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Dissociation of Synchronization and Excitability in Furosemide Blockade of Epileptiform Activity

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Furosemide, a chloride cotransport inhibitor, reversibly blocked synchronized burst discharges in hippocampal slices without reducing the pyramidal cell response to single electrical stimuli. Images of the intrinsic optical signal acquired during these slice experiments indicated that furosemide coincidentally blocked changes in extracellular space. In urethane-anesthetized rats, systemically injected furosemide blocked kainic acid–induced electrical discharges recorded from cortex. These results suggest that (i) neuronal synchronization involved in epileptiform activity can be dissociated from synaptic excitability; (ii) nonsynaptic mechanisms, possibly associated with furosemide-sensitive cell volume regulation, may be critical for synchronization of neuronal activity; and (iii) agents that affect extracellular volume may have clinical utility as antiepileptic drugs.

The two primary features that characterize seizure-associated ("epileptiform") electrical activity in the brain are hyperexcitability and hypersynchronization (*1*). Although either of these features alone might be sufficient for the development and spread of epileptiform activity, it has not been possible to dissociate them clearly in experimental models of epilepsy. Much basic research on epileptogenesis has focused on synaptic mechanisms underlying changes in excitability (*2*), but some studies have suggested that nonsynaptic mechanisms might be sufficient to produce hyperexcitability or hypersynchrony. For example, exposing hippocampal slices to Ca^{2+}-free medium abolishes synaptic transmission but produces synchronized burst discharges in the CA1 subfield (*3*). Investigators have argued that this synchronized activity is mediated by ephaptic interactions among the densely packed CA1 neurons (*4*). More generally, it has long been supposed that changes in the extracellular space (ECS) could play an important role in modulating normal activity and contributing to pathological levels of excitability or synchronization in the central nervous system (*5*).

A number of studies have indicated that a major component of ECS volume regulation under normal and pathological conditions is glial dependent (*6*), and that the furosemide-sensitive Na,K,2Cl cotransporter plays an important role in this function (*7*). In tissue slices, changes in ECS volume associated with synaptic activity can be monitored by measuring changes in tissue light-scattering or reflectance (intrinsic optical signal) (*8*). Stimulus-evoked changes in light transmission through the CA1 region of hippocampal slices are blocked by furosemide (*9*), but furosemide-treated tissue continues to show normal—or even enhanced—excitatory synaptic responses (*9–11*). Because the effect of furosemide on the ECS suggests a blockade of nonsynaptic synchronization mechanisms, but its effect on individual neurons suggests an enhancement of synaptic excitability, we performed a series of studies using hippocampal slices to investigate the effects of furosemide on epileptiform discharges. To this end, spontaneous epileptiform activity was elicited by a variety of treatments (*12*).

In the first of these procedures, episodes of afterdischarges were evoked by electrical stimulation of the Schaffer collaterals (*12, 13*), and the extracellular field response was monitored in the CA1 pyramidal cell region. The CA1 response to synaptic input was first tested by recording the field potential evoked by a single stimulus pulse. In

