Enhanced responses to somatostatin interneuron activation in developmentally malformed cortex

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Summary
Intractable epilepsy is commonly associated with developmental cortical malformations. Using the rodent freeze lesion model, we have sought the underlying circuit abnormalities contributing to the epileptiform activity that occurs in association with the structural pathology of four-layered microgyria. We showed previously that within the epileptogenic paramicrogyral region (PMR) surrounding the malformation, non–fast-spiking neurons commonly containing somatostatin (SSt) exhibit alterations, including having a greater maximum firing rate. Here we examined the output of SSt interneurons with optogenetics, using SSt-Cre mice mated to mice with floxed channelrhodopsin-2. Voltage clamp recordings from layer V pyramidal neurons in ex vivo slices had significantly enhanced SSt-evoked inhibitory postsynaptic currents in PMR cortex compared to control. In addition, under conditions of low- Mg2+ artificial cerebral spinal fluid (aCSF), light activation of the SSt neurons evoked field potential epileptiform activity in the PMR cortex, but not in control. These data suggest that within the PMR cortex, SSts have a significantly larger effect on excitatory neurons. Surprisingly, the network effect of this enhanced inhibition is hyperexcitability with propagating epileptiform activity, perhaps due to disinhibition of other interneuron cell types or to enhanced synchrony of excitatory cortical elements. This identification creates a new locus for potential modulation of epileptiform activity associated with cortical malformation.

KEYWORDS
Cortical inhibition, freeze lesion, malformation, microgyria, optogenetics

1 | INTRODUCTION
Disorders of cortical maldevelopment are often associated with pharmaco-resistant epilepsy. The cellular mechanisms underlying these sequelae in union are, however, poorly understood. One of the most common forms is polymicrogyria (PMR), distinguished by an increase of small cortical folds and irregular lamination. The form of four-layered microgyria has a continuation of layers I and II/III, a third cell-sparse layer, and a fourth layer similar to and contiguous with deep layer VI. The rodent neonatal transcranial freeze lesion model replicates this histopathology as well as the presence...
of epileptiform activity\textsuperscript{3} and thus provides means to unravel the presently unknown epileptogenic mechanisms associated with this malformation. Previous work in this model has demonstrated increased excitatory input to the PMR region within a millimeter of the induced sulcus that may be critical for the expression of epileptiform activity.\textsuperscript{4,5} However, these enhanced inputs are functional prior to the onset of epileptiform activity, suggesting that an additional factor is necessary to promote onset.\textsuperscript{6}

A second standard target of examination in epilepsy models is the inhibitory synaptic network. Although overall numbers of \(\gamma\)-aminobutyric acid (GABAergic) neurons appear unchanged in the PMR,\textsuperscript{7} neurons staining for parvalbumin (PV) are decreased in number specifically within layer V\textsuperscript{8} (but not when measured less specifically throughout the entire depth of cortex\textsuperscript{7}), whereas those staining for calbindin (some of which overlap with the somatostatin, SST, population) are increased.\textsuperscript{7} Because PV and SST interneuron subtypes are distinctly different in morphology, firing pattern, and location of their synaptic contacts on the postsynaptic neurons targeted, it is not surprising that they have consistently been suggested to carry unique functions within the cortical network. In fact PV neurons are powerful inhibitors positioned to control the spread of excitation across the cortex; whereas in contrast, the more modulatory inhibition of SST-containing interneurons is typically constricted to a single column while sometimes synapsing across layers.\textsuperscript{9} Within the PMR of the rodent, we previously examined these two populations based on their firing patterns of fast-spiking (FS, containing PV) and low-threshold-spiking (LTS, containing SST). The LTS interneurons had a slightly more depolarized membrane potential and fired at higher maximum frequencies in the PMR compared to control cortex.\textsuperscript{10} In contrast, the FS as a population fired at lower maximum frequencies in PMR cortex.\textsuperscript{10} We expected that with these intrinsic neuronal alterations, and synaptic and network effects would be produced. This initial study suggested that although the powerful PV/FS interneurons may be weakened, the SST/LTS interneurons might be strengthened. To test this idea we have used optogenetics to selectively activate the SST interneurons in PMR and control cortex. We find that the output from SST interneurons is indeed enhanced, and that this increased inhibition surprisingly can activate network hyperexcitability in the form of epileptiform activity.

