron development and survival.

Nevertheless, it is possible to maintain dissociated cultures of neurons under glial-free conditions (Pfrieger and Barres 1997; Ullian et al. 2001, 2004). Indeed, these isolated neuronal cultures have yielded important insights into the intrinsic and extrinsic developmental programs of excitatory networks. Importantly, results from recent studies suggest that the formation and function of glutamatergic synapses critically depend on astrocyte support (Christopherson et al. 2005; Eroglu et al. 2009; Kucukdereli et al. 2011; Allen et al. 2012). While the availability of methods to purify glutamatergic neurons have existed for some time, they have been only partially successful when used to purify GABAergic neurons (Baptista et al. 1994; Berghuis et al. 2004; Buard et al. 2010). Thus, a lack of effective protocols for producing suitably pure GABAergic cell cultures has led to a disparity in our understanding of how these two major neuronal subtypes develop.

To address this problem, we have developed methods for obtaining and culturing highly pure GABAergic neurons *in vitro*, allowing their intrinsic developmental program to

be studied in detail. To generate cultures of purified GABAergic neurons we employed fluorescence activated cell (FAC) sorting to isolate fluorescently-labelled cells from neocortical and hippocampal brain regions of transgenic rats, which were engineered to express the yellow fluorescent protein “Venus” specifically in GABAergic neurons (Uematsu et al. 2008). We then performed a detailed analysis of the survival, morphological and electrophysiological development, and synaptic properties of these purified GABAergic cell cultures.

Our results reveal a fundamental difference in the way GABAergic and glutamatergic neurons establish synaptic transmission. While both cell types depend on glial secreted signals for their growth and survival, only glutamatergic neurons depend on these signals to establish synaptic transmission. In contrast, purified GABAergic neurons establish functional synaptic transmission in the absence of glia.

Materials and methods (2531 words)

Transgenic animals

VGAT-Venus-A Wistar rats selectively express a yellow fluorescent protein variant (Venus) in >95% of cortical GABAergic neurons (Uematsu 2008). NexCre mice selectively express Cre recombinase in cortical glutamatergic neurons (Goebbels 2006). Ai9 mice have a loxP-flanked STOP cassette preventing the transcription of the downstream red fluorescent protein variant (tdTomato, Madisen 2010). To fluorescently label glutamatergic neurons, homozygous NexCre females (C57BL/6) were crossed with hemizygous Ai9 male mice (C57BL/6J) to produce NexCre;Ai9 offspring in which postmitotic cortical glutamatergic neurons express tdTomato. All experiments were performed in accordance with institutional (Charité - Universitätsmedizin Berlin; University of Freiburg, Freiburg, Germany), local (LaGeSo, Berlin, T 0215/11) and national guidelines (German Animal Welfare Act; ASPA, United Kingdom Home Office). Animals were housed and cared for at the central animal facility, Charité - Universitätsmedizin Berlin.

Cell culture and fluorescent activated cell (FAC) sorting

Dissociated cells were prepared from neocortical-hippocampal brain regions of individual VGAT-Venus-A Wistar rats or NexCre;Ai9 mice (0 - 3 days postnatal). Under sterile conditions, cortical and hippocampal regions were removed and transferred to chilled (4˚C) cell culture buffer. The tissue was then chopped several times before being incubated at 37˚C for 25 minutes in 5 ml cell culture buffer (cell culture buffer: 116 mM NaCl, 5.4 mM KCl, 26 mM $NaHCO_3$, 1.3 mM NaH_2PO_4, 1 mM $MgSO_4.7H_2O$, 1 mM

$CaCl_2.2H_2O$, 0.5 mM EDTA.2Na.$2H_2O$ and 25 mM D-glucose, pH = 7.4) containing 1.5 mg/ml Papain (Sigma, St. Louis, USA). The Papain-digested tissue was then triturated with a fine-tip Pasteur pipette (Neolab, Heidelberg, Germany) in three separate 15 ml falcon tubes, each containing 4 ml of 10 mg/ml bovine serum albumin (BSA) (Sigma), dissolved in cell culture buffer. Dissociated cells were then pooled into a single 12 ml solution, before being centrifuged at 3000 RPM for 3 minutes. The resultant cell pellet was re-suspended in 3 ml complete HA-LF medium for Venus-sorting and 2 ml complete HA-LF for tdTomato-sorting (complete HA-LF medium: Hibernate-A low fluorescence medium; BrainBits, Springfield, USA), supplemented with with B27 (at 1X concentration), GlutaMAX (at 1X concentration) and Penicillin-Streptomycin (100 U/ml) (all from Gibco, Waltham, USA). To remove large cell clumps cells were passed through a 30 µm Partec CellTrics filter (Sysmex, Kobe, Japan) into polypropylene round bottom sample tubes (BD, Franklin Lakes, USA).

Fluorescent neurons were FAC sorted using a BD Influx cell sorter (BD biosciences, San Jose, USA). The fluorescent reporter proteins Venus and tdTomato were excited using a blue (488 nm wavelength) or green (531 nm wavelength) laser, respectively. Emitted light was detected through 530/40 and 575/30 emission filter sets. Cells were sorted through a 100 µm nozzle at 15 psi sheath pressure at a rate of 5000 – 6000 events per second. Venus-positive cells were collected at a rate of ≈320 events per second, up to 1.7×10^6 cells per animal. TdTomato cells were collected at a rate of ≈420 cells per second, up to 8×10^5 cells per animal. Sorted cells were collected in BSA coated polystyrene round bottom tubes. Collections tubes were coated in 200 mg/ml BSA dissolved in complete HA-LF medium; excess BSA solution was removed and replaced

with 500 µl complete HA-LF medium before sorting. During FAC sorting cells were maintained at 4˚C.

For the culture of FAC sorted cells, unsorted or positive sorted cell samples were collected and centrifuged at 3000 RPM for 3 minutes. Resultant cell pellets were then re-suspended in complete NBA medium (37˚C) (complete NBA medium: Neural basal-A medium; Gibco) supplemented with B27 (at 1X concentration), GlutaMAX (at 1X concentration) and Penicillin-Streptomycin (100 U/ml) (Gibco) and cell densities were adjusted to 1000 fluorescent cells/µl. Cells were plated at 1000 cells/µl in a 10 µl droplet. This cell density was chosen because higher cell densities resulted in more cell "clumping" and reduced the overall general quality of the cultures at or around DIV 14, without improving the long-term survival of the cultures (data not shown). The relative proportion of Venus-positive cells within the overall unsorted cell population was estimated by FAC sorting to be between 15-20%. In experiments where Venus and tdTomato cells were mixed, Venus positive cells were adjusted to 1000 cells/µl and tdTomato cells were adjusted to 4000 cells/µl, in the same solution. Cells were applied as a 10 µl droplet to 12 mm, PLL-coated coverslips (Menzel-Gläzer; PLL solution: 20 µg/ml Poly-L-Lysine hydrobromide (Sigma, St. Louis, USA) dissolved in sterile water (Ampuwa, Bad Homburg, Germany). To facilitate cell adherence, coverslips were incubated at 37˚C / 5% CO_2, in a 24 well plate for 1 hour, before addition of 500 µl complete NBA medium. Cells were fed weekly by removing 100 µl of conditioned media and replacing it with 200 µl of fresh complete NBA medium. In these "mixed" co-cultures, both glutamatergic and GABAergic synapses were formed between GABAergic and

glutamatergic neurons in the presence of glia, indicating that species differences are not a barrier to synapse formation (**Fig. 5**).

