“TOR”RENTS OF EXCITEMENT OVER RAPAMYCIN’S ANTIEPILEPTOGENIC POTENTIAL

Response of a Neuronal Model of Tuberous Sclerosis to Mammalian Target of Rapamycin (mTOR) Inhibitors: Effects on mTORC1 and Akt Signaling Lead to Improved Survival and Function. Meikle L, Pollizzi K, Egnor A, Kramvis I, Lane H, Sahin M, Kwiatkowski DJ. *J Neurosci* 2008;28(21):5422–5432. Tuberous sclerosis (TSC) is a hamartoma syndrome attributable to mutations in either TSC1 or TSC2 in which brain involvement causes epilepsy, mental retardation, and autism. We have reported recently (Meikle et al., 2007) a mouse neuronal model of TSC in which Tsc1 is ablated in most neurons during cortical development. We have tested rapamycin and RAD001 [40-O-(2-hydroxyethyl)-rapamycin], both mammalian target of rapamycin mTORC1 inhibitors, as potential therapeutic agents in this model. Median survival is improved from 33 d to more than 100 d; behavior, phenotype, and weight gain are all also markedly improved. There is brain penetration of both drugs, with accumulation over time with repetitive treatment, and effective reduction of levels of phospho-S6, a downstream target of mTORC1. In addition, there is restoration of phospho-Akt and phospho-glycogen synthase kinase 3 levels in the treated mice, consistent with restoration of Akt function. Neurofilament abnormalities, myelination, and cell enlargement are all improved by the treatment. However, dysplastic neuronal features persist, and there are only modest changes in dendritic spine density and length. Strikingly, mice treated with rapamycin or RAD001 for 23 d only (postnatal days 7–30) displayed a persistent improvement in phenotype, with median survival of 78 d. In summary, rapamycin/RAD001 are highly effective therapies for this neuronal model of TSC, with benefit apparently attributable to effects on mTORC1 and Akt signaling and, consequently, cell size and myelination. Although caution is appropriate, the results suggest the possibility that rapamycin/RAD001 may have benefit in the treatment of TSC brain disease, including infantile spasms.

Rapamycin Prevents Epilepsy in a Mouse Model of Tuberous Sclerosis Complex. Zeng LH, Xu L, Gutmann DH, Wong M. *Ann Neurol* 2008;63(4):444–453. **OBJECTIVE:** Tuberous sclerosis complex (TSC) represents one of the most common genetic causes of epilepsy. TSC gene inactivation leads to hyperactivation of the mammalian target of rapamycin signaling pathway, raising the intriguing possibility that mammalian target of rapamycin inhibitors might be effective in preventing or treating epilepsy in patients with TSC. Mice with conditional inactivation of the Tsc1 gene primarily in glia (Tsc1^{GFAP}CKO mice) develop glial proliferation, progressive epilepsy, and premature death. Here, we tested whether rapamycin could prevent or reverse epilepsy, as well as other cellular and molecular brain abnormalities in Tsc1^{GFAP}CKO mice. **METHODS:** Tsc1^{GFAP}CKO mice and littermate control animals were treated with rapamycin or vehicle starting at postnatal day 14 (early treatment) or 6 weeks of age (late treatment), corresponding to times before and after onset of neurological abnormalities in Tsc1^{GFAP}CKO mice. Mice were monitored for seizures by serial video-electroencephalogram and for long-term survival. Brains were examined histologically for astrogliosis and neuronal organization. Expression of phospho-S6 and other molecular markers correlating with epileptogenesis was measured by Western blotting. **RESULTS:** Early treatment with rapamycin prevented the development of epilepsy and premature death observed in vehicle-treated Tsc1^{GFAP}CKO mice. Late treatment with rapamycin suppressed seizures and prolonged survival in Tsc1^{GFAP}CKO mice that had already developed epilepsy. Correspondingly, rapamycin inhibited the abnormal activation of the mammalian target of rapamycin pathway, astrogliosis, and neuronal disorganization, and increased brain size in Tsc1^{GFAP}CKO mice. **INTERPRETATION:** Rapamycin has strong efficacy for preventing seizures and prolonging survival in Tsc1^{GFAP}CKO mice.