2 | METHODS

All animal and experimental protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. To achieve expression of the light-sensitive channelrhodopsin2 (ChR) in SST-expressing interneurons, mice with floxed ChR (Ai32) were bred with SST-Cre mice (both strains from Jackson Laboratory, Farmington, CT; strains 012569 and 013044). Mice were housed with the dam and litter siblings and maintained on a 12/12 hour light/dark cycle at 72°F, with continuous access to standard rodent chow and water. The Agmon lab has demonstrated that this breeding strategy results in a rate of \(\sim\)10% ChR-expression in neurons other than SST\textsuperscript{11}; thus, in both control and experimental tissue, 90% of the light-emitting diode (LED)-evoked responses are due to SST interneurons. Microwire were induced in male and female rodents, with bilateral transcranial freeze lesions under hypothermic anesthesia on postnatal day (P) 1 as described previously.\textsuperscript{10} Briefly, mice were covered in ice for 4-6 minutes and maintained in ice-water slurry thereafter for the duration of the surgery. An anterior-posterior incision was made in the skin overlying the skull to expose the skull above somatosensory cortex. A circular probe \(-0.2\) mm in diameter that had been cooled to \(-50°C\) was placed on the skull for 5 seconds, after which the skin was sutured and the pup was rewarmed and returned to the dam. Acute brain slices from mice between 12 and 21 days of age were prepared from freeze-lesioned pups and naive sibling controls, with mice being fully anesthetized with isoflurane until unresponsive to tail and toe pinch, and then decapitated. Coronal slices, 0.3 mm thick, were made in standard sucrose slicing solution as described previously.\textsuperscript{10} Slices were maintained in artificial cerebrospinal fluid (aCSF) containing (in mmol/L): 126 NaCl, 3.5 KCl, 1.25 NaH2PO4, 1.2 CaCl2, 1 MgSO4, and 26 NaHCO3, maintained at pH 7.3 and 290 mOsm. For cellular recordings, the same aCSF was used with the addition of DL-2-Amino-5-Phosphonopentanoic acid (APV) (50 \(\mu\)mol/L) and 6,7-Dinitroquinoxaline-2,3-dione (DNQX) (20 \(\mu\)mol/L). Whole-cell patch-clamp recordings were obtained using glass micropipettes (2.8-4.5 M\(\Omega\)) containing a “high chloride” \((E_{Cl}\textsuperscript{−} = -15 \text{mV})\) intracellular solution (in mmol/L): 70 K-glucurate, 10 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES), 4 Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 70 KCl, 4 Adenosine 5′-triphosphate (ATP) disodium salt hydrate, and 0.2 Guanosine 5′-triphosphate sodium salt hydrate, with 0.25%-0.5% biocytin; 280-290 mOsm, pH 7.3. Recordings were made with a Multiclamp 700B amplifier and digitized with data acquisition instrument (Digidata 1440; both from Molecular Devices, San Jose, CA). Recordings were made from pyramidal and SST neurons within layer V of somatosensory cortex, 0.5-1.0 mm from the induced sulcus or in homotopic control cortex. Neuronal type was identified initially based on neuronal morphology under differential interference contrast (DIC) optics, where an apical dendrite was identified for pyramidal neurons and for the SST neurons, via the fluorescent green fluorescent protein (GFP) indicator associated with ChR. This was confirmed in some cases with fluorescent avidin staining of the biocytin-filled cells. The effect of selective activation of SST interneurons was attainable via blue-range LED at 460 nm (bLED) passing through...
a 60× objective, initially placed directly above the recorded cell. LED strength was controlled via the X-Cite system and Lumen Dynamics software (20%, producing 1.5 mW, continuously, Excelitas Technologies, Waltham, MA). A stimulation intensity series for the activation of SSt interneurons was generated by varying light duration from 0.1 to 2 msec (11 intensities, see Figure 1) and measurements were made from averages of three stimulus presentations. Field potential recordings from layer II/III were obtained with glass pipettes containing 1 mol/L NaCl. All recordings were digitized at either 10 or 20K with pClamp software (Molecular Devices).

