Selective, state-dependent activation of somatostatin-expressing inhibitory interneurons in mouse neocortex

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ABSTRACT

The specific functions of subtypes of cortical inhibitory neurons are not well understood. This is due in part to a dearth of information about the behaviors of interneurons under conditions when the surrounding circuit is in an active state. We investigated the firing behavior of a subset of inhibitory interneurons, identified using mice that express green fluorescent protein (GFP) in a subset of somatostatin-expressing inhibitory cells (“GFP-expressing inhibitory neurons”, GIN cells). The somata of the GIN cells were in layer 2/3 of somatosensory cortex, and had dense, layer 1-projecting axons that are characteristic of Martinotti neurons. Interestingly, GIN cells fired similarly during a variety of diverse activating conditions: when bathed in fluids with low-divalent cation concentrations, when stimulated with brief trains of local synaptic inputs, when exposed to group I metabotropic glutamate receptor agonists, or when exposed to muscarinic cholinergic receptor agonists. During these manipulations, GIN cells fired rhythmically and persistently in the theta frequency range (3-10 Hz). Synchronous firing was often observed and its strength was directly proportional to the magnitude of electrical coupling between GIN cells. These effects were cell type-specific: the four manipulations that persistently activated GIN cells rarely caused spiking of regular-spiking (RS) pyramidal cells or fast-spiking (FS) inhibitory interneurons. Our results suggest that supragranular GIN interneurons form an electrically coupled network that exerts a coherent 3-10 Hz inhibitory influence on its targets. Because GIN cells are more readily activated than RS and FS cells, it is possible that they act as “first responders” when cortical excitatory activity increases.
INTRODUCTION

Inhibitory interneurons of the neocortex are diverse, and they have been implicated in many distinct cortical functions. Inhibition plays important roles in the feedforward control of sensory information (Cruikshank et al. 2007; Simons 1978; Simons and Carvell 1989; Swadlow et al. 1998; Swadlow and Gusev 2000), the overall control of cortical tone (Chagnac-Amitai and Connors 1989), the generation of oscillatory activity at a range of frequencies (Beierlein et al. 2000; Blatow et al. 2003; Whittington et al. 1995), and synchronization of spiking in excitatory pyramidal neurons (Cobb et al. 1995; Long et al. 2005). However, it has been very difficult to assign particular cortical functions to specific interneuron types.

To understand the roles of subsets of inhibitory neurons, we will need to know more about how these cells fire under active states of the cortex and their different neurochemical milieus. In slices of neocortex under standard conditions, cells of all types are largely quiescent; the network is in an inactive state. In awake, behaving animals cortical cells generally display spontaneous firing; the network is in a more active state. It is critical to know how interneurons fire under activated conditions because this provides a context for interpreting the functions of their dynamic properties. For example, recent work has shown that somatostatin-expressing Martinotti cells, similar to the cells we studied here, are powerfully excited by synaptic contacts from repetitively firing pyramidal cells; the Martinotti cells can in turn generate powerful inhibition on those pyramidal cells (Kapfer et al. 2007b; Silberberg and Markram 2007). These findings lead directly to an important question about Martinotti cells: what are their naturalistic firing properties and relevant synaptic dynamics?
Here, we investigated the firing properties and synaptic dynamics of a specific subtype of neocortical inhibitory neuron identified by its expression of green fluorescent protein (GFP) in the cerebral cortex of a transgenic mouse line, known as the GIN line (Oliva et al. 2000), which express GFP in a subset of somatostatin-positive inhibitory neurons. In order to characterize the activity profile of the GIN cells, we examined them under a variety of neurochemical and physiological conditions. Our data show that GIN cells of neocortical layer 2/3, but typically not other major neuron types, are readily activated by diverse manipulations, fire persistently and rhythmically when active, and can generate a sustained inhibitory influence on their targets. These characteristics suggest that GIN cells are activated steadily during states of enhanced cortical firing, and could act as an important regulator of normal and/or pathological cortical activity.

METHODS

Slice preparation: Thalamocortical slices from the mouse somatosensory cortex were prepared as previously described (Agmon and Connors 1991) using tissue from the GIN strain of mice (Jackson Labs, Bar Harbor, ME; (Oliva et al. 2000)). Animals were aged postnatal days 13-18, and included both sexes. All procedures were conducted in accordance with the animal use regulations and approval of the IACUC of Brown University. Tissue was sliced in artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 26 NaHCO₃, 10 dextrose, and 2 CaCl₂, saturated with 95%O₂/5%CO₂. Slices were stored in ACSF of the same composition at 32°C for 30-45 min, then maintained at room temperature until used for recording. Slices were 400 µm thick.
**Recordings:** Whole-cell current clamp recordings were performed using micropipettes (4-7 MΩ) filled with internal solution containing (in mM): 135 K-gluconate, 4 KCl, 2 NaCl, 10 HEPES, 0.2 EGTA, 4 ATP-Mg, 0.3 GTP-Tris, and 14 phosphocreatine-Tris (pH 7.25, 280 mOsm). Membrane potentials reported here are not corrected for the liquid junction potential. In order to detect IPSPs, some recordings were performed with a high [Cl⁻] solution in the pipettes (same as the internal solution above except with 0 mM K-gluconate and 130 mM KCl). Recordings were conducted at 32°C. When patching, cell attached seal resistances were > 1 GΩ, and series resistance after achieving whole-cell configuration was between 5-25 MΩ. Data were collected using an Axoclamp 2B amplifier, and an in-house data collection program written in Labview (National Instruments Corporation, Austin, TX; J. Gibson). Data were analyzed using in-house programs written in Labview (J. Gibson) and Matlab (Mathworks, Natick, MA; E. Fanselow).