COMMENTARY

Rapamycin, as its name suggests, had humble beginnings as an antifungal agent. It lost favor among infectious disease specialists as a result of its immunosuppressive side effect; however, this effect was useful to transplant surgeons in preventing

organ rejection, so the drug remained on the market. Meanwhile, molecular biologists were assessing resistance of fungi to the growth-inhibitory properties of certain antibiotics and determined that there is a serine–threonine protein kinase in *Saccharomyces cerevisiae* (i.e., Brewer’s yeast) that, if mutated, confers resistance to rapamycin. The investigators creatively named this kinase target of rapamycin (TOR) (1). Mammalian studies subsequently revealed that humans have a similar kinase; the mammalian analog, hence, was named “mammalian target

of rapamycin" (mTOR). Rapamycin's efficacy appeared to be dependent on its ability to inhibit TOR.

TOR is a phosphatidylinositol kinase-related kinase involved in the second-messenger intracellular cascade that regulates protein synthesis. Studies of TOR suggest that all eukaryotes have this protein, but yeast have two TOR proteins and higher eukaryotes possess only one (2). Biochemical isolation of *TOR1* and *TOR2* from yeast resulted in the identification of two TOR protein complexes, TORC1 and TORC2. The TORC1 complex is the rapamycin-sensitive one and regulates cell growth. The TOR pathway is activated upstream by a number of major signals, including growth factors and insulin (which activate TOR), as well as by energy state and environmental stress (which inhibit TOR signaling). In mammals, these effects appear to be mediated via the tuberous sclerosis complex (TSC) proteins, TSC1 and TSC2—heterodimers that, when phosphorylated, negatively regulate mTOR signaling. Without TSC1 or TSC2, the mTOR pathway runs amuck, inducing excessive cell growth and proliferation. It has been determined that mutation of *TSC1* or *TSC2* genes accounts for tuberous sclerosis, a disease in which patients develop hamartomas in multiple organ systems. In the brain, the effects can be devastating, with mental retardation, epilepsy, and developmental difficulties being prominent features (see the Basic Review article by Crino in this issue of *Epilepsy Currents*). Because rapamycin inhibits TOR, one might expect it to be useful to patients who have excessive TOR activation that is due to mutation of *TSC* genes.

Advances in genetic engineering have resulted in the development of new animal models of disease utilizing mice with induced select genetic deletions or mutations (3). However, introduction of such genetic changes often turns out to be embryonically lethal or results in compensatory up- or down-regulation of other gene products, confounding interpretation of the resultant phenotype. Conditional knockouts therefore have been favored in recent years. In these animals, representing the second generation of genetic mouse models, targeted techniques are used to allow tissue-specific control of gene function (4). The two studies reviewed here both utilize a conditional allele of *Tsc1* in combination with various brain-specific cre recombinase alleles to produce, in one case, a neuronal *Tsc1* knockout mouse model, and in the other, an astrocytic *Tsc1* knockout mouse line.

The neuronal *Tsc1* knockout mouse develops brain pathology, including enlarged dysplastic ectopic neurons, persistently reduced myelination, and high expression of phospho-S6 (a protein downstream of mTORC1). Phenotypically, the mice exhibit hyperactivity, tremulousness, seizures, poor weight gain, and a median survival of 33 days. Many of these clinical and pathological features approximate those found in patients with tuberous sclerosis (5,6). In a series of elegantly designed studies

by Meikle et al., the *Tsc1* neuronal model was used to test the efficacy of early treatment of these mice with rapamycin and the rapamycin derivative RAD001. The data are very carefully and systematically analyzed, and a combination of genetic, histologic, biochemical, and pharmacokinetic studies are presented. After demonstrating substantial penetration of rapamycin into the CNS, with increased penetration in younger mice, the investigators determined that an appropriate dose for the studies was 6 mg/kg every other day, and treatment was begun at postnatal day 7–9 (P7–P9). The therapy dramatically prolonged life span, with over 90% of animals surviving to at least 80 days and most beyond 100 days; there was a reduction in tremor and other abnormal motor behaviors as well. Pathologically, myelination improved and cell enlargement was reduced in the rapamycin-treated animals, but the dysplastic features and migration defects of the neurons appeared unchanged, probably because these developmental aspects are completed before age P7. Importantly, when treatment with rapamycin was terminated at P30 (i.e., after only 23 days of treatment), the beneficial effects persisted, with continued phenotypic improvement as well as median survival of 78 days.