**FIGURE 1**  Effect of activating somatostatin (SSt) interneurons via channelrhodopsin (ChR) in malformed and control cortex. A series of increasing durations of blue light (bLED) were applied through the 60× objective. The objective was placed above the recorded layer V pyramidal neuron for A-C, within layers II/III superficial to the recorded layer V pyramidal neuron for D, and directly above the recorded layer V SSt interneuron for E. A, B Examples of responses within an individual control (A) and paramicrogyral region (PMR) (B) neuron; traces are averages of three presentations. C, A significantly larger light-evoked inhibitory postsynaptic current (IPSC) was produced in PMR compared to control when the light was placed in layer V (two-way repeated-measures analysis of variance (ANOVA), $P = 0.03$, $n = 19$ control and 14 PMR neurons from 8 and 9 mice, respectively). Note inset shows that in this case the light stimulation was placed over the recorded layer V neuron. D. This effect was lost with light stimulation of layers II/III ($n = 7$ control and 7 PMR neurons from 6 and 5 mice, respectively). Note inset shows that in this case the light stimulation was placed within layer II/III, superficial to the recorded layer V neuron. E. The light produced a similar number of action potentials in control and PMR SSt interneurons ($n = 9$ control and 7 PMR neurons from 4 and 2 mice, respectively). The SSt neurons were identified via fluorescence and had typical adapting firing patterns in response to intracellular depolarization (top inset). Green line shows level of first afterhyperpolarization. Bottom inset shows an example of an action potential evoked by 0.2 msec of light, in control tissue.

Analyses were primarily performed via home written programs (Visual Basic in Excel; Microsoft Corp., Redmond, WA). The peak current was averaged over 0.3 msec, with the largest single point at the center of that period. The response area was considered all points that were $>2$ standard deviations (SD) larger than the prestimulation baseline. Data are reported as mean ± standard error of the mean (SEM). Repeated-measure analysis of variance (ANOVA) was used to test for differences between control and PMR across the intensity series.

### 3 | RESULTS

The ability to produce light activation of SSt interneurons was confirmed by direct recordings from neurons expressing the ChR as identified by the GFP reporter. A series of depolarizing pulses in current clamp mode showed that these neurons had a firing pattern typical of LTS interneurons ($>1$ msec duration action potentials, with the frequency adapting over the 400 msec depolarizing pulse, and where the first afterhyperpolarization was lower, meaning more negative, than the last). The number of action potentials evoked in SSt interneurons was similar for control and PMR neurons, for the duration range of 0.2-2 msec, with the light centered on the recorded neuron (Figure 1E). Based on the response of SSt neurons to this range of LED durations, recordings were made from layer V pyramidal neurons using
the same intensity series of light applied through the 60× objective centered above the recorded neuron and producing a series of amplitude-graded light-evoked inhibitory postsynaptic currents (IPSCs) (Figure 1A,B). Measured across the population of neurons, the peak amplitude response was significantly larger for cells recorded within the PMR compared to controls (Figure 1C, −167.5 ± 40.5 and −510.5 ± 130.4 pA for control and PMR, respectively, two-way repeated-measures ANOVA, group P = 0.03). The area of the response was also significantly larger at 2 msec of light stimulation (in A*s: 40.9 ± 10.7 control vs −167.1 ± 47.5 PMR, t test, P = 0.006). When the light application was moved to layers II/III, directly superficial (toward the pia) to the recorded neuron, the PMR responses were not significantly different from those in controls (Figure 1D). To examine the effect of this SSt enhancement on the network, field potential recordings were made in response to the intensity series of light application. Under normal aCSF conditions, this did not produce epileptiform field potentials, but in some cases small graded fields were evoked (Figure 2B). To increase overall network excitability, we examined the effect of a low-Mg2+ aCSF (same as standard, but with the MgSO4 omitted). Following a 30-minute wash with this modified aCSF, the light intensity series was again applied. In control slices, this failed to change the lack of responsiveness to the activation of SSt interneurons, whereas within PMR, interictal-like epileptiform field potentials (deviation from baseline >2 SD below the prestimulus baseline) were evoked on an average of 34.2% of the light applications per slice, with every experimental slice showing some epileptiform activity (Figure 2A,C).