Glial cultures were prepared from brains of VGAT-Venus-A Wistar rat pups (2-5 days postnatal). Two animals provided sufficient cells for culture in a single PLL-coated six well plate. Under sterile conditions, neocortical and hippocampal regions were removed from excised whole brains and transferred to a 15 ml falcon tube containing 5 ml chilled (4˚C) Hank's Balanced Salt Solution (HBSS) (Biochrom, Berlin, Germany). Tissue was then triturated several times using a 1 ml Pasteur pipette (Alpha laboratories, Eastleigh, UK), before vigorous trituration using a fine-tip Pasteur pipette. Dissociated cells were then centrifuged at 800 RPM for 5 minutes, before the resultant cell pellet was re-suspended in 5 ml pre-warmed (37˚C) complete ACM (complete ACM: Opti-MEM reduced serum medium with GlutaMAX supplement, 10% fetal bovine serum (Biochrom, Berlin, Germany) and Penicillin-Streptomycin (100 U/ml). Cells were again vigorously triturated using a fine-tip Pasteur pipette, before further centrifugation at 800 RPM for 5 minutes. The resultant cell pellet was re-suspended in 12 ml pre-warmed (37˚C) complete ACM. Dissociated cells were transferred to a PLL-coated six well plate, at 2 ml cell solution/well. Cell cultures were fed weekly by total replacement of the cell culture medium with fresh complete ACM. Unless otherwise stated, imaging and electrophysiology experiments were performed between 12 and 16 DIV.

For cell detachment and passage, confluent glial cell cultures were washed twice with DPBS (no calcium, no magnesium) (Gibco) before application of 1 ml/well Trypsin/EDTA (0.25%/0.02% wt/vol) (Biochrom) solution and incubated at 37˚C / 5% CO_2 for 3 –

5 minutes. Detached cells from two wells were pooled together and gently dissociated using a fine-tip Pasteur pipette, before centrifugation at 3000 RPM for 3 minutes. The resultant cell pellet was re-suspended in 12 ml complete ACM. Passaged cells were transferred to a PLL-coated six well plate, at 2 ml cells/well. Cells reached confluence after approximately seven days. To culture glial cells on cell culture inserts, confluent glial cells were detached by trypsin digestion (as above) before centrifugation at 3000 RPM for 3 minutes; the detached cells were then re-suspended in prewarmed (37°C) complete NBA medium. Cell densities were estimated using a haemocytometer. Glial cells were seeded onto 0.4 µm transparent PET membrane culture inserts (24 well plate inserts, Falcon, Corning, USA) at 40,000 cells/insert, by applying a 500 µl droplet of cells. Membranes were then transferred to 500 µl complete NBA medium in a 24 well plate and incubated at 37˚C / 5% CO_2 for up to 24 hours before use. Inserts were then transferred to a 24 well plate containing adhered positive sorted neurons. Excess media was removed from the cell culture inserts, leaving the surface of the insert only slightly submerged. Glial-neuron co-cultures were fed weekly by removal of 100 µl conditioned media and replacement with 200 µl of fresh complete NBA medium. As demonstrated previously, rat glial cells are suitable support cells for neurons derived from either mice or rats (Kaech and Banker, 2006).

Electrophysiology

For electrophysiological recordings cell cultures were placed in a submerged chamber and superfused with artificial cerebrospinal fluid (ACSF: 125 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 25 mM D-glucose; equilibrated with 95% O_2 and 5% CO_2 gas mixture at 30-32°C). Recording pipettes were

pulled from thick-walled borosilicate glass tubing (outer diameter = 2 mm, inner diameter = 1 mm) with a horizontal pipette puller (P-97, Sutter Instruments, Novato, USA). Pipettes were filled with an internal solution containing: 130 mM potassium gluconate, 10 mM EGTA, 10 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 2 mM NA_2ATP, 0.3 mM Na_2GTP, 1 mM $Na_2Creatine$ and 0.1% Biocytin (wt/vol) (Molecular Probes, Eugene, USA); pH was adjusted to 7.3 with KOH and osmolarity adjusted to 300 ± 2 mOsm. Filled pipettes had resistances between 2-10 MΩ. Voltage-clamp and current-clamp experiments were performed using a Multiclamp 700B amplifier under the control of the Axon MultiClamp Commander software V2.1.0.16 (Molecular Devices, Sunnyvale, USA). Signals were low pass-filtered at 6 kHz and digitized at a sampling rate of 20 kHz using a USB-6259 AD-converter interface (National Instruments, Austin, USA). Data acquisition and stimulus protocols were made using WinWCP V4.5.2. (courtesy of John Dempster, University of Strathclyde). Pipette capacitance and series resistance compensation (bridge balance) were applied during current-clamp recordings. Only cells with series resistances <30 MΩ were analyzed; recordings during which series resistances changed >20% were excluded from analysis.

In current-clamp mode, hyperpolarizing voltage responses, action potential (AP) discharge and discharge frequencies were tested by a series of current pulses (500 ms duration, -200 pA starting amplitude and 20 pA increments). Neurons not firing multiple action potentials were excluded from current-frequency analysis. To determine spontaneous AP firing frequency, cells were recorded at their resting membrane potential for 60 seconds. Despite being able to fire action potentials in response to current injections, the spontaneous AP firing frequency in all groups was low (Mean spontaneous AP firing frequencies were: Unsorted: 0.05 +/- 0.018 Hz [36 cells, 7

cultures analyzed]; Positive sort: 0.02 +/- 0.018 Hz [46 cells, 11 cultures analyzed]; Glial support: 0.06 +/- 0.050 Hz [35 cells, 6 cultures analyzed]). The frequency of events was significantly higher in unsorted neurons vs. positive sorted neurons ($P < 0.01$), all other comparisons were not significant. AP and after hyperpolarization (AHP) amplitudes were measured from the AP threshold and their duration was measured at half-height (half-width). AP threshold was taken as deflection point at the foot of the AP, where the first derivative of the voltage signal exceeded 20 mV/ms (**Supplementary fig. 2e).**

In voltage-clamp mode, series resistance, capacitance and input resistance were estimated from the current response to a -5 mV voltage pulse of 20 ms duration (100 repetitions). Capacitance was estimated by determining the integral of the capacitive transient (as described by Golowasch et al, 2009). To determine the frequency and amplitude of spontaneously-occurring PSCs, recordings were made at 0 mV, -50 mV or -70 mV for 60 seconds. Spontaneous synaptic events were extracted by template matching (Clements and Bekkers, 1997); events smaller than three standard deviations of the baseline noise were ignored. Series resistance in these voltage-clamp recordings was uncorrected. IPSC amplitude and kinetic parameters derived from our voltage-clamp recordings are likely to be influenced by limited space clamp and differences in the electrotonic structure of the cells in the different culture types (Williams and Mitchell, 2008).