Lacking in the Meikle data is electrophysiological studies of seizures accompanying tuberous sclerosis complex. In fact, it is not clear how the Meikle group distinguished the whole body tremors they observed from seizures. Fortuitously, an unrelated investigation on rapamycin's effect on seizures was published by Zeng, almost simultaneously with Meikle's publication. Like Meikle et al., Zeng and colleagues performed genetic and histologic studies, but they also collected electrophysiological data, employing video-EEG monitoring to compare rapamycin- and vehicle-treated knockout mice. Their studies used the astrocytic *Tsc1* conditional knockout mouse, in which astrogliosis develops throughout the brain beginning at 3 weeks of age, with the neocortex and hippocampus being most severely affected. These animals have disorganization in the pyramidal cell layer of the hippocampus and reduced astrocytic expression of the glutamate transporters Glt-1 and GLAST, effects that may have epileptogenic sequelae. Phenotypically, these animals develop seizures, progressive encephalopathy, interictal EEG abnormalities, and premature death (7).

Importantly, Zeng et al. compared initiation of treatment at P14 (prior to onset of neurological abnormalities) to initiation at 6 weeks of age (late treatment, after clinical seizures appear). Their data support the benefits of early treatment with rapamycin, which was successful both in preventing astrogliosis and increasing Glt-1 expression, toward control levels. While seizures were routinely detected in the knockout mice by 1–2 months of age, no seizures were recorded in the early-treatment group (which was monitored up to 17 weeks). The progressive EEG changes were prevented, and

the decreased feeding accompanying encephalopathy was reduced in rapamycin-treated animals, although weight gain was somewhat better in vehicle-treated normal controls than in rapamycin-treated controls, suggesting rapamycin itself may have some systemic gastrointestinal side effects. While all untreated knockout mice died by 4 months of age, over 90% of the treated mice survived for at least 6 months. Regrettably, in contrast to the data presented in the preceding paper, seizures in the early treatment group did appear as soon as treatment ceased, indicating that continued long-term treatment would be necessary to sustain the benefits. However, the bigger surprise here was that late treatment was not entirely ineffective. In fact, in epileptic knockout animals in which treatment was initiated only at 6 weeks of age, seizures were dramatically suppressed, EEG abnormalities were reduced, and survival was markedly improved (i.e., no deaths while on treatment). Furthermore, although astrogliosis was not reversed, it appeared to be stopped in its tracks.

The potential for clinical application of these findings is expedited by the fact that rapamycin is an agent that is already FDA approved and available. In fact, its use has already expanded beyond immunosuppression for transplant patients, with rapamycin and rapamycin-derivatives gaining popularity as anticancer agents (8). Clinicians must proceed with caution, of course, since the agent has not been routinely used in children, and it may severely impact normal growth and development. Cognitive side effects also might be predicted, since the mTOR pathway participates in synaptic transmission (9) and plasticity (10). Nevertheless, considering the limited options for a child with tuberous sclerosis, rapamycin may offer, for the first time, a means to prevent epilepsy—the dream of every epileptologist and epilepsy researcher. With continued studies using these animal models, the future of epilepsy research and

the potential for prevention (the only true cure for epilepsy) are bright indeed.

by Lisa R. Merlin, MD

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WHEN IT COMES TO GABAERGIC RESPONSES AND NEONATAL SEIZURES—SEX MATTERS!

Dissociated Gender-Specific Effects of Recurrent Seizures on GABA Signaling in CA1 Pyramidal Neurons: Role of GABA_A Receptors. Galanopoulou AS. *J Neurosci* 2008;28(7):1557–1567. Early in development, the depolarizing GABA_Aergic signaling is needed for normal neuronal differentiation. It is shown here that hyperpolarizing reversal potentials of GABA_Aergic post-synaptic currents (E_{GABA}) appear earlier in female than in male rat CA1 pyramidal neurons because of increased potassium chloride cotransporter 2 (KCC2) expression and decreased bumetanide-sensitive chloride transport in females. Three episodes of neonatal kainic acid-induced status epilepticus (3KA-SE), each elicited at postnatal days 4 (P4)–P6, reverse the direction of GABA_Aergic responses in both sexes. In males, 3KA-SE trigger a premature appearance of hyperpolarizing GABA_Aergic signaling at P9, instead of P14. This is driven by an increase in KCC2 expression and decrease in bumetanide-sensitive chloride cotransport. In 3KA-SE females, E_{GABA} transiently becomes depolarizing at P8–P13 because of increase in the activity of a bumetanide-sensitive NKCC1 (sodium potassium chloride cotransporter 1)-like chloride cotransporter. However, females regain their hyperpolarizing GABA_Aergic signaling at P14 and do not manifest spontaneous seizures in adulthood. In maternally separated stressed controls, a hyperpolarizing shift in E_{GABA} was observed in both sexes, associated with decreased bumetanide-sensitive chloride cotransport, whereas KCC2 immunoreactivity was increased in males only. GABA_A receptor blockade at the time of 3KA-SE or maternal separation reversed their effects on E_{GABA} . These data suggest that the direction of GABA_A-receptor signaling may be a determining factor for the age- and sex-specific effects of prolonged seizures in the hippocampus, because they relate to normal brain development and possibly epileptogenesis. These effects differ from the consequences of severe stress.