**Cell visualization and identification:** Cells were viewed under infrared-differential interference contrast (IR-DIC) illumination using a Nikon E-600FN microscope and a Dage IR-1000 CCD-camera. GIN cells were identified by visualization of GFP under epifluorescence illumination. In addition, when injected with 600 ms suprathreshold current steps, these cells showed spike rate adaptation and the first afterhyperpolarization (AHP) in a train of spikes was the largest in the train (Beierlein et al. 2003). In some, but not all, cases these cells demonstrated an I₈ current-induced sag in the voltage trajectory during hyperpolarizing current steps (87%, n = 100 cells). In contrast, inhibitory fast-spiking cells did not express GFP in these mice, and did not
show spike-rate adaptation during suprathreshold current steps. RS cells, the excitatory pyramidal cells, also did not express GFP in these mice, showed adapting spike firing rates during supra-threshold current steps, and during a train of action potentials the first AHP was smaller than subsequent AHPs (Beierlein et al. 2003). These criteria for classifying neurons are the same as those described by Beierlein et al. (2003). In addition, the action potential widths differed among these cell types, as reported in the Results.

**Extracellular stimulation:** Trains of extracellular stimuli were applied using a bipolar stimulating electrode (Fredrick Haer, Bowdoinham, ME) placed in the tissue 100-200 µm ventral to the cell bodies being recorded (i.e. closer to the white matter), in either layer 4 or lower layer 2/3. Stimulus trains consisted of 20 pulses, presented at 1-500 Hz, with intensities ranging from 5-90 µA. Pulses were monophasic with a duration of 200 µsec.

**Pharmacological agents:** Drugs used in this study included DL-2-amino-5-phosphonovaleric acid (APV; Sigma), carbamylcholine chloride (carbachol; Sigma), (S)-3,5-dihydroxyphenylglycine hydrate (DHPG; Sigma), 6,7-dinitroquinoxaline-2,3(1H, 4H)-dione (DNQX; Sigma), SR 95531 hydrobromide (gabazine; Tocris), LY367385 (Tocris), (±) muscarine chloride (Sigma), and picrotoxin (PTX, Sigma).

**Biocytin staining:** Thirty GIN cells were filled with biocytin (0.2-0.4% in internal solution) in order to assess cell morphology. Cells were recorded for an average of 30 min. The electrode was then carefully removed and the slice was put into fixative. To process the
biocytin, 350-400 μm slices were fixed in 4% paraformaldehyde /0.1 M phosphate buffer (PB) 7.4 pH for 48 hours followed by immersion in 30% sucrose/4% paraformaldehyde /0.1 M PB for several days for cryoprotection. All slices were then resectioned at 80 μm. Tissue was washed in 0.1 M PB 3 times followed by incubation in 0.5% H₂O₂/0.1 M PB for 45 min to remove endogenous peroxidases. Tissue was rinsed in 0.1 M phosphate buffered saline (PBS) 2 times then rinsed once in PBS. Following the rinses, sections were incubated in 0.25% Triton/ PBS for 45 min followed by a 5 min rinse in 0.1% Tween/PBS prior to incubation overnight at 4°C in ABC kit (Vector, Burlingame, CA). The following day, tissue was rinsed 3 times in 0.1% Tween/PBS followed by 1 rinse in 50 mM Tris buffer, pH 7.4. Sections were presoaked in 0.05% DAB/0.3% nickel ammonium sulfate/Tris buffer for 20 min, then transferred to a solution of 0.05% DAB/0.3% nickel ammonium sulfate/Tris buffer also containing H₂O₂ (using the following ratio: 0.2 μl 30% H₂O₂/ml DAB/Ni/Tris solution) solution for visualization of biocytin. The reaction was stopped by several rinses in PB and slices were mounted from dilute PB (0.01 M PBS), dehydrated with increasing alcohols ending with xylenes and cover slipped using Krystalon (EM Science, Gibbstown, NJ).

After histological processing, photographs were taken of the filled cells in the slices. This was done by taking 20-25 serial images of a given cell in different focal planes, processing each image in Photoshop to remove the blurred regions in each focal plane, then performing a z-stack of all planes using Matlab to determine the minimum intensity at each pixel in RGB-mode. In addition, the contrast was adjusted in Matlab. The final figure was converted to grayscale and processed using Illustrator. After physiological recordings, low magnification pictures of the slices were taken using a Zeiss Axioscope...
microscope with a 2.5x objective, a Hammamatsu c2400 CCD camera and Winnov video capture software.

*Analysis methods:* The degree of electrotonic coupling between GIN cells was determined by applying twenty current steps (-100 pA) to each cell of a pair while measuring the resulting voltage deflections in the other cell. These twenty traces were averaged and the coupling coefficient was calculated as the ratio of the voltage deflection in the nonstimulated cell to the voltage deflection in the stimulated cell. The coupling coefficient was measured in both directions (i.e. each cell was used separately as the stimulated and nonstimulated cell) and the reported coupling coefficient was the average of these two values.

To quantify the degree of spiking synchrony between electrically coupled neurons, we calculated cross-correlograms of action potential times for 1 min segments of firing from pairs of simultaneously recorded GIN cells. The cross-correlograms were normalized to the number of action potentials in the index cell and binned at 20 ms. In addition, to determine which cross-correlation peaks were statistically significant, we shuffled the action potentials 100 times within a 1 min trace (in 3 sec segments) and calculated the cross correlation of these shuffled trials. Cross-correlation values were considered statistically significant if they were more than 3 standard deviations above the shuffled trial values calculated over the 20 ms before and after the central cross-correlation peak. Then, we calculated the cross-correlation index as the magnitude of the central peak in the cross-correlation, minus the value of the shuffled trials for that 1 min segment of data.
To assess whether IPSPs in RS cells were triggered directly by GIN neurons during DHPG application, we calculated average traces of RS cell activity, triggered by action potentials occurring in a presynaptic GIN cell. We also created control traces by “shuffling” the data; that is, we used action potential times from one 30 sec segment of data to trigger average traces in a subsequent 30 sec segment of data.

Action potential thresholds were determined by finding the average voltage at which the 2nd derivative of the trace was maximal, within 5 ms preceding the action potential peak.

For statistical comparisons, an ANOVA was performed for each data set and t-tests were used for post-hoc pair-wise comparisons. Results are reported as mean ± standard error of the mean.