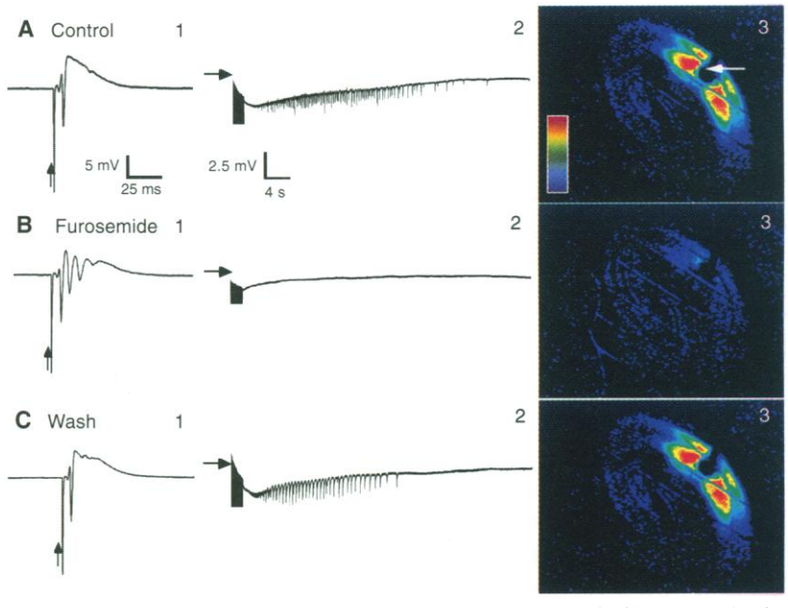

Fig. 1. Effect of furosemide on stimulation-evoked afterdischarge activity. (**A**) Two seconds of stimulation-elicited afterdischarge activity. (A1) The CA1 field response to a single 200-μs test pulse (artifact at arrow) delivered to the Schaffer collaterals. (A2) A typical afterdischarge episode recorded by the extracellular electrode (baseline shown by horizontal arrow). (A3) A map of the peak change in optical transmission through the tissue (reflecting a decrease in extracellular space) evoked by Schaffer collateral stimulation; the region of maximum optical change (red, yellow) corresponds to the apical and basal dendritic regions of CA1 on either side of the stimulating electrode (position indicated by white arrow). (**B**) Responses to stimulation after 20 min of perfusion with medium containing 2.5 mM furosemide. (**C**) Restoration of initial response patterns after 45 min of perfusion with normal bathing medium. For this and the following electrophysiological recordings, negative polarity is downward.

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normal bathing medium, Schaffer collateral stimulation evoked a single population spike (Fig. 1A1); tetanic stimulation evoked ~30 s of afterdischarge (Fig. 1A2) associated with a large change in intrinsic signal (*14*) (Fig. 1A3). After 10 min of perfusion with 2.5 mM furosemide added to the bathing medium, afterdischarge activity and associated optical changes were completely blocked (Fig. 1, B2 and B3). However, at the same time, multiple population spikes were elicited by the single test pulse (Fig. 1B1). Furosemide washout reversed these changes (Fig. 1C). These opposing effects of furosemide—blockade of the stimulation-evoked afterdischarges and a concomitant increase of the synaptic response to a test pulse—illustrate the two key results that were observed in all of our in vitro models: (i) furosemide blocked epileptiform activity, and (ii) synchronization (as reflected in spontaneous epileptiform activity) and excitability (as reflected in the response to a single synaptic input) were dissociated. Additional experiments in which the dose dependency of furosemide was examined determined that a minimum bath concentration of 1.25 mM was required to block both the afterdischarges and optical changes.

In a second series of slice experiments, spontaneous synchronized bursting activity was generated in slices perfused with high-K^+ (10 mM) bathing medium (*15*). Slices were perfused with high-K^+ medium until extended periods of spontaneous interictal-like bursting were recorded simultaneously in CA1 and CA3 pyramidal cell regions (Fig. 2A1). After 15 min of perfusion with furosemide-containing medium (2.5 mM furosemide), the burst discharges increased in magnitude (Fig. 2A2). However, after 45 min of furosemide perfusion, the bursts were blocked in a reversible manner (Fig. 2, A3 and A4). During this entire sequence of furosemide perfusion, the synaptic response to a single test pulse delivered to the Schaffer collaterals was either unchanged or enhanced during the furosemide block. The initial increase in discharge amplitude, as well as the enhanced synaptic response to single stimuli, likely reflected a furosemide-induced decrease in inhibition resulting from a depolarizing shift in the chloride equilibrium potential (*10, 11*).

Similar studies were performed with a variety of other commonly used in vitro models of epileptiform discharge (*3, 16–19*). Hippocampal-entorhinal slices were bathed in medium from which Mg^{2+} had been omitted (0 Mg^{2+}) and recordings were obtained from entorhinal cortex (Fig. 2B1). After 3 hours of perfusion with 0 Mg^{2+}, slices developed bursting patterns that appeared similar to previously described "anticonvulsant resistant" bursts (*16*). One hour after the addition of furosemide to the bathing medium, these bursts were blocked (Fig. 2B2). Furosemide also blocked spontaneous burst discharges observed with the following additions or modifications to the bathing medium: (i) addition of 200 to 300 μM 4-aminopyridine (4-AP, a potassium channel blocker) (*17*) (Fig. 2C); (ii) addition of 50 to 100 μm bicuculline [a γ-aminobutyric acid A ($GABA_A$) antagonist] (*18*) (Fig. 2D); (iii) removal of Mg^{2+} (0 Mg^{2+}) (*19*)—1-hour perfusion (Fig. 2E); and (iv) removal of Ca^{2+} plus extracellular Ca^{2+} chelation (0 Ca^{2+}) (*3*) (Fig. 2F). With each of these manipulations, spontaneous interictal-like patterns were simultaneously recorded from the CA1 and CA3 subfields (Fig. 2, C and D, shows only the CA3 trace, and Fig. 2, E and F, shows only the CA1 trace). In the 0 Ca^{2+} experiments, 5 mM furosemide blocked the bursting with a latency of 15 to 20 min. For all other protocols, bursting was blocked by 2.5 mM furosemide with a latency of 20 to 60 min. Although each model of epileptiform activity is thought to involve different physiological mechanisms (*1, 2*), furosemide blocked the spontaneous bursting activity in all experiments (Fig. 2, C to F).