COMMENTARY

Neuronal development requires the precise interplay of ionic currents and neurotransmitters, some of which are under hormonal control, that is, they differ by gender (1). The depolarizing action of GABA early in development is well established and likely plays an essential trophic role in neuronal maturation (2,3). Before about postnatal day 14 (P14) in the rat, activation of GABA_A receptors elicits neuronal depolarization rather than hyperpolarization, which emerges later. The early GABAergic depolarization permits calcium influx, which subsequently activates a wide variety of intracellular processes critical for normal neuronal proliferation, migration, and differentiation. This effect is due to differential expression over time of two different chloride (Cl^-) transporters. In the neonatal period, the Cl^- transporter, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter isoform 1 (NKCC1), is predominantly expressed, leading to intracellular Cl^- accumulation and depolarization upon activation of GABA_A receptors. Gradually, over the first 4 weeks of development, the expression of NKCC1 decreases and potassium chloride cotransporter 2 (KCC2) increases. KCC2 transports Cl^- out the cell, resulting in reduction of intracellular chloride concentration so that when GABA_A receptors are activated, Cl^- efflux and hyperpolarization occur.

There has been a recent explosion of research on the developmental regulation of Cl^- as it relates to seizure activity (4).

The most vulnerable age at which seizures occur, the first 1–2 weeks of life in the rat and the first couple of months in the humans, have been attributed, at least partially, to this ontogenetic Cl^- regulatory mechanism. In both species, transition from the seizure-prone state correlates with appearance of classic hyperpolarizing GABA responses. Importantly, pharmacologic alteration of these innate responses could lead to novel treatment for human neonatal seizures, for which an ideal therapy does not currently exist. GABAergic drugs, long used to treat human neonatal seizures although often ineffective in suppressing them, could have gender-, age-, and region-specific actions (5). Bumetanide, a diuretic that blocks NKCC1, can prevent excessive GABA depolarization and avert the neuronal hyperexcitability underlying neonatal seizures (6). Therefore, this class of drug shows promise for a potential anticonvulsant effect in neonates.

Until recently, little research has been performed to investigate a relationship between GABA responses and gender. Evidence now exists that males have a longer duration of depolarizing GABAergic responses than females, related to differential timing in the expression of the KCC2 and NKCC1 Cl^- cotransporters (7,8). The current paper describes a number of surprising and potentially important findings, showing that in regard to GABA responses and neonatal seizures, sex does matter! Galanopoulou investigated several aspects of the maturation of the GABAergic system with reference to animal gender. In neonatal rat hippocampal slices, the gramicidin perforated patch-clamp technique was used to study CA1 pyramidal neurons. The author first demonstrated that the GABA

reversal potential (E_{GABA}) and the difference between the E_{GABA} and E_{GABA} resting potential ($E_{\text{GABA}} - V_r$, reflecting the driving force on Cl^- ions) was larger (i.e., more negative) for females than males. In fact, the driving force changed from depolarizing to hyperpolarizing on P14 in males yet was hyperpolarizing at *all* postnatal ages checked (P4–18) for females. If verified, this finding would have profound implications, as it suggests that in postnatal females (at least over P4), GABA elicits only hyperpolarizing responses. The question of whether females *ever* have depolarizing GABA responses is not answered by this or any other study to date. The implications of this finding are that females might have less overall depolarization than males and therefore, less tendency to develop seizures because of more pronounced early GABA_A ergic inhibition (9). Previous animal studies used only male rats or did not specify the gender, making any claim for timing of the depolarizing/hyperpolarizing GABA switch uncertain.

In terms of human neonates, these results could explain, in part, the greater propensity of male babies to seize. While definitive data are lacking, extant literature suggests that neonatal males are more prone to neurological disorders of several types than females (10,11). Etiology plays a major role in neonatal seizure occurrence, but male infants suffer a disproportionately higher frequency of many early brain insults, including seizures, independent of other comorbidities (12). It would be important to control for etiology and determine whether a GABA/gender difference plays a role in epilepsy pathogenesis.