RESULTS

Characteristics of GIN cells

We filled a subset of our GIN cells (n = 30) with biocytin. A reconstruction of a typical GIN cell, showing the dendritic and axonal arbors, is shown in Fig. 1C. The dendritic patterns were either multipolar (23/30) or bitufted (Fig. 1A, C, D). The recording sites of the cells in Fig. 1A and D are shown in Fig. 1B and E, respectively. The average soma size was 11.0 ± 1.2 µm in the shortest dimension and 24.3 ± 7.7 µm in the longest dimension. The average number of dendritic processes emanating from each soma was 5.3 ± 0.5. The laminar distribution of GIN cells recorded in this study is shown in
Fig. 1F. The morphology of these cells (ovoid, vertically-oriented somata, multiple dendritic branches from the soma) was similar to those shown by Amitai et al. (2002) to be somatostatin-positive (SS+) cells in the rat neocortex. Amitai et al. (2002) also showed that these SS+ cells are distributed throughout layers 2-6, with the highest density in layer 5. Because the GIN cells we observed here were distributed largely in layer 2/3, but were not found as frequently in other layers, it is likely that they represent a subset of the SS+ cells reported by these authors.

In cells with successfully filled axons (n = 16/30), the axons projected densely to layer I (Fig. 1C), as Ma et al., (2006) also observed for GIN cells. Other cells either did not have an axon projecting to layer I, or the axonal destination could not be determined due to inadequate filling or high background staining. Such morphology is associated with Martinotti cells in the neocortex, which have been shown to be somatostatin positive (Ma et al. 2006), show spike rate adaptation (Ma et al. 2006) and have been implicated in robust intracortical inhibition (Kapfer et al. 2007a; Silberberg and Markram 2007). The 16 layer I-projecting neurons in our sample included both multipolar and bitufted cells, but there was no correlation between soma-dendritic patterns and axonal projections to layer I.

The injection of subthreshold and suprathreshold current steps allowed us to identify several characteristics that differentiated GIN cells from RS and FS cells. When we plotted the cumulative probabilities of threshold current levels for each cell type, it was clear that GIN cells were much more sensitive to stimuli than either FS or RS cells, as substantially less current was required to elicit action potentials (Fig. 2A). Average
thresholds for the first spikes were lowest in GIN cells (GIN: $44 \pm 2.6$ pA, FS: $248 \pm 18$ pA, RS: $93 \pm 5$ pA; all values significantly different from one another, $p < 0.0001$). In addition, the gain of the current-frequency relationship was lowest in RS cells (Fig 2B; slope = 0.09 Hz/ pA step) calculated for all RS values. The gain for FS cells (slope = 0.28 Hz/pA step; calculated for all FS values) and for the initial current steps that elicited action potentials in GIN cells (slope = 0.31 Hz/pA step; calculated for first 6 values in GIN cell data) were similar. With stronger current steps, GIN cell firing reached a maximum steady frequency of about 50 Hz. Their firing rates under the other activating conditions described in this paper (see below) were considerably slower. It should be noted that the magnitudes of the current steps used to create Fig. 2B began at 20 pA, and were incremented by 20 pA. Thus, they do not accurately reflect the absolute minimum firing rates of these cells. When current steps were presented that were carefully adjusted to be just-suprathreshold, GIN cells typically fired only once (data not shown). As the current was gradually increased from the just-suprathreshold level, multiple action potentials could be evoked. When using the minimum current for each cell that evoked multiple action potentials, the average firing frequency was 4.3±0.6 Hz (SEM), with a minimum of 1.9 Hz, and a maximum of 14.9 Hz ($n = 11$ cells).

With just-suprathreshold current pulses, FS cells often displayed a considerable delay before the first spike, whereas GIN cells did not (cf. Figs. 2C and D, upper panels). In addition, GIN cells often displayed an after-depolarization (ADP) following low frequency action potentials (Fig. 2C, inset; c.f. Halabisky et al., 2006). At higher levels of stimulus current, spike frequency adaptation was evident in GIN cells (Fig. 2D, lower panel), but not in FS cells (Fig. 2D, lower panel). Finally, at higher stimulus currents, the peak of
the first action potential in GIN cells was the most positive in the train, and the trough of
the first afterhyperpolarization (AHP) was the most negative (Fig. 2C, lower panel). By
contrast, the action potential heights and AHP magnitudes of FS cells changed little
under similar conditions (Fig. 2D, lower panel). The firing of GIN cells also differed from
that of RS cells, whose second action potential peak was substantially more negative
than the first, and whose first AHP was the most positive in the train (not shown; cf.
(Beierlein et al. 2003)). Finally, the mean widths of the action potentials measured at
half-height of the action potential were: GIN, 0.85 ± 0.02 ms; FS, 0.51 ±0.03 ms; RS,
1.39 ± 0.03 ms. These values are all larger than those reported by Beierlein et al.
(2003), which could be due to differences between layers 2/3 and 4. GIN cells share
many characteristics with inhibitory interneurons identified in other studies as bitufted
cells (Reyes et al. 1998), regular-spiking nonpyramidal somatostatin-expressing
neurons (Cauli et al. 2000) and LTS (low threshold-spiking) cells (Amitai et al. 2002;

Three intrinsic characteristics of GIN cells tend to make them more excitable than RS
and FS cells. First, as described above, GIN cells had lower firing thresholds in
response to injected current. Second, the resting membrane potential of GIN cells was
more depolarized than that of the other cell types (GIN: -56.6 ± 0.5 mV, Fig. 2E, H
arrow and dotted line; n = 45 cells; range: -65.0 to -50.6 mV; RS: -67.0 ± 1.0 mV, Fig.
2F, H arrow; n = 23 cells; range: -73.1 to -55.5 mV; FS: -64.1 ± 1.0 mV, Fig. 2G, H
arrow; n = 12 cells; range: -68.9 to -59.6 mV). The average resting membrane potentials
of all three cell types were significantly different from one another (p < 0.03). Third, GIN
cells had the most negative action potential threshold of the three neuron types (GIN: -
43.0 ± 0.4 mV; Fig. 2E, H; n = 45 cells; range = -49.1 to -35.3 mV; RS: -39.3 ± 0.6 mV; Fig. 2F, H; n = 23 cells; range = -43.4 to -32.9 mV; FS: -36.2 ± 0.7 mV; Fig. 2G, H; n = 12 cells; range: -41.7 to -33.6 mV). All three conditions were significantly different from one another (p < 0.01). Because of these factors, GIN cells at rest are substantially closer to their firing threshold than either RS cells or FS cells, and they are more responsive to stimulus currents. However, it should be noted that GIN cells do not fire spontaneously when at their resting membrane potential.