Given the generality of this furosemide effect in vitro, we carried out a parallel study on an in vivo model (*20*) in which epileptiform activity was induced by intravenous injection of kainic acid (KA) into anesthetized rats (*21*) (Fig. 3). In this model, intense electrical discharge (electrical "status epilepticus") can be recorded from the cortex 30 to 60 min after KA injection (Fig. 3B). Control experiments [and previous reports (*21*)] showed that this status-like activity was maintained for over 4 hours. Intravenous injections of furosemide blocked KA-induced seizure activity with a latency of 30 to 45 min, often producing a relatively flat electroencephalogram (EEG) (Fig. 3, C and D). Even 90 min after the furosemide injection, cortical activity remained near normal baseline levels (that is, that observed before the KA and furosemide injections). Pharmacokinetic studies of furosemide in the rat suggest that the dosages used in our experiments were below toxic levels (*22*).

These experiments show that furosemide, a chloride cotransporter antagonist and common loop diuretic, can block epileptiform activity in a wide variety of experimental models, even though a spectrum of different physiological mechanisms is thought to produce the spontaneous burst discharges associated with each of the models that we tested. This finding suggests that furosemide might act on a common mechanism that is necessary for the generation of synchronized bursts. Such a mechanism is likely to be nonsynaptic because furosemide did not block synaptic responses to electri-

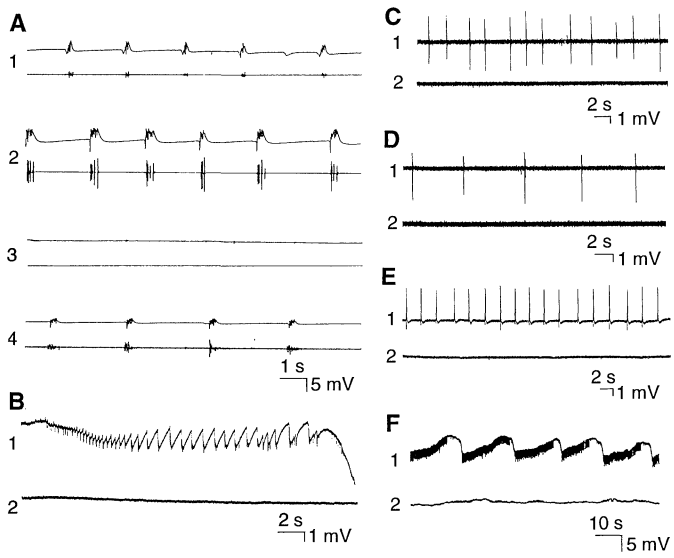

Fig. 2. Furosemide blockade of spontaneous epileptiform burst discharges in a variety of in vitro models. (**A**) Epileptiform activity was induced by slice perfusion with bathing medium containing 10 mM K^+. Field recordings were obtained simultaneously from CA3 (top trace) and CA1 (bottom trace) pyramidal cell layers. (A1) Spontaneous synchronous bursting was elicited before the addition of furosemide to the recording medium. (A2) After 15 min of perfusion with furosemide (2.5 mM), the bursts occurred with increased magnitude. (A3) After 45 min of furosemide perfusion, spontaneous bursts were blocked. (A4) Recovery of the high K^+–induced bursting pattern was seen within 1 hour of return to perfusion with normal bathing medium. (**B**) Prolonged periods of bursting activity were recorded extracellularly from the entorhinal cortex (B1) after 3 hours of perfusion with bathing medium containing no Mg^{2+}. This discharge pattern was blocked (B2) after 60 min of perfusion with medium containing 2.5 mM furosemide. (**C**) In bathing medium containing 200 μM 4-amino pyridine, spontaneous synchronous bursts were recorded in CA3 (C1); after 30 min of exposure to 2.5 mM furosemide, this activity was blocked (C2). (**D**) Bicuculline (100 μM; CA3 recording) and (**E**) 0 mM Mg^{2+} (1 hour; CA1 recording) treatments also gave rise to epileptiform discharges that were blocked by furosemide (2.5 mM). (**F**) Tissue treated with bathing medium containing no Ca^{2+} (and added EGTA) showed slow, rhythmic membrane potential shifts in CA1 (F1); these nonsynaptically mediated epileptiform events were completely blocked (F2) after perfusion with furosemide (5 mM).

cal stimulation, but did block epileptiform discharge in 0 Ca^{2+} (where synaptic activity is absent). Further, although furosemide treatment blocked spontaneous epileptiform activity, it was also often associated with a "hyperexcited" synaptic response (*10, 11*). Thus, furosemide dissociates a pure increase in synaptic excitability from the hypersynchronization that characterizes all forms of seizure activity.

