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Single-unit Analysis of Substantia Nigra Pars Reticulata Neurons in Freely Behaving Rats with Genetic Absence Epilepsy

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Summary: *Purpose:* The substantia nigra pars reticulata (SNpr) is assumed to be involved in the control of several kinds of epileptic seizures, an assumption based mostly on neuropharmacologic evidence. However, only very few neurophysiological recordings from the basal ganglia support neuropharmacologic data. We investigated the electrophysiologic activity of SNpr neurons in rats with genetic absence epilepsy.

Methods: Electrocorticography (ECoG) and multi-unit recordings using permanently implanted tetrodes were obtained in freely behaving rats. After spike sorting, auto- and cross-correlation analysis was used to detect oscillatory neuronal activities and synchronizations.

Results: During interictal periods, neither oscillation nor synchronization could be observed in the firing patterns of SNpr neurons. At the beginning of the absence seizure, the firing rate increased significantly. The SNpr neurons started firing in bursts of action potentials. Bursts were highly correlated to the spike-and-wave discharges (SWDs) in the ECoG, mainly after the spike component of the cortical spike-and-wave complex. Moreover, pairs of SNpr neurons tended to fire synchronously. Before the end of the seizure, the firing rate decreased progressively, and the burst-firing pattern ended at or before the end of the SWDs. Once the SWDs had stopped, the SNpr neurons resumed their basal firing pattern as before the seizure onset.

Conclusions: These results provide electrophysiologic evidence that firing patterns and synchronization of SNpr neurons are in phase with the occurrence of SWDs. The findings support the concept that nigral control mechanisms are involved in modulating the propagation of an ongoing generalized seizure. **Key Words:** Multi-unit recordings—Tetrode—Substantia nigra pars reticulate—Freely moving—Absence epilepsy.

Epileptic seizures are thought to be generated in various neuronal networks such as the thalamocortical circuit in absence seizures or limbic circuits in temporal lobe epilepsy (1). Propagation of the ictal activity from these generating networks through different pathways is thought to be responsible for the diversity in the clinical expression of seizures. Good evidence exists from electrophysiologic recordings in humans and animals that epileptic activity also is transmitted to the basal ganglia (2–6). Over the past 20-year period, basic research in animal models of epilepsy has contributed to the general view that the basal ganglia may act as critical structures in the modulation of seizures (for reviews, see 7 and 9). Several biochemical, neuropharmacologic, or metabolic data support that inhibition of the subthalamic nucleus or the substantia nigra pars reticulata (SNpr) has antiepileptic effects. For instance, animal studies have shown that pharmacologic inhibition of one of these two structures suppresses ictal activities (8,9). This is further supported by recent clinical data in patients with severe pharmacoresistant epilepsy in whom high-frequency stimulation of the subthalamic nucleus and SNpr significantly reduced seizure frequency and severity (10,11). However, most of the evidence supporting a role for the basal ganglia in the control of seizures arises from neuropharmacologic studies. However, whether this remote-control mechanism could be endogenously triggered cannot be assumed from such invasive manipulations. Moreover, the rare electrophysiologic data published were obtained in animal models in which seizures were not spontaneous but pharmacologically or electrically induced (3–5).

The neurophysiological mechanisms underlying the control of seizures by the basal ganglia system have so far not been elucidated. To address this issue, we used an animal model of spontaneous absence epilepsy in the rat (Genetic Absence Epilepsy Rat from Strasbourg; GAERS), in

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which the involvement of the basal ganglia in seizure control has already been documented by neuropharmacologic studies (9,12). In this strain of rats, the regularly occurring spontaneous nonconvulsive seizures allow investigations of the transition phases between ictal and interictal states by electrophysiological recordings. GAERS were permanently implanted with epidural electrodes and four-channel electrodes (tetrodes) aimed toward the SNpr, the main output structure of the basal ganglia. This approach was used to allow access to both the electrocorticogram (ECoG) and the single-unit activities in the freely behaving animal. The extracellular multi-unit activity from the SNpr was recorded, and multiple single-unit signals were extracted from tetrode recordings by using standard spike-sorting procedures (13). To investigate the interaction between cortex and SNpr, correlations between activities in these structures were studied during ictal and interictal periods.

MATERIALS AND METHODS

Animals

GAERS are an inbred strain of rats that display many of the characteristics of human absence epilepsy. Their phenotype is genetically determined, and the animals have no known neurologic or behavioral deficits aside from periodic absence-type seizures (12). Absence seizures in GAERS find expression in spontaneous spike-and-wave discharges (SWDs) in the ECoG and a concomitant behavioral immobility, rhythmic twitching of the vibrissae and facial muscles, with occasional gradual and slight lowering of the head. When the animals are maintained in a state of quiet wakefulness, SWDs last for 17 ± 10 s, occur 1.3 times/min on average, and their mean cumulated duration per minute is 25 ± 8 s (12). Four adults GAERS (3 to 5 months old, 280–340 g) were used in this study. They were housed in individual acrylic cages at constant room temperature ($22 \pm 1^{\circ}$C) and maintained on a 12-h light/dark cycle (lights on at 0800). Food and water were available ad libitum. All experiments were carried out in accordance with the German Animal Welfare Act and were approved by the local animal care committee (Regierungspräsidium Freiburg, G-01/57).