To determine the connectivity between GABAergic neurons, cell bodies of presynaptic GABAergic neurons, which were located <400 µm from the recorded cell, were stimulated by an extracellular monopolar electrode (stimulus amplitude range: 20 – 320 A; pulse width: 0.1 ms; frequency: 0.1 Hz). To determine the mean amplitude of

evoked responses, ≥12 stimulation repetitions were collected and analyzed. To measure evoked IPSCs, the postsynaptic GABAergic neuron was maintained at a 0 mV holding potential. All data was analyzed using Stimfit V0.14.7 (Guzman et al, 2014; https://github.com/neurodroid/stimfit/wiki/Stimfit) on Windows 7.

Immunocytochemistry

Cell cultures were fixed for 15 minutes in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (pH 7.4). Cells were then washed at room temperature for 10 minutes in 0.1 M PB before two subsequent 10 minute washes in phosphate buffered saline (PBS: 154 mM NaCl in 0.025 M PB). Primary antibodies were applied overnight at 4°C in PBS containing 0.1% Triton-X-100 (Serva, Heidelberg, Germany). Three 10 minute PBS washes removed primary antibodies before secondary antibody application. Secondary antibodies were applied overnight at 4°C in PBS containing 0.1% Triton-X-100. Three 10 minute PBS washes removed secondary antibodies before coverslips were mounted onto glass slides in Fluoromount-G mounting medium (Southern Biotech, Birmingham, USA) and sealed with varnish. Antibody incubation and washing steps were performed on a 3D orbital shaker.

The following primary antibodies were used: Mouse anti-GFP (1:1000, NeuroMab, Davis, USA), Rabbit anti-GFP (1:1000, Molecular Probes), Mouse anti-NeuN (1:1000, Chemicon, Billerica, USA), Mouse anti GFAP (1:1000, Sigma, St. Louis, USA), Mouse anti-Gephyrin (1:1000, SySy, Goettingen, Germany) and Rabbit anti-VGAT (1:500, SySy). The following secondary antibodies, all raised in goat, were used: Alexa Fluor 488 anti-Mouse (1:1000, Molecular Probes), Alexa Fluor 488 anti-Rabbit (1:1000,

Jackson, West Grove, USA), Alexa Fluor 546 anti-Mouse (1:1000, Molecular Probes) and Alexa Fluor 647 anti-Rabbit (1:1000, Jackson). In addition, Streptavidin-Alexa Fluor 647 conjugate (1:1000, Molecular Probes) was used to visualize biocytin-filled cells. To test for the survival *versus* loss of cells in some experiments, DRAQ7 (1:100, BioStatus, Shepshed, UK) was added to the final PBS washing step.

Imaging

Confocal images were collected on an upright microscope (BX61, Olympus, Tokyo, Japan). Endogenous Venus-expressing neurons or Alexa Fluor-488 secondary antibodies were excited at 488 nm by a multi-line Argon laser; a short-pass dichroic mirror (SDM, 560) directed emitted light through a barrier filter (BA, 505-525). TdTomato expressing neurons or Alexa Fluor-546 secondary antibodies were excited at 543 nm by a Helium-Neon (G) laser (emission path: SDM 560, BA 560-620). Alexa Fluor-647 secondary antibodies were excited at 635 nm by a diode laser (emission path: BA 655-755). Confocal images were captured using: x4 (0.16 N.A., 13 mm W.D.), x20 (0.75 N.A., 0.6 mm W.D.), x30 (silicon oil-immersion, 1.05 N.A., 0.8 mm W.D.) or x60 objectives (silicon oil-immersion, 1.35 N.A., 0.15 mm W.D., all from Olympus). Images were acquired using FluoView FV1000 V4.2 software (Olympus).

To assess the proportion of Venus-positive neurons in unsorted or positive sorted cultures, fixed cell cultures were immunolabeled for GFP and NeuN and imaged with a x4 objective (0.78 µm X/Y-resolution). A single region from the center of each coverslip was captured for analysis. For axonal and dendritic reconstructions, fixed biocytin-filled

cells were visualized with Streptavidin-647. For axonal reconstructions, cells were imaged with a x30 or x60 objective (at 0.2 - 0.4 µm X/Y-resolution, 0.5 - 0.8 µm Z-resolution). For dendritic reconstructions, cells were imaged with x20, x30 or x60 objectives (0.2-0.4 µm X/Y-resolution, 0.8 µm Z-steps).

During whole-cell patch clamp experiments, cell cultures were imaged with infrared (IR) oblique illumination (780 nm excitation LED, Scientifica, Uckfield, UK) through a x20 water-immersion objective (1.0 N.A., 2 mm W.D., Olympus) in combination with a c-mount video magnification changer and Dodt gradient contrast (DGC, Luigs and Neumann, Ratingen, Germany). Images were captured with a Retiga 2000R CCD camera (QImaging, Surrey, Canada) using Micro-Manager software V1.47F7 (Edelstein 2010). Venus- or tdTomato-expressing neurons were excited at 500 nm or 550 nm wavelengths respectively, using LED illumination (CoolLED, Andover, UK). YFP (F36-528, AHF) and Cy3/Trit-C (F46-016) filter sets were used to distinguish between Venus and tdTomato signals. Some tdTomato “bleed through” signal was observed in the YFP filter set. In figure 5b, to aid with visualization of Venus positive cells in mixed cultures, this tdTomato signal was subtracted from the YFP channel using the Cy3/Trit-C channel as a reference. Offline image subtraction was performed using the process>image calculator tool in the FIJI distribution of ImageJ software (Schneider 2012; http://fiji.sc).

Image analysis

Neuronal reconstructions were made in FIJI. Individual confocal image stacks of fluorescently-labeled biocytin-filled neurons were stitched together using the plugin 3D stitching (Preibisch 2009). Filled neurons were then reconstructed using the plugin

Simple Neurite Tracer (Longair 2011). A morphological analysis of the reconstructed cells was performed using L-Measure (Scorscioni 2008). Quantification of the proportion of Venus-positive neurons was performed in FIJI. Manual cell counts of Venus-positive and NeuN-positive cells were made using the "multipoint tool" and saved using the plugin ROI manager.

Statistics

In all figures, error bars represent the S.E.M. Measurements made from individual cells are shown superimposed on bar charts as open circles. The number of cells recorded is shown in brackets below the bar charts. Significance indicated: (*) = $P<0.05$, (**) = $P<0.01$ and (***) = $P<0.001$. A Wilcoxon matched-pairs nonparametric test was used to assess significant differences for pairwise comparisons; a Mann-Whitney nonparametric test was performed to assess for a difference between two independent samples; a Kruskal-Wallis nonparametric test, with Dunn's posttest, was used to assess for significant changes between multiple groups. A Fisher's exact test was performed to test for differences in connectivity between culture types. All graphs and statistics were performed and prepared in Graph Pad Prism V5.0.1.