Dynamic responses of GIN→RS and RS→GIN synapses

During paired recordings, spikes in GIN cells triggered inhibitory postsynaptic potentials (IPSPs) in monosynaptically connected RS target cells (Fig. 3A; (Beierlein et al. 2003)). To make the IPSPs more visible, their reversal potentials were shifted more positive with high [Cl⁻] internal solutions in some experiments. During presynaptic stimulus trains, IPSPs from GIN→RS synapses displayed short-term depression at frequencies from 3-40 Hz (Fig. 3A,C). Spikes in RS cells often triggered excitatory PSPs (EPSPs) in monosynaptically connected GIN cells (Fig. 3B). In contrast to the IPSPs of GIN→RS connections, the EPSPs of RS→GIN connections showed substantial frequency-dependent facilitation in the 3-40 Hz frequency range (Fig. 3B,D; (Beierlein et al. 2003; Reyes et al. 1998)).

Responses of GIN, RS and FS cells to low-divalent ACSF

The standard ACSF used in many electrophysiological studies of brain slice preparations (including this one) contains 2 mM Ca²⁺ and 2 mM Mg²⁺. However, biological CSF and the extracellular fluid that bathes central neurons in vivo contains
concentrations of about 1 mM of each of these divalent cations, (Somjen 2004). When the divalent ion concentrations of the extracellular fluid are reduced, neurons tend to become more excitable (Frankenhaeuser and Hodgkin 1957) and slices of neocortex are much more likely to generate spontaneous, network-dependent activity (Sanchez-Vives and McCormick 2000; Shu et al. 2003). In order to study GIN cells under more natural conditions of activity, we lowered the [Ca\(^{2+}\)] and [Mg\(^{2+}\)] of our ACSF to 1 mM ("low-divalent ACSF"; other components of the ACSF were the same as described in the Methods section).

Bathing slices in low-divalent ACSF had several effects on layer 2/3 cells. First, 76.3% of GIN cells depolarized and fired for as long as the low-divalent ACSF was applied to the slice (Fig. 4A,B). This effect was reversible; when low-divalent ACSF was washed off, GIN cells ceased their firing. Only 9.0% of RS cells and no FS cells fired spontaneously during low-divalent ACSF application (Fig. 4C). During application of low-divalent ACSF, RS cells that did not fire depolarized an average of 3.9 ± 1.6 mV, and FS cells hyperpolarized an average of 1.4 ± 0.8 mV. GIN cells that did not fire depolarized an average of 3.5 ± 2.4 mV during application of low-divalent ACSF.

The steady-state firing rate of GIN cells (averaged for each of three 1 min segments from 3-6 min after application of low-divalent ACSF began) was 3.1 ± 0.1 Hz (n = 23 cells; range = 2.0-3.9 Hz; Fig. 4B). This persistent firing was not prevented by blockers of fast synaptic transmission (DNQX, 20 µM, to block AMPA receptors; APV, 50 µM, to block NMDA receptors; PTX, 20 µM, to block GABA\(_A\) receptors; n = 15 cells, data not shown), suggesting that such firing did not require chemical synaptic connections within
the local circuit. In addition to inducing the persistent firing of GIN cells described here, low-divalent ACSF caused sudden, brief depolarizations ("UP-states"; (Sanchez-Vives and McCormick 2000; Shu et al. 2003) in all three types of cells (Fanselow and Connors 2005). UP-state activity was excluded from all of the analyses we report here.

**Electrical coupling and synchronous firing among GIN cells**

Somatostatin-expressing interneurons are extensively coupled by gap junctions in neocortex (Amitai et al. 2002; Gibson et al. 1999). We found that GIN cells can also be electrically coupled to other GIN cells (n = 44/67 pairs, average coupling coefficient for coupled pairs was 0.05 ± 0.01; a pair was considered electrically coupled if the average coupling coefficient was ≥ 0.01). Thus, GIN cells, which appear based on morphology to be Martinotti cells in many cases, are often coupled to other Martinotti cells in layer 2/3 of the mouse somatosensory cortex. GIN cells were never found to be electrically connected to any other cell type (pairs tested RS-GIN: n = 0/28; FS-GIN: n = 0/11). The electrical synapses between inhibitory interneurons are capable of mediating robust spike synchrony (Mancilla et al. 2007). In order to assess the degree of synchrony between electrically coupled GIN cells under conditions of spontaneous network firing, we calculated a cross-correlation index for each coupled pair and selected only those pairs that yielded statistically significant cross correlation scores for further analysis (n = 9/17 pairs had significant cross correlation scores; see Methods). The average of the significant cross-correlation scores was 0.15±0.03, (range = 0.05-0.28), and the range of coupling coefficients from pairs that yielded significant cross correlation scores was 0.02-0.21. Because our sample of coupled pairs for each activating condition was small, we pooled the pairs across conditions (low-divalent ACSF, extracellular stimulus...
trains, DHPG and cholinergic agonists; see below) for this purpose. When the cross-correlation scores were plotted as a function of coupling coefficient there was a strong linear relationship ($R = 0.85$, $p = 0.003$, $n = 9$ pairs, Fig. 5). Thus, the higher the coupling coefficient between a pair of cells, the higher the likelihood that the cells involved would fire synchronously when activated. Examples of synchrony and the associated cross-correlogram of GIN cell pairs during low-divalent ACSF, after extracellular stimulation, during DHPG, and carbachol activation are shown in Figs. 4D and E, 6E and F, 8D and E and 9D and E, respectively.