We propose that the nonsynaptic mechanisms that underlie furosemide's action are related to cell volume regulation, and in particular to glial swelling (*7, 9*). This hypothesis is supported by our observation that stimulation-evoked changes in optical transmission through the slice tissue were blocked coincidentally with the furosemide block of spontaneous discharges, and is consistent with previous findings that alterations in osmolarity can block spontaneous bursts in some models of epilepsy (*15, 23–26*). Indeed, hyperosmotic conditions have been shown to decrease electrographic brain activity and reduce the likelihood of the development of seizure activity (*24–26*), whereas hypo-osmotic conditions have the opposite effects (*27*). In experimental models of status epilepticus, seizure activity can be blocked by intravenous injection of mannitol (*24*); this effect is similar to that achieved by intravenous injection of urea in human patients (*25*). The treatment in each of these cases increases the osmolarity of the blood and extracellular fluid, resulting in water efflux from cells and an increase in ECS. Because furosemide is used safely as a commonly prescribed diuretic, our results suggest that it or other related compounds might find clinical utility as antiepileptic agents. For example, acetazolamide (ACZ), another diuretic with a different mechanism of action (inhibition of carbonic anhydrase), has been studied experimentally as an anticonvulsant and is used clinically on a limited basis (*28*).

Several hypotheses could explain how changes in cell volume might affect neuronal synchronization and excitability: (i) Cell swelling and resultant ECS shrinkage may cause an increased resistivity of the ECS and thus enhance ephaptic interactions (*4*); (ii) ion channels—in either glia or neurons—may be mechanosensitive so that changes in osmotic or hydrostatic pressure could lead to altered voltage- or ligand-gated channel properties (*29*); and (iii) cell swelling may mediate the release or uptake of neuroactive substances by glial cells (*30*). Although underlying mechanisms and consequences of glial and neuronal cell swelling and extracellular volume changes are not yet completely understood, our studies suggest that modulation of the ECS can play a critical role in neuronal synchronization.

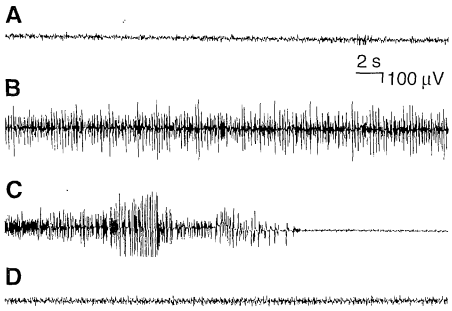

Fig. 3. Furosemide blockade of KA-evoked electrical "status epilepticus" in a urethane-anesthetized rat. Cortical EEG was recorded continuously during 3- to 4-hour experiments. (**A**) Baseline EEG activity before KA injection. (**B**) Seizure-like EEG pattern, 60 min after KA injection (12 mg/kg, intravenous). (**C**) Furosemide blockade of seizure-like EEG activity, 120 min after KA injection and 20 min after furosemide administration (two injections, each 20 mg/kg). (**D**) Complete furosemide blockade of the seizure-like EEG activity, 30 min later (150 min after KA injection). The KA-induced seizure-like EEG pattern was associated with an increase in heart rate (*31*); furosemide had no further effect on ECG.

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ECG) was monitored continuously. The jugular vein was cannulated on one side for intravenous (i.v.) drug administration. Rats were placed in a Kopf stereotaxic device (with the top of the skull level), and a bipolar stainless-steel microelectrode insulated to 0.5 mm of the tip was inserted to a depth of 0.5 to 1.2 mm from the cortical surface to record electroencephalographic (EEG) activity in the fronto-parietal cortex. Data were stored on VHS videotape and analyzed off-line. After the surgical preparation and electrode placement, animals were allowed to recover for 30 min before the experiments were initiated with an injection of kainic acid (10 to 12 mg/kg, i.v.). Intense electrographic seizure activity, an increased heart rate, and rapid movements of the vibrissae were induced with a latency of about 30 min. Strong nociceptive stimulation of the hind paw was performed intermittently to ensure that no response was produced, indicating that a sufficient level of anesthesia was maintained [S. C. Baraban, R. L. Stornetta, P. G. Guynet, *Brain Res.* **676**, 245 (1995); R. S. Sloviter, *Hippocampus* **1**, 31 (1991)]. Once stable electrical seizure activity was evident, furosemide was delivered in 20 mg/kg boluses every 30 min up to a total of three injections. Experiments were terminated with the i.v. administration of urethane. Animal care was in accordance with NIH guidelines and approved by the University of Washington Animal Care Committee.