Results (2905 words)

Establishing purified GABAergic cell cultures

To establish cell cultures of purified GABAergic neurons, we used fluorescent activated cell (FAC) sorting to collect fluorescent Venus-positive cells from the neocortex and hippocampus of VGAT-Venus-A rats (see Methods; **Fig. 1a, b and c**). To assess the ability of FAC sorting to generate purified GABAergic cell cultures, we estimated the proportion of GABAergic neurons in positive sorted cell cultures, immunolabeled for the Neuronal Nuclei marker (NeuN, **Fig. 1d**), a Fox-1 protein found in cortical neurons (Kim et al 2009, Mullen et al. 1992), after 2 weeks (12 - 16 DIV) and compared this to standard unsorted primary neocortical neuronal cultures. This analysis revealed that in unsorted cultures Venus-positive cells represented 27 ± 0.4% the total neuron cell population [6 coverslips, 3 culture batches analyzed], while in positive sorted cultures Venus-positive cells comprised 99 ± 0.4% of all neurons (10 coverslips, 6 cultures analyzed, $P < 0.0001$, **Fig. 1e**), demonstrating the high efficiency and purity achieved by FAC sorting. At this time point, the expression levels of neurochemical markers such as parvalbumin (PV), somatostatin (SOM) and cholecystokinin (CCK) were low or absent in both unsorted and positive sorted cultures (data not shown), precluding a characterization of interneuron subtypes in these cultures.

To check for possible astrocyte contamination we also immunolabeled unsorted and positive sorted cell cultures for the astrocyte specific protein Glial Fibrillary Acid Protein (GFAP; **Fig. 1f**). This analysis confirmed the absence of astrocytes in positive sorted cultures [12 coverslips, 3 cultures analyzed]. Taken together this data

demonstrates that purified GABAergic neurons are viable to at least 16 DIV, despite the absence of other neuron and glial cell types.

Limited survival of purified GABAergic neurons is improved by glial support

Having first established purified GABAergic cell cultures, we next examined the ability of these cultures to grow over extended periods (**Fig. 2a and b**). First, we compared the survival of GABAergic neurons in positive sorted and unsorted cell cultures by plating cells at equal densities ($1x10^4$ Venus-positive cells / coverslip) and then assessing their survival up to DIV 42 (**Fig. 2b**). This analysis revealed that unlike unsorted neurons, positive sorted neurons had only limited survival beyond 16 DIV, and were incapable of growing to DIV 42. To test whether positive sorted neurons lack certain essential factors that originate from glial cells, we grew purified GABAergic neurons and glial cells in a non-contact co-culture arrangement and assessed their survival. Indeed, this co-culture arrangement led to a marked improvement in the survival of neurons, with supported cells being able to grow beyond 42 DIV (**Fig. 2b**). These results therefore underpin the notion that glial secreted factors directly improve the long-term survival of GABAergic neurons.

Reduced growth and morphological development of purified GABAergic neurons is recovered by glial support

To examine the ability of purified GABAergic neurons to grow and develop in cell culture, we intracellularly-filled and reconstructed Venus-immunolabeled neurons from positive sorted and unsorted cell cultures and compared their neuroanatomical characteristics (**Fig. 2c**). A morphometric analysis of unsorted and positive sorted neuronal

reconstructions revealed that positive sorted neurons have lower total lengths of both axons (unsorted 22831 ± 4258 µm [8 cells, 3 cultures analyzed] vs. positive sort 5469 ± 840 µm [9 cells, 3 cultures analyzed], $P < 0.05$) and dendrites (unsorted 3627 ± 568.3 µm [18 cells, 3 cultures analyzed] vs. positive sort 870 ± 120 µm [16 cells, 3 cultures analyzed], $P < 0.001$, **Fig. 2d, e**). A dendritic Sholl analysis further demonstrated that purified GABAergic neurons were not only smaller than unsorted neurons, but also notably less complex in their morphology, having significantly fewer and shorter branched dendrites (unsorted vs. positive sort; two-way ANOVA, $P < 0.0001$, **Fig. 2f**). Additional analysis of dendritic segments by branch order can be found in **Supplementary Fig. 1**. Taken together, these data, indicate that purified GABAergic neurons are considerably smaller and less developed than unsorted neurons.

To test whether glial cells support the morphological maturation of purified GABAergic neurons, we repeated the above morphometric analysis in glial supported cultures. This analysis revealed that glial supported neurons had greater axon lengths (37215 ± 9229 µm [6 cells, 4 cultures analyzed], $P < 0.01$) and dendrite lengths (2277 ± 321.0 µm [16 cells, 5 cultures analyzed], $P < 0.01$) when compared to positive sorted neurons, and possessed a complex and more elaborate dendritic tree (positive sort vs. glial support; two-way ANOVA, $P < 0.0001$, **Fig. 2d-f**). However, although similar, glial supported neurons were still less complex than unsorted neurons (unsorted vs. glial support; two-way ANOVA, $P < 0.0001$), a possible indication that factors other than glia-secreted signals may be required for the normal morphological development of GABAergic neurons.

Overall, these data indicate that while purified GABAergic neurons can develop a neuron-like morphology in isolation, glial-secreted signals play an important role in their

neurite outgrowth, development and maintenance.

Development of intrinsic electrophysiological properties of purified GABAergic neurons

To examine the consequences of purification on the passive and active membrane properties of GABAergic neurons, we performed whole-cell patch-clamp recordings from identified Venus-positive cells in each culture configuration (**Fig. 3a**). We found that positive sorted neurons maintained a resting membrane potential (-55.6 ± 1.4 mV [85 cells, 13 cultures analyzed]) that was slightly more depolarized, but not significantly different from unsorted neurons (-60 ± 0.89 mV [74 cells, 10 cultures analyzed], $P > 0.05$, **Fig. 3b**). Furthermore, in response to long depolarizing current pulses, purified neurons were capable of firing repetitive trains of action potentials. Consistent with their smaller size (**Fig. 2c-f**), purified GABAergic neurons possessed a markedly reduced membrane capacitance (50.8 ± 2.5 pF [80 cells, 13 cultures analyzed]) when compared to unsorted neurons (110.5 ± 6.3 pF [73 cells, 10 cultures analyzed], $P < 0.0001$, **Fig. 3c**). Surprisingly however, despite their small size, we observed no significant difference in the input resistance of positive sorted and unsorted neurons (unsorted 170.6 ± 8.9 MΩ [73 cells, 10 cultures analyzed] vs. positive sort 197.2 ± 10.7 MΩ [80 cells, 13 cultures analyzed], $P > 0.05$, **Fig. 3d**). These results therefore suggest that the membranes of purified GABAergic neurons may have a lower specific resistance (i.e. higher conductance) than in unsorted neurons, plausibly reflecting the presence of more open “leak” ion channels.

To examine the influence of glial secreted factors on the electrophysiological properties of purified GABAergic neurons, we performed the above whole-cell patch-

clamp experiments on glial supported cultures. We observed no difference in the resting membrane potential of glial supported neurons (-62 ± 1.6 mV, [38 cells, 6 cultures analyzed]) relative to unsorted neurons ($P > 0.05$), but did observe a slight hyperpolarization of their resting membrane potential relative to positive sorted neurons ($P < 0.05$). We found that glial-supported neurons had a markedly increased membrane capacitance (100.9 ± 5.7 pF [37 cells, 6 cultures analyzed]) when compared to positive sorted neurons ($P < 0.0001$, **Fig. 3c**), but were similar to unsorted neurons ($P > 0.05$), which is in good agreement with our morphometric analysis (**Fig. 2c**). Interestingly however, glial supported neurons had significantly lower input resistances (91 ± 6.9 MΩ [37 cells, 6 cultures analyzed]) when compared to both unsorted ($P < 0.0001$) and positive sorted ($P < 0.0001$) neurons (**Fig. 3d**). These data indicate that the membranes of glial supported purified GABAergic neurons, similar to purified GABAergic neurons, have a higher conductance than unsorted neurons.

