Does Plasticity of the $GABA_A$ Reversal Potential Contribute to Epileptogenesis?

Disrupted Dentate Granule Cell Chloride Regulation Enhances Synaptic Excitability during Development of Temporal Lobe Epilepsy. Pathak HR, Weissinger F, Terunuma M, Carlson GC, Hsu FC, Moss SJ, Coulter DA. *J Neurosci* 2007;27(51):14012–14022. $GABA_A$ receptor-mediated inhibition depends on the maintenance of intracellular Cl^- concentration ($[Cl^-]_{in}$) at low levels. In neurons in the developing CNS, $[Cl^-]_{in}$ is elevated, E_{GABA} is depolarizing, and GABA consequently is excitatory. Depolarizing GABAergic synaptic responses may be recapitulated in various neuropathological conditions, including epilepsy. In the present study, rat hippocampal dentate granule cells were recorded using gramicidin perforated patch techniques at varying times (1–60 d) after an epileptogenic injury, pilocarpine-induced status epilepticus (STEP). In normal, non-epileptic animals, these strongly inhibited dentate granule cells act as a gate, regulating hippocampal excitation, controlling seizure initiation and/or propagation. For 2 weeks after STEP, we found that E_{GABA} was positively shifted in granule cells. This shift in E_{GABA} altered synaptic integration, increased granule cell excitability, and resulted in compromised "gate" function of the dentate gyrus. E_{GABA} recovered to control values at longer latencies post-STEP (2–8 weeks), when animals had developed epilepsy. During this period of shifted E_{GABA}, expression of the Cl^- extruding K^+/Cl^- cotransporter, KCC2 was decreased. Application of the KCC2 blocker, furosemide, to control neurons mimicked E_{GABA} shifts evident in granule cells post-STEP. Furthermore, post-STEP and furosemide effects interacted occlusively, both on E_{GABA} in granule cells, and on gatekeeper function of the dentate gyrus. This suggests a shared mechanism, reduced KCC2 function. These findings demonstrate that decreased expression of KCC2 persists for weeks after an epileptogenic injury, reducing inhibitory efficacy and enhancing dentate granule cell excitability. This pathophysiological process may constitute a significant mechanism linking injury to the subsequent development of epilepsy.

COMMENTARY

Readers of *Epilepsy Currents* understand the importance of uncovering the processes that lead to epilepsy after brain injury. Many recent reports have demonstrated that the reversal potential for $GABA_A$ synaptic responses (E_{GABA}) becomes more positive after various forms of intense neuronal activity (1–4). Now Pathak et al. report that E_{GABA} is changed in dentate granule cells following pilocarpine-induced status epilepticus. The direction of the change in E_{GABA} could compromise inhibition and potentially contribute to epileptogenesis. Curiously, E_{GABA} only changed during the first week after brain injury. This time period is shorter than the latency to onset of spontaneous seizures, so E_{GABA} likely normalizes before the onset of epilepsy. The finding is a potentially important clue to understanding epileptogenesis, but measuring E_{GABA} and the transport of the ions that flow through the GABA channel is difficult and there are a few caveats to consider.

The reversal potential for a synaptic response ought to be a straightforward measurement: evoke a synaptic response, change the neuron's membrane potential by injecting current,

Epilepsy Currents, Vol. 8, No. 4 (July/August) 2008 pp. 107–110
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and then evoke another response. Thus, a plot of the size of the synaptic response versus the membrane potential would provide the reversal potential. However, it is surprisingly easy to end up with a spurious value of E_{GABA}. The most common pitfall occurs during the process of recording and changing the membrane potential, which frequently changes the balance of ions that flow through the $GABA_A$ receptor. This alteration was well known to early investigators who used sharp electrodes filled with molar concentrations of potassium salts; some of the anions used permeated the $GABA_A$ receptor channel and altered the reversal potential (5). The subsequent development of whole-cell recording techniques utilizing pipettes filled with "physiological" concentrations of potassium and chloride salts to measure E_{GABA} initially seemed ideal (6). However, the whole-cell recording technique presented as many problems as had the sharp electrodes, because the low-access resistance of the pipette allowed free dialysis of the neuronal soma and to a lesser extent, the dendrites (7). The dialysis disrupts not only the ionic balance but also every second messenger system as well. What to do?