To determine the effects of neonatal seizures on GABA_A ergic responses as a function of gender, the author induced three episodes of kainic acid (KA) status epilepticus on consecutive days from P4–P6. GABA became hyperpolarizing at an earlier age (around P9 vs P14) in males than females with three KA-induced seizures. Paradoxically, GABA elicited depolarizing responses between P8 and P13 in males before becoming hyperpolarizing at P14. This most surprising result, suggesting a gender-specific shift in GABA_A ergic postsynaptic responses that are due to neonatal seizures, requires explanation. Galanopoulou begins this search by examining the expression of the two chloride cotransporters in neonatal rats of each gender. Control females had higher immunoreactivity of KCC2 at earlier ages, corresponding to their earlier GABA hyperpolarizing effects. The three KA seizures affected each gender differently. Males with seizures had greater KCC2 immunoreactivity than controls, again correlating with their enhanced GABA hyperpolarizing response. In pups with seizures, there also were gender-specific effects of the NKCC1 blocker bumetanide on

chloride transport: it was decreased in males and increased in females. The GABA antagonist bicuculline reverses the effects of KA seizures on E_{GABA} in rats of both genders, verifying a role of GABA_A receptors in this effect.

Emerging evidence suggests that gender is a critical variable in physiological responses in the developing brain. Investigators have long ignored this issue. However, the possibility that there could be both age- and sex-specific treatments for neonatal seizures is enticing and warrants further exploration.

by Carl E. Stafstrom, MD, PhD

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THE SPINE LOSS PARADOX: CLUES TO MECHANISMS AND MEANING

A Cellular Mechanism for Dendritic Spine Loss in the Pilocarpine Model of Status Epilepticus. Kurz JE, Moore BJ, Henderson SC, Campbell JN, Churn SB. *Epilepsia* 2008 May 8. [Epub ahead of print] **PURPOSE:** Previous studies have documented a synaptic translocation of calcineurin (CaN) and increased CaN activity following status epilepticus (SE); however, the cellular effect of these changes in CaN in the pathology of SE remains to be elucidated. This study examined a CaN-dependent modification of the dendritic cytoskeleton. CaN has been shown to induce dephosphorylation of cofilin, an actin depolymerization factor. The ensuing actin depolymerization can lead to a number of physiological changes that are of interest in SE. **METHODS:** SE was induced by pilocarpine injection, and seizure activity was monitored by video-EEG. Subcellular fractions were isolated by differential centrifugation. CaN activity was assayed using a paranitrophenol phosphate (pNPP) assay protocol. Cofilin phosphorylation was assessed using phosphocofilin-specific antibodies. Cofilin-actin binding was determined by coimmunoprecipitation, and actin polymerization was measured using a triton-solubilization protocol. Spines were visualized using a single-section rapid Golgi impregnation procedure. **RESULTS:** The immunoreactivity of phosphocofilin decreased significantly in hippocampal and cortical synaptosomal samples after SE. SE-induced cofilin dephosphorylation could be partially blocked by the preinjection of CaN inhibitors. Cofilin activation could be further demonstrated by increased actin-cofilin binding and a significant depolymerization of neuronal actin, both of which were also blocked by CaN inhibitors. Finally, we demonstrated a CaN-dependent loss of dendritic spines histologically. **DISCUSSION:** The data demonstrate a CaN-dependent, cellular mechanism through which prolonged seizure activity results in loss of dendritic spines via cofilin activation. Further research into this area may provide useful insights into the pathology of SE and epileptogenic mechanisms.

COMMENTARY

For many years—indeed, more than a 100 years—it has been known that neurons in the brains of humans and animals with epilepsy have a decreased density of spines on dendrites (1). Dendritic spines are the major sites of excitatory synaptic transmission onto hippocampal and neocortical pyramidal cells. The loss of spines might be interpreted as a reduction in the number of excitatory synapses impinging on these cells and therefore, a decrease in neuronal excitability. Yet, just the opposite is observed in epilepsy, a chronically hyperexcitable state—thus, the paradox. How can one reconcile an apparent decrease in excitatory synaptic transmission with epilepsy? Most often, network, cellular, and molecular remodeling are studied in an attempt to explain this apparent paradox.