To understand how these altered membrane properties influence AP firing, we injected incremental current pulses and recorded AP firing frequency from cells in each culture condition (**Fig. 3e**). We found that at lower current-pulse amplitudes (0 - 340 pA) positive sorted neurons and unsorted neurons had surprisingly similar AP firing rates (two-way ANOVA, $P > 0.05$). These firing rates were markedly higher than in glial supported neurons (**Fig. 3e**). Analysis of the current-pulse amplitude required to evoke APs (rheobase), revealed that positive sorted neurons and unsorted neurons were similarly excitable (132 ± 11 pA [75 cells, 10 cultures analyzed] and 137 ± 13 pA [78 cells, 13 cultures analyzed], respectively, $P > 0.05$), but that glial supported neurons required approximately 2.5x higher stimulus intensity to begin to generate APs (340 ± 36 pA [40 cells, 6 cultures analyzed], $P < 0.0001$, **Supplementary Fig. 2f**).

To investigate this further, we analyzed the kinetics of single APs evoked at or near threshold. We found that the maximal AP rise rate (mV/ms) in unsorted neurons was significantly higher than in either positive sorted or glial supported neurons (unsorted 283.7 ± 13.0 mV/ms [77 cells, 10 cultures analyzed] vs. positive sort 191.3 ± 11.6 mV/ms [81 cells, 13 cultures analyzed] vs. glial support 200.2 ± 15.19 mV/ms [45 cells, 6 cultures analyzed], $P < 0.0001$, **Supplementary Fig. 2b**), as was the peak AP amplitude from threshold (unsorted 63.6 ± 1.27 mV [78 cells, 10 cultures analyzed] vs. positive sort 53.7 ± 1.4 mV [81 cells, 13 cultures analyzed] vs. glial support 53.2 ± 2.07 mV [45 cells, 6 cultures analyzed], $P < 0.0001$, **Supplementary Fig. 2a**). However, the maximal decay rate (unsorted 91.0 ± 4.0 mV/ms [76 cells, 10 cultures analyzed] vs. positive sort 80.4 ± 3.6 mV/ms [80 cells, 10 cultures analyzed] vs. glial support 100.2 ± 9.6 mV/ms [44 cells, 6 cultures analyzed], $P > 0.05$, **Supplementary Fig. 2c**) and AP half-width (unsorted 0.76 ± 0.02 ms [78 cells, 10 cultures analyzed] vs. positive sort 0.84 ± 0.04 ms [81 cells, 13 cultures analyzed] vs. glial support 0.80 ± 0.06 ms [45 cells, 6 cultures analyzed], $P > 0.05$, **Supplementary Fig. 2d**) remained unchanged. Similarly, afterhyperpolarization (AHP) amplitude and kinetics following single APs were not significantly different in the three culture types either (**Supplementary Fig. 2g or h)**. Taken together, these results suggest that sodium currents, but not potassium currents, are significantly reduced and/or shunted in positive sorted and glial supported neurons.

GABAergic synaptic transmission is established in purified cell cultures without glia

To determine the ability of purified GABAergic neurons to form functional synaptic connections and to assess the influence of glia on the establishment of spontaneous

synaptic activity we performed voltage-clamp recordings from GABAergic neurons in unsorted, positive sorted and glial supported cell cultures. These recordings revealed the presence of large numbers of spontaneous IPSCs in positive sorted cultures, albeit with lower amplitudes, when compared to unsorted and glial supported cultures (**Fig. 4a, b and Supplementary Fig. 3g**). Immunocytochemistry for the presynaptic and postsynaptic markers, VGAT and gephyrin, respectively, confirmed the presence of GABAergic synaptic contacts among purified GABAergic neurons (see **Supplementary Fig. 3a**). Importantly, IPSCs were abolished in the presence of the selective $GABA_A$ receptor antagonist gabazine (SR-95531, 10 µM; **Supplementary Fig. 3b,c and d**), leaving no residual glutamatergic activity (**Supplementary Fig. 3e and f**). Further analysis confirmed that the amplitudes of IPSCs in unsorted cultures and glial supported cultures were significantly larger than in positive sorted cultures (unsorted 125.1 ± 19.7 pA [25 cells, 5 cultures analyzed] vs. positive sort 57.6 ± 3.7 pA [36 cells, 5 cultures analyzed] vs. glial support 154.8 ± 34.4 pA [18 cells, 3 cultures analyzed], $P < 0.05$, **Fig. 4b**). The mean frequency of synaptic events recorded from GABAergic neurons in all groups was: unsorted 2.7 ± 0.64 Hz [25 cells, 5 cultures analyzed], positive sort 3.7 ± 0.56 Hz [36 cells, 5 cultures analyzed] and glial support 1.9 ± 0.38 Hz [18 cells, 3 cultures analyzed], Statistics: Kruskal-Wallis test, $P = 0.0387$; Dunns multiple comparison test, $P > 0.05$ for all comparisons). Interestingly, the high frequency of synaptic events observed in cultures of purified GABAergic neurons indicates that there is no essential requirement for glia or glial derived factors for the establishment of functional synaptic transmission.

Given the high frequency and low amplitude of synaptic events observed in positive sorted cultures (**Fig. 4a and b**) we speculated that this might be the result of

neurons receiving large amounts miniature synaptic events (that is, AP-independent synaptic events, mediated by single vesicle release). To test this hypothesis we bath applied the voltage gated sodium channel blocker tetrodotoxin (TTX, 100 nM), in order to abolish APs in positive sorted and unsorted cultures (**Supplementary Fig. 3g**). As predicted, TTX application reduced the amplitude of IPSCs in unsorted cultures, (control 135.5 ± 23.10 pA vs. TTX 46.5 ± 2.81 pA [13 cells, 3 cultures analyzed], $P < 0.001$), but not in positive sorted cultures (control 49.6 ± 5.12 pA vs. TTX 48.4 ± 6.66 pA [12 cells, 3 cultures analyzed], $P = 0.79$, **Supplementary Fig. 3h**). In addition, TTX application also reduced the frequency of IPSCs in unsorted cultures (control 2.8 ± 0.92 Hz vs. TTX 1.6 ± 0.67 Hz [15 cells, 3 cultures analyzed], $P < 0.0001$), but not in positive sorted cultures (control 5.2 ± 1.27Hz vs. TTX 4.8 ± 1.28 Hz [12 cells, 3 cultures analyzed], $P = 0.42$, **Supplementary Fig. 3i**). Interestingly, while the resultant amplitude of mIPSCs in both groups was comparable ($P = 0.61$, **Supplementary Fig. 3h**), positive sorted neurons received approximately 3-fold more mIPSCs than unsorted neurons ($P = 0.0008$, **Supplementary Fig. 3i**). An analysis of the kinetics of synaptic events before and after TTX application is included in **Supplementary Fig. 3j, k and l**. Taken together, these results suggest that mIPSCs are the dominant form of synaptic transmission between purified GABAergic neurons.