**Responses of GIN, RS, and FS cells to repetitive synaptic input**

When intracortical pathways were tetanically stimulated (20 stimuli, 100 Hz), 89% (24/27) of GIN cells generated the following response sequence: 1) a short latency period of excitation during the stimulation, during which cells fired action potentials, followed by 2) a period of inhibition (average duration: $5.4 \pm 0.5$ sec), during which the cells hyperpolarized by an average of $10$ mV and did not fire, and then 3) a period of persistent depolarization (average $6.1$ mV; range = 1.9-9.9 mV) during which the cells fired rhythmically for tens of seconds (average duration for 90% of spikes after stimulus was presented: $32.15 \pm 2.06$ sec, Fig. 6A, B). The average firing rate of GIN cells during persistent activity following extracellular stimulation was $3.1 \pm 0.5$ Hz (range = 1.3-6.0 Hz). In contrast, following similar stimulus trains, RS and FS cells fired during the stimulus train, then occasionally fired briefly following the stimuli (data not shown), but remained silent during the period of persistent firing observed in GIN cells (persistent firing was not observed in 25 RS and 5 FS cells; Fig. 6C). Fig. 6D, E and F show that the persistent firing of GIN cells evoked by synaptic input trains was rhythmic as well as
synchronous among electrically coupled GIN cells; this is similar to the effect of low-divalent ACSF.

The synaptically evoked persistent activity of GIN cells, as well as the depolarization associated with it, were not blocked by antagonists of AMPA (DNQX, 20 µM) NMDA, (APV, 50 µM) and GABA\(_A\) (PTX, 20 µM) receptors (Fig. 7 A,B; \(n = 13\) cells). These antagonists did block the initial firing and subsequent hyperpolarization that normally occur during and after the stimulation, respectively, indicating that these two components are mediated by AMPA, NMDA and GABA\(_A\) receptors. Both the persistent activity and the depolarization, however, could be blocked reversibly by the specific mGluR1a antagonist LY367385 (200 µM; Fig. 7 C-E; \(n = 6/8\) cells). This suggests that the depolarization and persistent firing were mediated, at least in part, by activation of mGluR1a receptors following brief trains of synaptic input.

**Responses of GIN cells to application of DHPG**

When the group I mGluR agonist, DHPG, was applied to the bathing solution (0.25-100 µM), 95% of GIN cells (\(n = 19/20\)) fired rhythmically and persistently (Fig. 8A-C). This effect was reversible. During DHPG application, few RS cells (14%; \(n = 2/14\)), and no FS cells (\(n = 0/1\)) fired (Fig. 8C). When we recorded from electrically coupled pairs of neighboring GIN cells during DHPG application, their activity was synchronous (e.g. Fig. 8D,E). The average firing rate during DHPG application from 3-6 min after application and across concentrations was \(5.8 \pm 0.5\) Hz (range: 0.1-21.2 Hz; \(n = 19\) cells).
The firing frequency of GIN cells was somewhat dependent upon the concentration of DHPG, but reached median firing levels at ≥2 µM DHPG (Fig. 10A-C). Interestingly, however, median firing rates tended to be either 0 Hz or between 4.3-8.9 Hz. This suggests that the persistent activity of GIN cells was bistable; cells were either silent or firing in the range of about 4-9 Hz (Fig. 10 A-B). The steady-state firing rates of GIN cells during DHPG application (3-6 min following application of each different concentration) are plotted in Fig. 10A-B. The spike-triggered average IPSP in an RS cell was calculated while evoking spikes in a GIN cell at 40 Hz under control conditions (Fig. 8G, thick black line); the spike-triggered average IPSP evoked by GIN cell spikes during DHPG application is similar (Fig. 8G, thin black line). In contrast, when averages were calculated from shuffled data (see Methods), no IPSPs were evident (grey line in Fig. 8G).

**Responses of GIN cells to stimulation of cholinergic receptors**

We also applied two cholinergic agonists, carbachol (1-20 µM) and muscarine (0.25-100 µM) separately to gauge their effect on GIN cell firing. During application of either agonist, nearly all GIN cells fired persistently (carbachol n = 17/18, muscarine n = 9/9; Fig. 9 A,B,C), whereas RS cells did not (carbachol n = 0/4, muscarine = 0/1; FS cells not tested). The average firing frequency during muscarine application was 7.4 ± 0.2 Hz (range = 0.2-16.5 Hz, n = 8 cells). Synchrony between coupled GIN cells was also observed during application of carbachol (Fig. 9D-E). Firing rates during muscarine application depended mildly on concentration, reaching maximum median values around 0.5 µM (Fig 10C-D). The average steady-state firing rates for GIN cells during
muscarine application (3-6 min following each concentration presented) are shown in Fig. 10F.

**DISCUSSION**

Our study showed that GIN cells, a subset of somatostatin-expressing, dendrite-targeting inhibitory interneurons, fired rhythmically and persistently in the theta frequency range during several mechanistically diverse conditions that activated the neocortex. These conditions included low-divalent ACSF (which increases the intrinsic excitability of excitable membranes presumably due to surface charge effects; (Frankenhaeuser and Hodgkin 1957; Somjen 2004)), trains of local synaptic input (which activate neuromodulatory systems mediated by mGluRs and other receptors), and direct activation of mGluR1a or muscarinic cholinergic receptors with agonists. In contrast, neither pyramidal (RS) cells nor the major type of inhibitory interneuron (FS cells) typically fired under these conditions. The high sensitivity of GIN cells is due, at least in part, to their relatively depolarized resting membrane potential and relatively negative spike threshold, compared to RS and FS cells.

*Identity of GIN cells*

Based on the fact that many GIN cells have dense axonal projections to layer I (Halabisky et al. 2006; Ma et al. 2006); this study), they can generally be categorized morphologically as Martinotti cells, though we did find some GIN cells that did not project to layer I. In addition, these cells share multiple characteristics with other
described Martinotti cells, including being somatostatin expressing (Halabisky et al. 2006; Ma et al. 2006; Oliva et al. 2000; Wang et al. 2004; Xu et al. 2006), receiving facilitating input from pyramidal cells (Silberberg and Markram 2007), and showing spike rate adaptation (Silberberg and Markram 2007; Wang et al. 2004). The cells discussed here were often electrically coupled with one another via gap junctions. While the connexin(s) responsible for coupling in GIN cells is unknown, it is likely that connexin36 is involved, as previous studies have shown connexin36 to be responsible for electrical coupling in similar inhibitory interneurons in the neocortex (Deans et al. 2001).