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Discrete Cortical Regions Associated with Knowledge of Color and Knowledge of Action

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The areas of the brain that mediate knowledge about objects were investigated by measuring changes in regional cerebral blood flow (rCBF) using positron emission tomography (PET). Subjects generated words denoting colors and actions associated with static, achromatic line drawings of objects in one experiment, and with the written names of objects in a second experiment. In both studies, generation of color words selectively activated a region in the ventral temporal lobe just anterior to the area involved in the perception of color, whereas generation of action words activated a region in the middle temporal gyrus just anterior to the area involved in the perception of motion. These data suggest that object knowledge is organized as a distributed system in which the attributes of an object are stored close to the regions of the cortex that mediate perception of those attributes.

During our lifetimes we acquire knowledge about a tremendous number of concrete objects. This knowledge includes not just the name, but also the physical features (form and color) and functional properties (uses) that define each object. When an object is seen or its name read, knowledge of these attributes is activated automatically and without conscious awareness (*1*). In addition, the ability to retrieve information about a specific attribute of an object can be selectively disrupted by a focal brain lesion (*2*, *3*). These findings suggest that object knowledge is stored in the brain as a distributed network of discrete cortical areas (*4*). However, direct evidence for the existence of such a network in the normal human brain has not been reported, nor have the component areas of the network been identified. We show, using positron emission tomography (PET), that the attributes that define an object are represented close to the cortical regions that mediate perception of those attributes.

We chose to study knowledge of color and action because evidence from monkeys (*5*) and humans (*6*–*8*) suggests that the perception of these attributes is mediated, in part, by discrete regions of the posterior cortex. In humans the syndrome of acquired color blindness, or achromatopsia, has been found after damage to the fusiform and lingual gyri on the ventral surface of the occipital lobes (*6*, *7*), whereas acquired motion blindness, or akinetopsia, follows a more dorsally located lesion at the junction of the occipital, parietal, and temporal lobes (*7*, *8*). Converging evidence that these regions are specialized for the perception of color and motion, respectively, has been provided by functional brain imaging studies of normal individuals (*9*, *10*). Moreover, reports of patients with selective difficulty retrieving information about object-associated color (*2*) or action (*3*), without corresponding deficits in perception, suggest that knowledge of these attributes also may be mediated by distinct brain areas (*11*).

In the first study (*12*), achromatic line drawings of common objects were present-

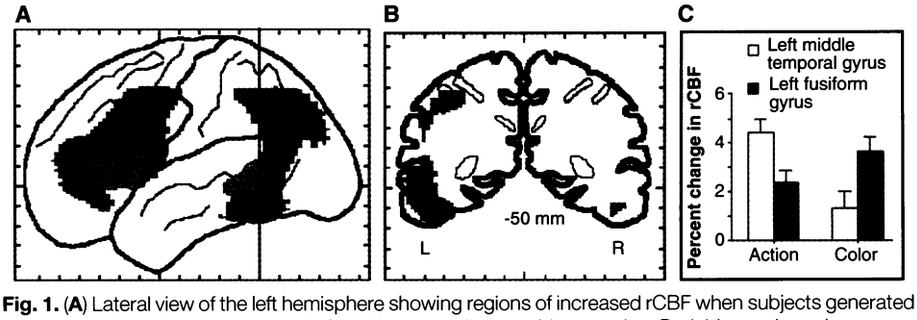

Fig. 1. (**A**) Lateral view of the left hemisphere showing regions of increased rCBF when subjects generated color words (green) and action words (gray) in comparison to object naming. Dark blue regions show areas of overlap. (**B**) Coronal section 50 mm posterior to the anterior commissure showing locations of bilateral fusiform and left parietal lobe activation during color word generation, and left temporal and parietal activations during action word generation. Shown are all pixels that exceeded a threshold of $Z = 2.58$ ($P < 0.005$, one-tailed). (**C**) Percent change in rCBF, relative to object naming, at the site of peak activity in the left middle temporal gyrus (open bar) (−50, −50, 0) and left fusiform gyrus (closed bar) (−46, −46, −12) shown in (B). Bars represent mean percent change in rCBF ± SEM. Analysis of variance indicated that rCBF at these sites was modulated by the type of word that subjects generated [site X task interaction $F(1,11) = 21.66$; $P < 0.001$].

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