The high frequency of AP-independent (miniature) IPSCs recorded in positive sorted GABAergic cultures raised the question of whether these cells were also capable of producing evoked, action AP-dependent neurotransmitter release. To test this, we performed whole-cell recordings on Venus-positive neurons, in each culture type, and recorded evoked IPSCs (eIPSCs), by minimally stimulating neighboring GABAergic neurons (stimulated cells were <400 µm away from the recorded neuron, **Fig. 4d**). Using

this stimulation protocol we were able to evoke synaptic responses in 92% of all unsorted GABAergic neurons tested, significantly higher than in positive sorted cultures (49%, $P > 0.001$). Notably, glial support improved the connectivity between purified GABAergic neurons to 95% ($P > 0.001$), to a level similar to that in unsorted cultures ($P > 0.05$, **Fig. 4f**). Interestingly, despite apparent differences in connectivity, these experiments revealed that IPSCs could be evoked at comparable amplitudes in all culture types (unsorted 513 ± 72 pA [25 cells, 3 cultures analyzed] vs. positive sort 524 ± 94 pA [18 cells, 3 cultures analyzed] vs. glial support 529 ± 82 pA [28 cells, 3 cultures analyzed], $P > 0.05$, **Fig. 4e**). In addition, they also revealed that short term plasticity, in the form of paired pulse depression, was similar between all groups ($P > 0.05$, **Supplementary Fig. 3m,n and o**). These results, thus, demonstrate that although spontaneous AP-mediated synaptic transmission is rare between purified GABAergic neurons, these cells are nevertheless capable of producing evoked neurotransmitter release.

Mixed GABAergic and glutamatergic neuronal networks show only GABAergic transmission in the absence of glia

Having ascertained that purified GABAergic neurons were able to establish mutual synaptic contacts in the absence of glia, we next tested whether they were also capable of establishing synapses onto glutamatergic neurons. Therefore, we first obtained purified glutamatergic neurons by FAC sorting neocortical-hippocampal neurons from NexCre;Ai9 mice (**Supplementary Fig. 4a-c)**. Despite being smaller and more excitable than unsorted neurons, purified glutamatergic neurons were able to grow for up to 16 DIV and matured sufficiently to maintain repetitive trains of actions potentials in

response to depolarizing current pulses (**Supplementary Fig.4d-l** for morphological and electrophysiological properties). We then combined these neurons with purified GABAergic neurons and cultured them at a ratio of 4:1 (glutamatergic: GABAergic; **Fig. 5a and b**), to create a mixed-neuron culture system lacking glia. In this culture system, we examined the establishment of synaptic transmission by performing voltage-clamp recordings from both GABAergic (Venus-positive) and glutamatergic (tdTomato-positive) neurons (for IPSCs V_h = 0 mV; for EPSCs V_h = -50 mV; **Fig. 5c and f**). These experiments revealed that, in the absence of glia, it was possible to observe high frequencies of IPSCs in both Venus positive (2.7 ± 0.57 Hz [14 cells, 4 cultures analyzed]) and tdTomato positive neurons (3.2 ± 0.60 Hz [12 cells, 4 cultures analyzed]). However, despite glutamatergic neurons outnumbering GABAergic neurons 4 to 1, EPSCs were low or absent, regardless of type of the recorded neuron (Venus positive: 0.40 ±0.27 Hz [11 cells, 4 cultures analyzed]; TdTomato positive: 0.29 ± 0.22 Hz [12 cells, 4 cultures analyzed]).

To examine how glial secreted factors influence the synaptic transmission in these combined cultures, we performed the same voltage-clamp experiments, but in cells co-cultured with glia (**Fig. 5d and g**). We found that glial support did not strongly affect the frequency of IPSCs (Venus positive, 1.5 ± 0.32 Hz [19 cells, 4 cultures analyzed], $P > 0.05$; TdTomato positive neurons, 3.0 ± 0.58 Hz [15 cells, 4 cultures analyzed], $P > 0.05$) **(Fig. 5e)**, but instead significantly increased the frequency of EPSCs in both cell types (Venus positive: 6.5 ± 1.16 Hz [15 cells, 4 cultures analyzed], $P < 0.001$; TdTomato: 1.8 ± 0.40 Hz [14 cells, 4 cultures analyzed], $P < 0.05$, **Fig. 5g,h**). Surprisingly, in these co-cultures, glutamatergic neurons received a greater number IPSCs than GABAergic neurons (IPSC frequency, Glutamatergic>GABAergic, $P < 0.05$),

but less EPSCs (EPSC frequency, GABAergic>glutamatergic, $P < 0.01$).

Overall, our results support the idea that in mixed neuronal networks, glial secreted substances play an important role in the establishment of glutamatergic synaptic transmission. In stark contrast, GABAergic transmission onto both glutamatergic principal cells and GABAergic neurons is established even in the absence of glia cells.

Discussion (1592 words)

While methods for purifying glutamatergic neurons have existed for some time (Pfrieger and Barres 1997; Ullian et al. 2001, 2004), methods for establishing purified primary GABAergic cell cultures have only been partially successful (Baptista et al. 1994; Berghuis et al. 2004; Buard et al. 2010). This has led to a disparity in our knowledge of how these two major cell classes develop and establish neuronal networks. To reconcile this issue, we established methods for purifying and culturing GABAergic neurons *in vitro*. An analysis of the development of these cultures has revealed that similar to glutamatergic neurons, GABAergic neurons directly depend on glial secreted substances for their growth and long-term survival. However, in stark contrast to glutamatergic neurons, GABAergic neurons establish robust synaptic transmission even in the absence of glia (**Fig. 6**). This fundamental difference between the two dominant cortical neuron classes has important implications for our understanding of how neuronal networks develop. In particular, it may help to explain why GABAergic synaptic transmission is able to establish before glutamatergic synaptic transmission during early development (Gozlan and Ben-Ari 2003).

Glial secreted factors promote the survival and neurite development of purified GABAergic neurons, but not their aberrant electrical properties

As demonstrated here, we were able to establish pure and viable cell cultures composed of GABAergic neurons only, by FAC sorting fluorescent neurons from a transgenic rodent line expressing Venus under the control of the VGAT promoter (Uematsu et al. 2008). We find that these cultures are viable to at least 16 DIV, but that their long-term culture (to 42 DIV) is dependent on support from glial cells (**Fig. 2b**). We also find that

both axon and dendrite growth is strongly potentiated by glial support (**Fig. 2c-f**). Presumably, purified neurons lack trophic signals and/or metabolic support, which are required to sustain their growth and survival (Banker 1980; Engele et al, 1991; Magistretti 2006). Our results suggest that glial cells, through the release of secreted factors, are largely responsible for promoting the growth and survival of GABAergic neurons. Importantly, these results further emphasize the essential role played by glia in the development and maintenance of neuronal networks (Lindsay 1979; Banker 1980; Le Roux and Reh 1994, Hughes et al, 2010), but also specifically demonstrate the roles that glial cells play in the growth and survival of GABAergic neurons.