The exploitation of antibiotics that can form pores in cell membranes to allow passage of only ions overcame the second messenger problem. The antibiotic gramicidin was hailed as the solution to the measurement of E_{GABA}, because the pores that it forms allow passage of only cations, so that anions that permeate the GABA channel are not affected. However, the balance of anions in a cell is determined by the balance of cations, through the action of cation–anion cotransporters. In adult neurons, cation–anion cotransport is generally handled by the K^+/Cl^- cotransporter, KCC2, which links the export of one potassium and one chloride ion from the cell, resulting in a low chloride concentration and an E_{GABA} that is usually more negative than the resting membrane potential (RMP). Gramicidin recordings are accurate in adult neurons because the intracellular potassium concentration is high and the potassium reversal potential (which determines the reversal potential for Cl^-, via KCC2 cotransport) does not change much if intracellular potassium is altered by a few mM; however, this is not the case with Cl^- cotransport in immature neurons where chloride transport is handled by NKCC1, a cotransporter that depends on a low intracellular Na^+ concentration to maintain a high intracellular Cl^- concentration by linking the import of Na^+, K^+, and Cl^- ions. Gramicidin recordings in such neurons cause major artifacts by altering the intracellular Na^+ concentration (4).

Pathak et al. used gramicidin perforated patch recordings to measure E_{GABA} from adult animals; thus, as long as only KCC2 is expressed in these cells, the gramicidin whole-cell recording technique would be an appropriate method to measure E_{GABA}. However, there is ample evidence from resected brain tissue that NKCC1 also is expressed in neurons from patients with intractable epilepsy (8). Pathak et al. studied hippocampal dentate granule cells during the first week after brain injury, which might antedate the expression of NKCC1 in epileptic tissue. Furthermore, they looked for biochemical evidence of NKCC1 and did not find it. So, why do concerns persist regarding gramicidin and NKCC1?

Dentate granule cells have a very negative RMP, about −80 mV (9). While most cells have a $GABA_A$ reversal potential that is negative to RMP (as a result of KCC2 cotransport), granule cells have a $GABA_A$ reversal that is more positive than the RMP; Pathak et al. found an E_{GABA} of about −75 mV. Although they did not report the RMP in these cells, a figure published in the study (Figure 6) demonstrates depolarizing $GABA_A$ receptor responses, suggesting that indeed E_{GABA} was positive to the RMP. It is difficult to explain E_{GABA} in terms of KCC2 when E_{GABA} is more positive than the RMP. As mentioned, KCC2 cotransports K^+ and Cl^- and thus, comes to equilibrium when $E_{Cl} = E_K$. E_K is at least −100 mV and is substantially more negative than the RMP. If KCC2 activity is completely blocked, Cl^- will be passively distributed and E_{Cl} will equal the RMP. So how does E_{GABA} become positive relative to RMP? It would require either a substantial conductance of another anion or an active Cl^- accumulation mechanism, such as NKCC1.

GABA receptors are slightly permeable to the anion, HCO_3^-, which has a much less negative reversal potential of approximately −12 mV (10). Thus, studies measuring Cl^- transport by KCC2 or NKCC1 are best performed in the absence of CO_2 and HCO_3^-, for example, by buffering the media with HEPES and removing CO_2 from the artificial CSF (4,11). Is the HCO_3^- permeability sufficient to explain why E_{GABA} is positive to the RMP in granule cells? The answer also would tell us whether another cation–anion cotransporter, such as NKCC1, is active in these cells. Although Pathak et al. performed some experiments in the absence of HCO_3^-, the impact of HCO_3^- flux was not systematically studied, so it cannot be assumed that only KCC2-mediated Cl^- transport is active. In some experiments, furosemide was used to block KCC2; this diuretic also blocks NKCC1 and carbonic anhydrase (an enzyme that facilitates HCO_3^- regeneration) at the same or lower concentrations used by Pathak and coworkers (12) and also blocks some $GABA_A$ receptors (13). Unfortunately, no more specific KCC2 antagonist has been identified, making it difficult to be sure that changes in E_{GABA} are solely responsible for the disinhibition produced by furosemide (which was shown in a figure [Figure 7] published in the Pathak et al.'s study).

There are more accurate electrophysiological means to measure E_{GABA} and RMP, but they have limited time resolution and involve painstaking recordings of the reversal potential of channel currents measured from cell-attached electrodes (14). The intracellular Cl^- concentration also can be measured using ion-sensitive dyes, including genetically expressed

dual-wavelength dyes, such as Clomeleon. To date, these dyes have restricted regions of expression in the CNS (but, fortunately they are expressed in the hippocampus!), limited affinity for Cl^-, exquisite pH sensitivity (which can be a problem in light of the HCO_3^- permeability of the GABA receptor), and must be calibrated to a known Cl^- concentration at the end of the experiment (15).