**GIN cells have preferred persistent firing rates around the theta range**

Because GIN cells responded similarly to a range of activating conditions, they may constitute a generic theta-frequency inhibitory mechanism that operates under a variety of normal cortical conditions. Under all of the activating conditions used in this paper, GIN cells fired the large majority of their spikes at 3-10 Hz, approximately the theta frequency range. However, these rates are not limited by the intrinsic dynamic range of GIN neurons. When provoked with even modest levels of injected current, GIN cells could fire at sustained rates up to ~50 Hz, showing that GIN cells are *capable* of readily firing at high rates and had not simply topped out at their maximum firing rates during the activating manipulations used here. Instead, under relatively natural activating conditions they settled into a preferred rate within the theta frequency range.

Theta frequency oscillations have been observed in the neocortex both *in vitro* (Bao and Wu 2003; Flint and Connors 1996; Lukatch and Maclver 1997; Silva et al. 1991) and *in*
vivo (Canolty et al. 2006; Kahana et al. 2001; Raghavachari et al. 2006) and have been proposed to play a role in functions such as working memory and synaptic plasticity (Kahana et al. 2001; Raghavachari et al. 2006; Werk and Chapman 2003). However, the mechanism(s) that generate neocortical, as well as hippocampal theta rhythms are not well elucidated (reviewed in (Buzsaki 2002)) and our results suggest GIN cell activity could be a novel mechanism for inducing or supporting theta oscillations by exerting a network-wide inhibitory theta-range rhythm. Because GIN cells are extensively electrically coupled to one another (Amitai et al. 2002; Gibson et al. 1999), their coherence could be widespread (Beierlein et al. 2000), supporting long-range coordination of theta-range oscillatory firing. Another subtype of inhibitory interneurons, the parvalbumin-expressing multipolar bursting cells, may also play a role in theta oscillations as they fire at a theta frequency during application of carbachol (Blatow et al. 2003).

A second implication of our study is that because GIN cells were more readily activated than were RS and FS cells, GIN cells may act as “first responders” that activate when there are large, relatively sustained increases in cortical excitatory activity. In this way, the GIN cell network may act as a negative feedback loop to control cortical firing.

**Effects of synapse dynamics on the role of GIN cells**

In order to understand how a given subset of interneurons responds during normal activity within a circuit, it is critical to take into account how synaptic dynamics affect firing properties. The dichotomy in synaptic dynamics between FS and GIN cells suggests that these inhibitory interneurons react best to opposite types of presynaptic
firing and act as differential filters for neocortical activity (see Pouille and Scanziani 2004 for similar results in hippocampus).

FS cells initially respond readily to excitatory input (Cruikshank et al. 2007), but because they receive dramatically depressing synapses from upstream excitatory neurons (Beierlein et al. 2003; Gibson et al. 1999), they would not be able to sustain their response during ongoing activity. Thus, they seem to be ideal for responding to sudden, brief changes in cortical activity such as incoming sensory information (Simons 1978; Simons and Carvell 1989; Swadlow et al. 1998; Swadlow and Gusev 2000), but not to slow changes in ongoing activity. In this respect, they act as a sort of filter of incoming excitatory activity, faithfully transmitting abrupt increases in input, but not persistent firing (Abbott and Regehr 2004). This characteristic is typical of synapses that display an initial high probability of neurotransmitter release (Abbott and Regehr 2004; Reyes et al. 1998; Watanabe et al. 2005).

In contrast, because GIN cells receive initially weak but dramatically facilitating inputs from their upstream excitatory neighbors, they would be best recruited only when there was a gradual, tonic increase in excitatory activity in the surrounding tissue. In this way, RS\rightarrow GIN synapses, which likely have an initial low probability of neurotransmitter release, as do similar cortical synapses (e.g. pyramidal\rightarrow bitufted interneurons; (Reyes et al. 1998; Watanabe et al. 2005)), act as a type of filter that only responds when input is persistent at or above a sufficient frequency (Abbott and Regehr 2004).
Likewise, it is important to consider the dynamic nature of the output of each cell type. A critical finding in this paper is that while GIN→RS synapses are depressing, this depression is frequency-dependent. Importantly, in the firing range the GIN cells tended to display under all of the activating manipulations tested here (~3-10 Hz), the degree of depression was minimal. Surprisingly, the average IPSP size during tonic DHGP-evoked firing was not very different from that during unitary input, suggesting that these synapses did not depress substantially. This means that once activated to fire persistently, GIN cells would not lose their ability to exert an inhibitory influence on their targets. In contrast, FS→RS synapses depress substantially (Beierlein et al. 2003; Gibson et al. 1999), falling to ~55% of the initial IPSP amplitude after 8 spikes at 10 Hz, so FS cells would likely not be able to sustain an inhibitory drive to their targets even if they were persistently active.

*Activation of GIN cells*

The fact that GIN cells acted similarly under all of the activating conditions used in this study suggests that they might fire similarly in the intact cortical circuit under conditions of increased excitatory neuron activity. Increased firing of excitatory neurons could occur under several conditions that are related to the mechanisms used in this study to activate GIN cells. First, activation of mGluRs would presumably occur under conditions of sustained excitatory neuron firing which would cause sustained glutamate release. Such sustained basal firing levels are known to occur *in vivo*, especially during alert waking states (Fanselow and Nicolelis 1999). Another condition that might increase GIN cell activity *in vivo* is increased cholinergic activity. The neocortex receives cholinergic input from the basal forebrain nuclei (for review see (Mufson et al. 2003; Sarter and
Bruno 2000), which become active during waking states and conditions that involve attention and memory (Hasselmo 2006; Sarter and Bruno 2000; Sarter et al. 2003). Muscarinic effects on neocortical neurons are complex, but are thought mainly to affect multiple types of K+ currents (e.g. $I_M$ and $I_{K,L}$), which cause depolarization, increased excitability, and reduced firing rate adaptation (McCormick 1992; McCormick and Prince 1986). Our results suggest that when cholinergic systems are engaged, the cholinergic output of that system could activate the GIN cell network.