Interestingly, our results also reveal that, despite their vital importance, glial cells are not able to recover all aberrant changes that occur as a result of neuronal purification. One obvious example of this is the altered action potential kinetics (**Fig. 2c-e; Supplementary Fig. 2**) and lower than expected (given their small size) input resistance values measured from purified GABAergic neurons (**Fig. 1c and Fig. 2c**). Notably, these altered membrane properties, which are likely caused by an increase in open, “leak” ion channels in the membranes of purified GABAergic neurons, were not recovered by glial support. In fact, following glial support, which improves the growth of purified cells, glial supported GABAergic neurons were significantly less excitable than unsorted neurons (**Fig. 3e**). Their large size, coupled to an increase in their membrane conductance, may explain their markedly decreased excitability.

Presently, the exact mechanisms that regulates these altered membrane properties are not clear. However, given that glutamatergic neurons are absent from both purified and glial supported GABAergic cultures, it is possible that signaling from glutamatergic neurons may regulate these membranes properties. Indeed, glutamatergic

neurons have already been shown to shape the activity of neighbouring cells and to provide trophic support (Berghuis et al. 2004; Elmariah et al. 2005; Andreska et al. 2014; Chang et al. 2014). Future studies will be needed to determine the exact signalling mechanisms that lead to these changes. Nevertheless, what these results demonstrate is that, in addition to glial derived signals, other cell-to-cell interactions are required to correctly shape neuron development.

The establishment of Glutamatergic and GABAergic synaptic transmission differentially depend on glial secreted factors

Several reports have previously described how glial conditioned media and glial secreted factors (namely proteins) are able to directly promote synapse formation and synaptic transmission between glutamatergic neurons (Pfrieger and Barres 1997; Ullian et al. 2001; Christopherson et al. 2005; Pfrieger 2009; McKellar et al. 2009; Allen et al. 2012; but see Steinmetz et al, 2006). However, it has not been clear if the same holds true for GABAergic neurons. In recent years, several astrocyte secreted proteins have been identified which play a central role in directing glutamatergic synapse formation (for a review, see Chung et al, 2015). However, no astrocyte secreted proteins regulating the the development of GABAergic synapse formation have so far been identified. Interestingly, the thrombospondin (TSP) family of extracellular matrix (ECM) proteins, which play a critical role in the establishment of glutamatergic synapses, have little or no effect on the establishment of GABAergic synapses *in vitro* (Christopherson et al, 2005; Hughes et al, 2010), further indicating that GABAergic and glutamatergic neurons may differentially depend on glial signalling for synapse formation. Our results strongly support this notion. We find that while glutamatergic synaptic transmission is low or

absent in cultures lacking glial secreted factors, GABAergic synaptic transmission is nevertheless able to persist (**Fig. 6a**). Even when culturing glutamatergic and GABAergic neurons, we observed almost no glutamatergic synaptic transmission in the absence of glia, but we did observe robust GABAergic synaptic transmission regardless of the postsynaptic neuron type recorded. Importantly, when glial support was provided to mixed neuron cultures, glutamatergic synaptic transmission was dramatically increased, but GABAergic synaptic transmission was not further potentiated (**Fig. 6b**). Thus, we find that the presence of glial secreted factors correlates strongly to the level of glutamatergic synaptic transmission, but not GABAergic synaptic transmission. Further supporting these findings, is our observation that the local stimulation of purified GABAergic neurons produces evoked IPSCs of comparable amplitudes, even in the absence of glial support (**Fig. 4d and e**). Our results therefore indicate that glial secreted substances are important for the establishment of glutamatergic synaptic transmission, but are not essential for the establishment of GABAergic synaptic transmission in cortical networks.

For the same reason, it is also difficult to explain precisely why glial support leads to an increase in the number of synaptically coupled GABAergic neurons (seen in **Fig. 4f**). One possibility is that glial support directly promotes an increase in synapse formation. Alternatively, increased connectivity may simply be a consequence of increased survival and morphological development (**Fig. 2c-f**). It will be necessary, in future studies, to investigate whether glial secreted factors are able to directly modulate GABAergic synaptic transmission.

In addition, it may also be useful to focus on other recently identified astrocyte secreted proteins, such as glypican 4/6, Hevin and SPARC, which have been shown to

play key roles in the regulation of glutamatergic synapse formation (Chung et al, 2015), but have not yet been investigated with regards to GABAergic synapse formation and function. Importantly, our purified GABAergic cell cultures should prove to be a valuable tool for investigating this, in the same way purified glutamatergic neuronal cultures significantly aided in the identification of glypicans 4 and 6 as important synaptogenic protein (Allen et al, 2012). In the future, using a similar approach of protein fractionation and mass spectroscopy, as described by Allen et al, 2012, it may be possible to identify novel glial derived factors that also influence the development of GABAergic neurons.

In summary, our results support the idea that glia are important for the establishment of glutamatergic synaptic transmission (Segal 1991; Verderio et al. 1999; Christopherson et al. 2005; McKellar et al, 2009; Allen et al. 2012). They also provide important insights into the development of GABAergic neuronal networks, and help reconcile conflicting reports regarding the influence of glia on the establishment GABAergic synaptic transmission (Liu et al. 1996; Elmariah et al. 2005; Steinmetz et al. 2006; Kaczor et al. 2015).

Implications for network formation and function

Neuronal networks require a delicate balance of excitation and inhibition for their proper function, as uncontrolled excitation can lead to seizures, glutamate toxicity and cell death. To avoid this, developing cortical networks initially rely on GABA as their primary neurotransmitter (Ben-Ari 2001, 2002). While being both excitatory and inhibitory at different stages of development (Katz and Shatz 1996; Holmes and Ben-Ari 1998; Ben-Ari 2002), GABAergic transmission, in general, acts to stabilize cell excitability, as unlike glutamate receptors the reversal potential for $GABA_A$ receptors is close to the resting

membrane potential of most neurons (Staley and Mody 1992; Kaila 1994; Banke and McBain 2006). *In vitro* slice recordings made from mouse cortex and excised at embryonic or early postnatal stages, indicate that GABAergic synaptic transmission is established prior to glutamatergic synaptic transmission (Tyzio et al. 1999; Gozlan and Ben-Ari 2003; Ben-Ari et al. 2004), at a time when there are few mature astrocytes in the developing cortex (Qian et al. 2000; Yuasa 2001). The emergence of GABAergic synaptic transmission prior to glutamatergic transmission might be explained by the early maturation of GABAergic neurons (Gozlan and Ben-Ari 2003), consistent with the different developmental origins of the two cell types (Marín and Müller 2014). However, more recently, it has been suggested that GABAergic neurons, unlike glutamatergic neurons, may not require glial secreted signals to establish synapses (Steinmetz et al. 2006). This idea is now directly supported by our experimental findings and represents an attractive mechanism for ensuring the formation of GABAergic networks prior to glutamatergic networks during development. This mechanism requires three conditions: First, glutamatergic synapse formation depends on glial secreted factors (Ullian et al. 2001); second, glial proliferation and maturation occur late in embryonic development (Yuasa 2001); finally, GABAergic synapse formation is independent of glial support. These conditions provide GABAergic neurons with the opportunity to establish synaptic connections prior to glutamatergic neurons, during early development (Gozlan and Ben-Ari 2003).