In this paper, Kurz and colleagues use biochemical and anatomical techniques to investigate the mechanisms of acute dendritic spine loss following pilocarpine-induced status epilepticus (SE) (2). Video-EEG recordings stage the time course and severity of SE in each rat, ensuring that rats entered into the study had comparable seizures. The authors report that the calcium-regulated phosphatase, calcineurin, is increased in synaptic membrane fractions shortly after (15–20 minutes) the onset of SE, and this effect results in the dephosphorylation of proteins that are found in spines. One of these proteins, cofilin, is a small peptide that is thought to regulate actin in dendritic spines. Dephosphorylation of cofilin leads to enhanced binding to filamentous actin and to its depolymerization, which con-

ceivably could result in the collapse of dendritic spines since filamentous actin is the major cytoskeletal protein in spines. Importantly, the effects of SE on cofilin phosphorylation and its binding to actin are blocked by two different calcineurin inhibitors, FK-506 and cyclosporine A. Moreover, decreases in spine density on hippocampal and neocortical neurons produced by 1 hour of SE were prevented by FK-506.

These results implicate that the activation of calcineurin during prolonged seizures in dendritic spine loss and suggest it is a key molecular participant in this phenomenon. The findings are complemented by another recent report by Zeng and colleagues that also showed that dendritic spine loss during SE was suppressed by pretreatment with the calcineurin inhibitor, FK-506 (3). This study employed multiphoton imaging in intact mice that express green fluorescent protein (GFP) in neocortical pyramidal cells, as previously described in an *Epilepsy Currents* commentary (4). One major difference between the results reported by Kurz et al. and Zeng et al. is that in the latter study a marked, but transient beading of dendrites also was observed. Indeed, the dendrites took on the appearance of a string of beads, at which time few spines were apparent. Kurz et al. do not report the presence of dendritic beading in their Golgi stained sections, leaving the reader to consider reasons for the disparate outcomes. A major difference between the two studies is that Kurz et al. used a rat pilocarpine model and Zeng et al. a mouse kainate model. Another distinguishing feature may be the severity of SE, since Zeng et al. only observed beading once an animal underwent 1 hour of stage 5 SE. Nonetheless, both studies report major decreases in dendritic spine density and implicate calcineurin in this process. While the Zeng et al. study is primarily an imaging study, Kurz and colleagues rely on

numerous biochemical assays to convincingly show calcineurin enrichment in synaptic membrane fractions following SE and to elucidate the downstream effects of this phosphatase on spine proteins.

The results of these studies revisit the long-standing question in epilepsy research: what is the meaning of spine loss, and for this particular study, of the acute effects of SE on dendrites? Instances of dendritic beading concurrent with spine loss might suggest excitotoxic dendritic injury; that is, spine loss may merely be a consequence of the severe seizures. However, for situations in which beading is not seen but spine loss is prominent, a neuroprotective or adaptive role for spine loss is possible and may be equated to a loss of excitatory synapses on dendrites. Earlier studies in dissociated neuronal cultures showed that when NMDA was briefly applied to neurons, spine loss occurred without beading (also in a calcineurin-dependent manner) (5). This finding suggests that neurons may attempt to compensate for excessive excitatory synaptic input by eliminating their spines.

There has been much speculation as to whether there are long-term consequences of spine loss following SE. One possibility is that spine loss is permanent and contributes to cognitive deficits seen in chronic epilepsy. A decrease in the normal complement of glutamatergic synapses on hippocampus or neocortex would be expected to impact learning and memory, given their roles in long-lasting forms of synaptic plasticity. However, an earlier study using the pilocarpine model suggested that spine number recovers after SE, only to decrease again once spontaneous seizures emerge (6). While these results have yet to be confirmed, they call into question the importance of acute spine loss in cognitive deficits seen in chronic epilepsy. In contrast, Kurz et al. suggest that acute spine loss could be an early step in epileptogenesis. Spines could regrow, yet the complement of spines might be quite different than that before SE, with remodeling of the molecular make-up and biophysical proper-

ties of spines contributing to the neuronal hyperexcitability of epilepsy.

While clearly there is much more to be learned, the importance of the Kurz et al. and similar studies is that they provide important clues to potential molecular mechanisms responsible for spine loss and offer experimental tools to unravel the role of spine loss in epileptogenesis and/or in cognitive deficits associated with some forms of epilepsy. Many experiments can be envisioned in this regard. For instance, would pretreatment with a calcineurin inhibitor not only prevent spine loss during SE but also the development of spontaneous seizures? Results from an earlier study suggest this may be the case (7). If so, this information would provide a unique point of entry for new molecular studies—hopefully leading to the day when the spine loss paradox is no longer paradoxical.

by John W. Swann, PhD

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SELECTIVE CHANGES IN HIPPOCAMPAL GABA_A RECEPTORS DURING STATUS EPILEPTICUS