Measuring the transport of Cl^- can be useful even if the exact measurement of E_{GABA} is likely to be offset by some of the factors described above (10). For example, if exclusively inward Cl^- transport is recorded, it can be concluded that E_{GABA} is positive to RMP, so that $GABA_A$ receptor activation will produce membrane depolarization. If the rate of inward transport increases after an experimental intervention, it can be assumed that the intervention caused E_{GABA} to become less negative. Pathak et al. measured Cl^- transport by evoking a train of synaptic $GABA_A$ receptor responses and then measuring the change in E_{GABA} at the end of the train. If the synaptic influx of Cl^- exceeds Cl^- efflux via KCC2, Cl^- should accumulate and E_{GABA} should become less negative. This shift in E_{GABA} provides a measure of KCC2 activity. Because this measure of KCC2 depends on the difference between synaptic GABA-evoked Cl^- influx and KCC2 Cl^- efflux, the outcome is affected by a number of factors that impact the synaptic GABA-evoked Cl^- influx, including the rate of IPSC decay and receptor desensitization, facilitation and depression of presynaptic GABA release, as well as the rate of GABA reuptake (which affects the spread of GABA to nearby extrasynaptic receptors). Thus, it is important to report the total IPSC current (i.e., the charge transfer during the train of responses that loads the Cl^-), which provides the actual amount of Cl^- that flowed into the cell and the rate at which it flowed (presuming the experiment is performed in bicarbonate-free solutions). This calculation removes all the synaptic variables from the measurement of KCC2 function and allows quantitation of KCC2 activity (4,10).

Another advantage of calculating the charge transferred during the train of IPSCs is that the value can be combined with the shift in intracellular Cl^- concentration, which can be calculated from the change in E_{GABA} using the Nernst equation. Dividing the amount of Cl^- influx by the change in Cl^- concentration gives the actual volume into which the Cl^- current flows (4). The resulting value is important, because the subcellular distribution of $GABA_A$ receptors that are active during the IPSCs is not known and smaller volumes will accumulate Cl^- more rapidly. Stated in another way, cells with a low total GABA activated conductance will need to have more $GABA_A$ receptor channels activated over a larger area to generate the same amplitude IPSC as a cell with a higher density of GABA receptors. Thus, in a cell with a lower density of $GABA_A$ receptors, the Cl^- currents will flow into a larger intracellular volume. The larger volume will effectively dilute the incoming Cl^-, decreasing the rate of intracellular Cl^- accumulation and decreasing the rate of change of E_{GABA}. The somatic versus dendritic balance of GABAergic innervation of hippocampal neurons changes during epileptogenesis (16), altering the balance of subcellular GABA flux—so calculation of the volume of distribution of the Cl^- influx is an important control in studies of Cl^- transport during epileptogenesis. The volume of distribution of the Cl^- influx also is important because diffusion operates in parallel with KCC2 to reduce the changes in subsynaptic intracellular Cl^- concentration caused by GABA-mediated Cl^- influx. Widely spread GABAergic synapses will facilitate such diffusion, leading to an overestimate of KCC2 function. Diffusion effects are best controlled by repeating the experiment after transport has been blocked and by subtracting the apparent rates of Cl^- transport under these two conditions to derive the transport-specific rate (4). Finally, calculating the volume into which the IPSC currents flow provides an answer to the question raised by Pathak et al. as to whether the nucleus of dentate granule cells should count as part of the volume into which Cl^- diffuses (i.e., whether or not Cl^- is freely diffusible across the nuclear membrane).

Using Western blots, Pathak et al. found a 25 percent decrease in KCC2 expression in the dentate after status epilepticus. Is this finding sufficient to explain the measured reductions in Cl^- transport? The investigators used furosemide, an inhibitor of KCC2, to reduce transport. Only maximal concentrations of furosemide, corresponding to 100 percent block of KCC2 (11), inhibited KCC2 to the same extent as was observed in granule cells after status epilepticus, suggesting that there is essentially no KCC2 function in the granule cells. As the authors conclude, this finding further suggests that the 25 percent decrease in KCC2 expression is not sufficient to cause the observed reduction in KCC2 function and that posttranslational modifications, membrane trafficking, and phosphorylation also are likely to be important determinants of KCC2 function after status epilepticus.

To measure the impact of the change in E_{GABA} on signal processing, Pathak et al. elicited a postsynaptic GABA response (i.e., an IPSP) followed by somatic current injection; they found that more action potentials were triggered from granule cells by this protocol in the first week after status epilepticus. The subcellular location of the conductance evoked by the GABAergic IPSP is an important variable in this experiment. Depolarizing GABA-activated currents can both activate voltage-dependent cation currents in the axon hillock and shunt the resulting cation currents (17), so that the net results of $GABA_A$ receptor activation depend not only on E_{GABA} but also on the subcellular distribution of the evoked GABA-activated conductances. Because the subcellular distribution of GABA-activated conductances shifts in epileptic animals (15), it will be important to complement these experiments by Pathak and colleagues with

studies of the subcellular location of GABA-activated conductances after status epilepticus. In summary, Pathak et al. have identified an important potential mechanism of epileptogenesis. Clearly, the topic is complex and a promising area for many future investigations.

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