4 | DISCUSSION

These data demonstrate that the activation of SSt interneurons produces an enhanced response in malformed compared to control cortex. When recorded from layer V pyramidal neurons, the amplitude of the SSt-IPSC is enhanced only when evoked by stimulation of light within layer V, as the SSt-IPSC evoked with light application in layers II/III is not different between PMR and control (Figure 1D). Amazingly, this enhanced inhibitory response induces network hyperexcitability, at least under conditions of low-Mg2+ aCSF (Figure 2). Thus, increased output from SSt interneurons (firing at higher maximum rates) may be a contributor to the epileptogenesis in microgyral cortex.

The increased amplitude and area of the IPSC produced by light activation of SSt interneurons could be caused by a number of different mechanisms, including an increased number of SSt neurons present in PMR cortex (with the same number of terminals per cell as in control cortex), an increased number of terminals from each SSt interneuron onto their postsynaptic pyramidal neuron partners, increased GABA released by the terminals, or postsynaptic changes in the GABA receptors that receive input from SSt interneurons. Previously, we showed a decrease in neuronal PV immunohistochemistry within the PMR,8 and have hypothesized that SSt interneurons may survive effectively taking the place of vulnerable PV interneurons, which is consistent with counts of these neurons normalized to the overall number of glutamic acid decarboxylase (GAD)-stained interneurons (Jacobs, unpublished data). This phenomenon might also happen at the level of inhibitory terminals rather than full cells. Such a response by SSt interneurons or simply a failure to fully prune SSt interneurons could occur as a result of initial loss of PV interneurons or terminals, as homeostatic processes are known to occur via inhibitory synapses in a cortical network.12 These results could also be affected by the ~10% of non-SSt neurons present in both control and PMR groups. Agmon showed that when errors occur, they are typically PV interneurons.11 We are currently testing whether the response to selective activation of PV interneurons is different between PMR and control.

**FIGURE 2** Field potentials recorded in layer II/III during light stimulation of ChR-SSt within layer V in normal aCSF (A,C). A, A light stimulation intensity series was applied through the 60× objective within layer V by varying the duration of the light application at 20% LED strength. This series was repeated a total of three times for 33 total tests of the response. This produced epileptiform activity on an average of 34.2% of all stimulus presentations only within PMR (every mouse and every slice had some incidence of epileptiform activity) and never within control slices. N = 5 control and 7 PMR slices from 3 and 5 mice, respectively. B, Examples of individual sweeps in normal aCSF for control (B1) and PMR (B2). C, Examples of individual sweeps in low magnesium aCSF for control (C1) and three successive sweeps from PMR (C2-4), showing the all-or-none nature of this activity.
It is likely that the epileptogenic potential of the malformation depends on a number of mechanisms altered by the aberrant development resulting from the initial insult. Certainly, enhanced excitatory input to the PMR is an important factor, although the timing of its onset does not coincide with the initiation of epileptiform activity in the rodent freeze lesion model. This suggests that other factors may be involved at a time when cortical inhibition is undergoing maturation.

Another consideration is increased synaptic inhibition, which could lead to network hyperexcitability via an increased synchrony of the excitatory neurons or via disinhibition due to SSt inhibiting other interneurons subtypes. Normally SSt inhibition onto pyramidal neurons is expected to be only weak or modulatory. It is possible, however, for these interneurons to synchronize the activity of surrounding pyramidal neurons during the activation of group I metabotropic glutamate receptors. Should this mechanism be strengthened, particularly within a column, this could result in the translaminar hypersynchrony typical of the epileptogenic brain. In addition, an increased synaptic connection from SSt to PV interneurons could produce network disinhibition. The current finding presents novel targets for the potential control of epileptiform activity in the malformed brain, including that of metabotropic glutamate receptors.

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DISCLOSURE

The authors have no conflicts of interest to disclose. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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