Subunit-Specific Trafficking of GABA_A Receptors During Status Epilepticus. Goodkin HP, Joshi S, Mtchedlishvili Z, Brar J, Kapur J. *J Neurosci* 2008 5;28(10):2527–2538. It is proposed that a reduced surface expression of GABA_A receptors (GABARs) contributes to the pathogenesis of status epilepticus (SE), a condition characterized by prolonged seizures. This hypothesis was based on the finding that prolonged epileptiform bursting (repetitive bursts of prolonged depolarizations with superimposed action potentials) in cultures of dissociated hippocampal pyramidal neurons (dissociated cultures) results in the increased intracellular accumulation of GABARs. However, it is not known whether this rapid modification in the surface-expressed GABAR pool results from selective, subunit-dependent or nonselective, subunit-independent internalization of GABARs. In hippocampal slices obtained from animals undergoing prolonged SE (SE-treated slices), we found that the surface expression of the GABAR $\beta 2/3$ and $\gamma 2$ subunits was reduced, whereas that of the δ subunit was not. Complementary electrophysiological recordings from dentate granule cells in SE-treated slices demonstrated a reduction in GABAR-mediated synaptic inhibition, but not tonic inhibition. A reduction in the surface expression of the $\gamma 2$ subunit, but not the δ subunit was also observed in dissociated cultures and organotypic hippocampal slice cultures when incubated in an elevated KCl external medium or an elevated KCl external medium supplemented with NMDA, respectively. Additional studies demonstrated that the reduction in the surface expression of the $\gamma 2$ subunit was independent of direct ligand binding of the GABAR. These findings demonstrate that the regulation of surface-expressed GABAR pool during SE is subunit-specific and occurs independent of ligand binding. The differential modulation of the surface expression of GABARs during SE has potential implications for the treatment of this neurological emergency.

COMMENTARY

Status epilepticus (SE) is a life-threatening condition that afflicts more than 150,000 people per year in the United States alone (1). Although the criteria for classifying a prolonged seizure or repetitive seizures as being definitively SE have been the subject of ongoing debate, it is clear that a longer duration of seizure, particularly the generalized convulsive type lasting 30 minutes or more, carries a much higher risk of neuronal injury and permanent neurological sequelae. Therefore, an understanding of the mechanisms that underlie the transition from typical brief, self-limited seizures to this dangerous form of seizure are important to understand as the knowledge may lead to more effective treatment or preventative strategies.

Neurons have a remarkable ability to acutely modify their excitability through alterations in the function or expression of ion channels and in receptors or other proteins. During prolonged seizures, some of these changes may be adaptive responses to high levels of activity, but others may be maladaptive and enhance seizure susceptibility. In the clinical setting, it is known that the longer a seizure lasts, the less likely it is to stop spontaneously and the more likely it is to require intensive medical intervention (2). In addition, longer seizures are more apt to be resistant to existing therapies, leading to even greater delays in successful treatment and poorer prognosis for recovery (3). These clinical findings have led to the hypothesis that pro-

longed epileptiform activity results in a collapse of the brain's inhibitory mechanisms, which is most likely due to changes in the GABA-mediated neurotransmission.

Changes in GABAergic neurotransmission have been explored in animal models of SE and of chronic epilepsy. Studies have revealed alterations in phasic (synaptic) inhibition as well as in tonic inhibition, mediated by ambient GABA acting at extrasynaptic receptors. Both types of inhibition have important but unique roles in limiting neuronal excitability, and they depend on distinct subsets of GABA_A receptors composed of different subunit combinations within the pentameric GABA_A receptor complex. The synaptic receptors comprise α and β subunits along with $\gamma 2$ subunits, while extrasynaptic receptors contain δ instead of $\gamma 2$ subunits and mostly utilize the $\alpha 4$ -subunit. Earlier studies (4,5) had demonstrated that GABA_A receptors are internalized and synaptic inhibition is reduced during SE, while tonic inhibition may be increased (5). Other animal studies have shown that prolonged seizures become resistant to the ability of benzodiazepines to stop them (6) and synaptic GABA responses become less sensitive to these drugs (7). Taken together, these studies have suggested that rather than nonspecific internalization of GABA_A receptors, SE may induce specific alterations in the complement of GABA_A receptors mediating the different types of inhibition. Thus, Goodkin and colleagues investigated the SE-induced changes in GABA_A receptors and the physiological consequences to address this possibility.