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Figure legends:

Figure 1. Purified GABAergic cell cultures produced by FAC sorting fluorescent neurons from VGAT-Venus-A Wistar rats. (**a**) A schematic of the VGAT-Venus-A bacterial artificial chromosome (BAC) construct. GABAergic neurons express Venus under the Vesicular GABA transporter (VGAT) promoter. (**b**) A schematic of the FAC sorting procedure used to collect either GABAergic neurons (positive sort), or all cells (unsorted), from the neocortex and hippocampus of VGAT-Venus-A rats. (**c**) Representative fluorescence intensity plots of WT (left) or VGAT-Venus-A (right) dissociated cells. Fluorescence intensities were measured through emission filters optimized for either Venus (X-axis) or red fluorescent protein (RFP, Y-axis). Fluorescent Venus-positive cells were collected for positive-sorted cultures (polygon area, black arrow). (**d**) Confocal images (maximal Z-projection) of Neuronal Nuclei protein (NeuN, magenta pseudocolor) and Venus (green) immunofluorescence, in unsorted (left panel) and positive-sorted cultures (middle panel, both DIV 15). White arrows indicate GABAergic neurons expressing both NeuN and Venus. Scale bar: 20 µm. (**e**) Bar chart summarizing the proportion of Venus positive neurons in unsorted (3 cell culture batches analyzed) and positive-sorted cultures (6 cell culture batches analyzed). (**f**) Confocal images of glial fibrillary acidic protein (GFAP, magenta) and Venus (green) immunofluorescence in unsorted (left panel) and positive-sorted cultures (right panel, both DIV 16). White arrow indicates a GFAP-positive process. Scale bar: 50 µm. Mann-Whitney nonparametric test. Significance indicated: (***) = $P<0.001$.

Figure 2. Glia are necessary for the long-term survival and morphological development of purified GABAergic neurons. (**a**) Schematic illustration of the three culture conditions used to study the survival of GABAergic neurons: unsorted cell culture (left), positive-sorted cell culture (middle), and positive-sorted cell culture with glial support (right, glia to neuron ratio 4:1). Glia were grown separately in the same medium on permeable cell culture inserts. (**b**) Confocal images (maximal Z-projection) of Venus-immunofluorescence, at the indicated time points. White arrows indicate surviving neurons. (**c**) Representative reconstructions of biocytin-filled GABAergic neurons in each culture type (Axon: colored, Dendrites: black) at DIV 14 ± 2. (**d ,e**) Summary bar charts of the total length of the axon (**d**) and dendrites (**e**) in the three culture types. (**f**) Sholl plot summarizing dendritic arbor distribution for each culture type (Sholl radius interval: 25 μm). Measurements were made from ≥3 cell culture batches. Kruskal-Wallis nonparametric test with Dunn's post test. Significance indicated: (*) = $P<0.05$, (**) = $P<0.01$, (***) = $P<0.001$.

Figure 3. Electrophysiological properties of cultured GABAergic neurons. (**a**) Representative examples of the recorded neurons from the three culture types. On the left, composite images of the IR-oblique illumination (grey) and fluorescent Venus signal (yellow) of the recorded GABAergic neurons. On the right, voltage responses of the neurons to hyperpolarizing and depolarizing current pulses (hyperpolarizing pulses: -200 pA to -20 pA in 20 pA steps, depolarizing pulses: +20 pA and +200 pA, 500 ms duration). The inset to the right shows the voltage response of the same GABAergic neuron to a 1000 pA current step in the glial supported culture. (**b-d**) Summary bar charts of the intrinsic electrophysiological properties, including: resting membrane

potential (Vm, **b**), whole cell capacitance (Cm, **c**) and input resistance (Ri, **d**). (**e**) Current-frequency plot of neurons recorded in the three culture types. The neurons were kept at resting membrane potential and injected with depolarizing current pulses (20 pA incremental current steps, 500 ms duration). Measurements were made from ≥6 cell culture batches. Unsorted: n = 80; positive sort: n = 66; glial support: n = 22. Means are ± S.D. Kruskal-Wallis nonparametric test with Dunn's post test. Significance indicated: (*) = P<0.05, (**) = P<0.01, (***) = P<0.001.

Figure 4. Purified GABAergic neurons establish synaptic transmission in the absence of glia. (**a**) Representative voltage-clamp recordings of spontaneous synaptic events (60 second duration) from unsorted (top), positive-sorted (middle), and glial supported cultures (bottom). At a holding potential (Vh) of 0 mV. (**b**) Summary bar chart of the peak amplitude of IPSCs recorded from GABAergic neurons in each cell culture type. Measurements were made from ≥3 cell culture batches. (**c**) Evoked IPSCs traces (averages of ≥12 individual responses each). Stimulus artifacts have been removed to aid visualization. (**d, e**) Bar charts summarizing mean eIPSC amplitudes (**d**) and the proportion of connected cells (**e**). Measurements were made from 3 cell culture batches. Statistics: Kruskal-Wallis nonparametric test with Dunn's post test (b and d) and Fisher's exact test (e). Significance indicated: (*) = P<0.05, (**) = P<0.01, (***) = P<0.001.

Figure 5. Glial secreted factors promote glutamatergic, but not GABAergic synaptic transmission. (**a**) A schematic of the "mixed" neuron culture configuration used to study the influence of glia on glutamatergic and GABAergic synaptic transmission. FAC sorted tdTomato-positive neurons (magenta) and Venus-positive neurons (yellow) were

cultured at a ratio of 4:1, in the presence or absence of glia (grey). (**b**) Fluorescent images of endogenous Venus expression (left), tdTomato expression (middle) and a merged composite (right) at DIV 0. (**c,d,f,g**) Representative voltage-clamp recordings (60 seconds) from neurons in “mixed” cell cultures in the absence (**c,f**) or presence of glia (**d,g**) at a holding potentials of 0 mV (**c,d**) to record IPSCs or -50 mV to record EPSCs (**f,g**). Hash symbols (#) highlight the presence of excitatory events. The patched cell type (tdTomato or Venus) is indicated in brackets below the recording trace. (**e, h**) Summary bar charts of the frequency of IPSCs (**e**) and EPSCs (**h**) recorded from each culture configuration. Measurements were made from 4 cell culture batches. Kruskal-Wallis nonparametric test with Dunn’s post test. Significance (*) = $P<0.05$, (**) = $P<0.01$, (***) = $P<0.001$.

Figure 6. Glial secreted factors are required for the establishment of functional glutamatergic synapses, but not for GABAergic synapses. (**a**) Schematic drawing illustrates GABAergic (yellow) and glutamatergic (magenta) neurons in the absence of glial secreted factors. In this configuration neuron growth is limited, and synaptic transmission is only established at GABAergic synapses. (**b**) Schematic showing the influence of glial secreted factors on the development of GABAergic and glutamatergic neurons. In this configuration, both neuron classes have improved neurite growth and survival, and synaptic transmission is established at both GABAergic and glutamatergic synapses.