Finally, pathological levels of activity such as seizures could also activate the GIN cell population. In our study, even brief trains of extracellular stimuli at high frequencies were sufficient to activate GIN cells for tens of seconds after the termination of the stimulus. Thus, it is possible that the onset of a seizure could evoke activity in the GIN cell population, which could presumably contribute to stopping the seizure activity (Dinocourt et al. 2003; Gorter et al. 2001).

In conclusion, the results of this study show that a network of electrically coupled GIN cells in layer 2/3 of the mouse neocortex fire persistently in the theta frequency range during a number of dissimilar activating manipulations. This suggests that GIN cells may display this type of firing under a range of natural conditions as well. Further, because their synapses onto RS cells are only mildly depressing in the theta range, GIN cells are able to maintain an inhibitory influence on their excitatory targets during this type of persistent firing. These findings, and the fact that the GIN cells receive initially mild but greatly facilitating synapses from presynaptic excitatory cells, suggest that this subtype of inhibitory interneuron could act as a brake for cortical activity, acting to keep cortical
activity under control when there are increases in excitatory tone. In addition, because GIN cells exert a rhythmic, network-wide theta-range rhythmic influence on their excitatory targets, it is possible that this group of inhibitory cells acts to coordinate neuronal firing in the theta range across wide areas of neocortex.
ACKNOWLEDGEMENTS

Thank you to Saundy Patrick for histological processing of the biocytin filled tissue and for general technical support. This work was supported by the following grants: NS2598 (NIH) and NS050434 (NIH) to BWC and NS046163 (NIH) and the Epilepsy Foundation through the generous support of the American Epilepsy Society and the Milken Family Foundation to EEF. Current address for EEF: Department of Neurobiology, University of Pittsburgh School of Medicine, W1458 Thomas E. Starzl Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15261

The authors have no potential conflicts of interest to disclose.
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FIGURE LEGENDS

Figure 1. GIN cell morphologies  A. Bitufted GIN cell in layer 2/3 of mouse neocortex filled with and stained for biocytin. B. Low magnification picture of slice in which cell in A was found. C. Reconstruction of GIN cell depicted in A created using a camera lucida microscope attachment. Cell body and dendrites are black and axon is red. D. Multipolar cell in layer 2/3 of mouse neocortex, also filled with and stained for biocytin. E. Low magnification picture of slice in which cell in D was found. In all cases, pial surface is up. F. Laminar distribution of GIN cells recorded in this study.

Figure 2. Characteristics of layer 2/3 neocortical GIN cells  A. Cumulative plots of the lowest current value (pA) that elicited action potentials for each cell type (i.e. firing threshold). B. Average firing frequencies of GIN, RS and FS cells as a function of stimulus current, measured during the last 200 ms of 600 ms constant current steps. Currents are normalized to the threshold (i.e. the lowest current amplitude that yielded action potentials for each cell). C. GIN cell responses to current steps at -100 and 40 pA. An example of the ADP characteristic of GIN cells is enlarged in the inset (from area above horizontal line in C). With injection of stronger currents (e.g. 300 pA in lower trace in C), GIN cells display spike frequency adaptation. In addition, characteristic action potential height and AHP profiles of GIN cells can be observed in this trace. Note that the peak of the first action potential is the most positive in the trace, and the depth of the first AHP is the most negative. D. FS cell responses to current steps at -100 and 180 pA (top panel) and 500 pA (bottom panel). The lack of an ADP is shown in the inset of the top panel (enlarged from area above horizontal line in D). Note also the delay in
firing in the top panel in D, which is characteristic of FS neurons with injections of just-suprathreshold currents. E. GIN cells have a more positive resting membrane potential (fine dashed line) and a more negative action potential threshold (course dashed line) than RS (F) and FS (G) cells. Arrows indicate resting membrane potentials for each cell type. H. Average resting membrane potentials and action potential thresholds.

**Figure 3. Dynamic responses at GIN→RS and RS→GIN synapses.** A. IPSPs recorded in an RS cell (high [Cl⁻] in RS cell) in response to stimulation at 10 Hz show minimal synaptic depression. B. In contrast, EPSPs recorded in GIN cells in response to RS cell stimulation show a large degree of synaptic facilitation. These findings are quantified in C and D for 3, 10, 20, and 40 Hz for each synapse type. The degree of depression at the GIN→RS synapse and the degree of facilitation at the RS→GIN synapse increased with increasing stimulus frequency. E. Degree of depression at the 8th IPSP for RS cell responses to presynaptic GIN cell stimulation as a function of stimulus frequency. F. Degree of facilitation for 8th EPSP for GIN cell responses to presynaptic RS cell stimulation as a function of stimulus frequency. Number of cell pairs tested at each frequency for GIN→RS synapses: 3 Hz = 12, 10 Hz = 12, 20 Hz = 7, 40 Hz = 15; for RS→GIN synapses: 3 Hz = 6, 10 Hz = 8, 20 Hz = 8, 40 Hz = 11.

**Figure 4. Application of low-divalent ACSF causes synchronous, persistent firing in GIN cells.** A. When low-divalent ACSF (1mM Ca²⁺ and Mg²⁺) was washed onto the slice, GIN cells fired persistently for as long as the low-divalent ACSF was presented. B. Instantaneous firing frequency during recording in A. C. 76.3% (58/76) of GIN cells in layers 2/3 fired persistently during application of low-divalent ACSF, whereas only 9% of
RS cells (4/44) and no FS cells (0/11) fired under these conditions. D. Synchronous persistent firing in pairs of GIN cells that were electrically coupled. E. Cross-corellogram for spike times across 18 min of low-divalent ACSF application, of which the trace in D is a sample. Bin width was 20 ms.

**Figure 5. Synchrony among coupled GIN cells is proportional to the coupling coefficient.** When a measure of firing synchrony (measured as the height of the main peak of the cross correllogram, see Methods) was plotted as a function of the coupling coefficient, there was a positive correlation between these two measures (R = 0.85). Data were pooled across activating conditions.