In their study, Goodkin et al. induced SE in rats using the muscarinic agonist, pilocarpine, then rapidly prepared

hippocampal slices for analysis. They used biochemical techniques to quantify GABA_A receptor subunits that were located on the surface of neurons and indeed, found subunit-specific changes. In particular, the surface expression of $\beta 2/3$ and $\gamma 2$ subunits were markedly reduced compared with control slices, but the δ subunit was unchanged. The total cellular content of all of these subunits remained unchanged, indicating that receptors containing $\beta 2/3$ and $\gamma 2$ subunits had been translocated from the surface to the interior of the cells. To show that the changes were functionally significant, the investigators used electrophysiological techniques to record inhibitory synaptic responses and tonic GABA-mediated currents in dentate granule cells of hippocampal slices. After SE, the amplitude and frequency of miniature inhibitory synaptic currents (i.e., the responses to release of single vesicles of GABA) were reduced compared with those from control animals. In contrast, the authors found no difference between the groups in the tonic currents recorded in dentate granule cells, in agreement with the lack of change in the surface δ subunit. However, when GABA uptake was blocked, the tonic current in the dentate granule cells from SE-treated rats was greater than that from control rats. There is no obvious explanation for this finding, except that there appears to be a change in the steady-state balance of GABA release and reuptake (by GABA transporters) during SE. Therefore, although there was no significant change in the surface expression of δ subunits that mediate most of the tonic GABA-mediated current, other alterations, possibly in GABA release, reuptake, or surface expression of other receptor subunits, are likely to occur in SE.

To further explore the cellular mechanisms underlying these changes in GABA_A receptors during SE, the authors used a model of hyperexcitability in cultured hippocampal neurons. Incubation of dissociated neuronal cultures with elevated extracellular potassium depolarizes neurons and increases network activity, and this treatment resulted in a decrease in surface $\gamma 2$, but not δ , subunits within 15 minutes, much like the *in vivo* SE results. In organotypic hippocampal cultures, which more closely resemble the synaptic organization of intact hippocampus, the investigators were able to achieve similar results to those using dissociated cultures, but the addition of NMDA to the elevated potassium solution was required for the effect. Replication of the *in vivo* results *in vitro* allowed the investigators to analyze the mechanisms of subunit-specific trafficking in more detail.

Similar to the trafficking of many other receptor types, clathrin-dependent endocytosis appears to be the major route of GABA_A receptor internalization and may thereby contribute to a dynamic regulation of inhibition through surface expression of receptors (8). Making the extracellular solution hyperosmolar by the addition of sucrose, which impairs clathrin-mediated

endocytosis, partially inhibited the high potassium-induced reduction in surface $\gamma 2$, suggesting that this mechanism was playing a role in SE-induced internalization of receptors. In addition, because a dependence on ligand binding for internalization is common among other receptor types, the authors considered whether activation of GABA_A receptors was the stimulus for their internalization. Incubation of organotypic cultures with either GABA, GABA and an uptake inhibitor, or muscimol (a GABA_A agonist) failed to reproduce the reduction in surface $\gamma 2$ seen with SE. Although these results do not rule out the possibility that activation of GABA_A receptors is required for internalization to occur, they indicate that nonspecific activation of receptors by bath application of GABA is not sufficient to explain the observed SE-induced changes.

The findings in this study may help to explain two phenomena occurring in experimental, and possibly human, SE. First, the tendency of prolonged seizures to continue rather than spontaneously terminate, as most seizures do, may result, at least partially, from weakened inhibition that is due to internalization of GABA_A receptors within inhibitory synapses. Second, the development of resistance to the therapeutic benefit of benzodiazepines may be related to the selective loss of surface $\gamma 2$ -containing receptors within the synapses and the relative preservation of δ -containing extrasynaptic receptors that are insensitive to benzodiazepines. Clearly, there are other adaptive and maladaptive changes that occur with the extreme stress incurred during SE. For instance, chloride homeostasis is altered, which also will influence GABA-mediated inhibition. The importance of the specific changes described in this study relative to the many molecular and cellular processes that accompany prolonged seizure activity is presently unknown. Moreover, many experimental animals and human patients who experience SE will subsequently develop epilepsy, and the role of these acute changes in GABA_A receptors in the epileptogenic process is not clear. In conclusion, while Goodkin et al. have demonstrated that there is a complex dynamic regulation of receptor cell surface expression during SE that alters the balance between synaptic and tonic inhibition, many questions still remain about how and why the changes occur and about their ultimate consequences. The findings they report and future studies in this area will undoubtedly influence the way SE is understood and treated in humans.

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