**Figure 6. GIN cell responses following brief trains of extracellular stimulation.** A. When a train of stimuli (here, 20 stimuli at 100 Hz, pulse duration 200 µsec) was presented using a bipolar stimulating electrode near the layer 4/layer 3 border, GIN cells responded with action potentials during the stimulation (arrow), then were inhibited for an average of 5.4 ±0.5 seconds, and subsequently fired for an average of 32.6±2.1 sec. The average pre-stimulus resting membrane potential was -51 mV, and the average minimum following the stimulus was -61 mV. B. Summary of the average membrane potential prior to stimulation, the average negative-most value during the inhibitory period, the average duration of the inhibitory period and the average duration of the persistent firing period (open bar; error bars indicate SEM). C. At resting membrane potentials, 84.7% (n = 83/98) of GIN cells, but no RS (n = 0/25) or FS (n = 0/5) cells showed persistent firing following trains of extracellular stimuli. D. When we recorded from strongly electrically coupled pairs of GIN cells following extracellular stimulation,
the firing in these cells was often synchronous. E. Expansion of firing during horizontal line in D. F. Cross-correllogram of activity across two min of activity following extracellular stimulation. Integration window was 20 ms.

**Figure 7. Persistent activity in GIN cells following trains of extracellular stimuli was not blocked by pharmacological blockade of fast synaptic transmission, but was blocked by the mGluR1a antagonist LY367385.** A. Persistent activity in a GIN cell in normal ACSF. B. Persistent activity in the same cell after washing on APV (50 µM), DNQX (20 µM) and PTX (20 µM) for 15 minutes. Note that the inhibitory period that typically followed the stimulus under normal conditions (A) was eliminated by application of these blockers (B). Scale bar for A also applies to B. C. Persistent activity in a GIN cell in normal ACSF (stimulus train presented at arrow above trace). D. Membrane depolarization and persistent firing were blocked by the application of LY367385 (200 µM). E. Membrane depolarization and persistent firing returned upon washout of LY367385. Scale bar for D also applies to C and E. Dotted line in all panels indicates baseline membrane potential.

**Figure 8. Application of the group 1 mGluR agonist. DHPG, causes persistent firing in GIN cells.** A. Persistent firing in a GIN cell during application of DHPG. B. Instantaneous firing frequency during the trace in A. C. 95.0% (n = 19/20) GIN cells, 14.3% of RS (n = 2/12) cells and no FS cells (n = 0/1) showed tonic firing during application of DHPG. D. During DHPG application, firing in pairs of strongly electrically coupled GIN cells showed a significant degree of synchrony. Action potential peaks were cut off in these traces to best show synchrony. E. Cross-correllogram of activity
during 7 min of firing in response to DHPG application, from which D is a sample. Bin width was 20 ms. F. Simultaneous recording from a GIN cell and an RS cell. The electrode for the RS cell contained 30 mM [Cl\(^-\)] so IPSPs could easily be observed at resting membrane potentials. G. Average IPSPs during intracellular GIN stimulation (thick black line), DHPG application (thin black line; traces triggered from action potentials below long horizontal line for GIN cell in F), and a control trace (grey) triggered by action potential times for the subsequent 30 sec period. H. Expansion of RS trace in F (above short bar) showing IPSPs.

**Figure 9. Application of cholinergic agonists to slices causes persistent firing in GIN cells.** A. Persistent firing during carbachol application in a GIN cell. B. Instantaneous firing rate during trace in A. C. During carbachol application, 94.4% of GIN cells fired (n = 17/18), and no RS cells did (0/4). FS cells were not tested with carbachol. Similarly, 100% of tested GIN cells (n = 9/9) fired in response to application of the muscarinic agonist, muscarine; no RS cells did (n = 0/1), and FS cells were not tested with muscarine. D. During application of carbachol, firing in strongly electrically coupled GIN cells was synchronous. E. Cross-correlogram of four min of firing in GIN cells during carbachol application, from which D is a sample. Bin width was 20 ms.

**Figure 10. Firing rates of GIN cells during DHPG or muscarine application show a mild degree of concentration dependence.** A. Firing rates of GIN cells during DHPG application from 0.25 to 100 µM. Circles indicate individual data points and horizontal lines indicate median values for each concentration. B. Histogram of firing frequency during DHPG application at steady-state firing levels for each cell. C. Firing rates of
GIN cells from 0.25 to 100 μM. Circles indicate individual data points and horizontal lines indicate median values. D. Histogram of firing frequency for steady-state firing levels for each cell.
Figure 1

Panel A shows a close-up view of the pial surface, with a scale bar of 50 µm. Panel B displays an expanded view of the pia layer, including Layer 1, with a scale bar of 1 mm. Panel C illustrates the pial surface and Layer 1 with a 50 µm scale bar. Panels D and E provide additional close-up images with similar scale bars. Panel F presents a histogram showing the distribution of distances from the pial surface to the bottom of Layer 3, with a scale indicating the number of recorded cells.
Figure 3

A. GIN and RS with High Cl- internal solution

B. RS and GIN

C. GIN→RS normalized PSP amplitude over stimulus number

D. RS→GIN normalized magnitude 8th PSP

E. Normalized magnitude of the 8th PSP over stimulus frequency (Hz)

F. Normalized magnitude over stimulus frequency (Hz)
Figure 4
Figure 5
Figure 6

A. Extracellular stimulus train

B. % cells showing persistent firing

C. GIN, RS, FS

D. GIN

E. GIN

F. Normalized cross correlation
Figure 7

A. Normal ACSF

B. APV + DNQX + PTX

C. Before LY367385

D. During LY367385

E. LY367385 washout
Figure 8

A

DHPG on

DHPG off

B

firing freq. (Hz)

10 mV

1 min

C

% cells showing persistent firing

GIN RS FS

D

normalized cross correlation

0.5

0.4

0.3

0.2

0.1

-1000 500 0 500 1000
time (ms)

E

GIN

RS

FS

GIN

RS

high Cl-

internal solution

F

DHPG on

GIN

RS

high Cl-

intracellular stimulation

G

intracellular stimulation

DHPG

RS

control

H

1 mV

1 sec

RS
Figure 9

A. Carbachol on 20 mV 30 sec

B. Firing freq. (Hz)

C. % cells showing persistent activity

D. GIN RS GIN RS

E. Normalized cross correlation

-1000     500        0       500   1000
time (ms)
Figure 10