Surgery

All animals were implanted under general anesthesia [diazepam (DZP; Valium, Roche), 4 mg/kg i.p., and ketamine (Ketamin 10%, Essex Pharma GmbH), 100 mg/kg i.p.]. Rectal temperature was maintained at 37.5°C by a thermostatically controlled heating blanket, and eyes were covered with cornea gel to prevent drying. Two single-contact cortical stainless steel electrodes were placed over the frontal left and parietal right cortex. A third electrode implanted behind the interaural line provided an electrical ground. A screw was fixed over the frontal right hemisphere to strengthen the structural support. A steel flexible wire was inserted into the neck muscles for standard monitoring of the electromyogram (EMG). The four-channels electrodes (tetrodes) consisted of four H-Formvar–insulated NiCr wires [size, 0.00072 inch (18-μm section); California Fine Wire, Grover Beach, CA, U.S.A.] twisted together with a noninsulated NiCr wire of the same diameter, which served as a reference (Isabellenhütte Heusler GmbH, KG, Germany). The twisted wires were inserted into a piece of silica tube (TSP075150; Polymicro Technologies, Phoenix, AZ, U.S.A.) and connected to a 12-pin microconnector (SLR 125; Bürklin OHG, Germany). The tetrode was then inserted and fixed in a home-made miniature microdrive consisting of a 20-G cannula made mobile (without rotation) by a micrometric screw (350-μm vertical drive per revolution); 2 mm of the tetrode tip extended out of the small silica tube. Animals were equipped with such a movable tetrode aimed at the dorsal border of the SNR [AP, 3.7 mm; ML, 2.2 mm; DV, 7.5 mm; lambda as the reference; coordinates derived from the atlas of Paxinos and Watson, 1986 (14)]. The mobile part of the implanted tetrode at the surface of the skull was embedded in a drop of bone wax, and the microdrive was then fixed to the skull by acrylic cement together with the cortical electrodes and the anchorage screw. A small aluminum box (12×12 mm, 20 mm high) connected to the ground electrode was adapted with acrylic cement around the microdrive to prevent damage and reduce noise during recordings. After soldering the EMG, ECoG, and ground electrodes to the 12-pin microconnector, the microconnector was then fixed with acrylic cement behind the caudal part of the aluminum box, and a female free-end microconnector (BLR 125; Bürklin OHG, Germany) was adapted to prevent bending of the connector pins. The animals were allowed a week for recovery and were handled daily for habituation.

Neurophysiologic recordings and data acquisition

Recording sessions lasted $\leq$3 h (between 08:00 and 18:00) and were performed over successive days. The animals were allowed unrestricted movements. In each recording session, one to two sets of multi-unit were recorded, allowing recording from up to five different tetrode positions in the same animal after completion of all sessions. Tetrodes were moved to a new position at the beginning of each recording session. Neurons were identified as SNpr neurons on the basis of their location and electrophysiologic features (15–17). Data were recorded for 2–15 min during periods of quiet wakefulness to allow spontaneous absence-seizure occurrence (12).

Tetrode, EMG, and ECoG signals were amplified by using a miniature headstage preamplifier (HA-8-I; voltage gain, $\times$4; frequency band, DC-5 KHz; weight without cable, 2.5 g) connected to a 16-channel filter amplifier (voltage gain, $\times$500; low cut-off frequency, 1.0 Hz; high cut-off frequency, 5 KHz) (Multi Channel Systems,

Reutlingen, Germany) and sampled at 14–17 kHz. Multi-unit activity (signal-to-noise ratio of ≥3:1) was collected on a personal computer via a CED interface (Cambridge Electronic Design, Cambridge, U.K.) by using the Spike 2 software, in parallel with analogue-to-digital sampling of ECoG signals. The digitized data were continuously streamed to the disk. Multi-unit activity was filtered off-line by using a high-pass filter (200–400 Hz). To separate signals from different neurons, clusters based on amplitude were defined manually by using "Spiker" software available in the public domain (http://mccoy.ucsf.edu) for off-line sorting of tetrode data (18).

Histology

On completion of the experiment, animals received an overdose of pentobarbital (60 mg/kg, i.p.), and each electrode track was marked with two to three electrolytic lesions (10 μA, 20 s of anodal current passing through one of the tetrode channels; distance between two lesions, 1.5 micrometric screw revolutions corresponding to 525 μm). The brain was cut on a freezing microtome into sections of 20-μm thickness. Sections were stained with cresyl violet. Recording tracks were reconstructed under light microscopy, and electrode-tip locations for recordings obtained on successive days were reconstructed by using a depth scale on the microdrive assembly.

Data analysis

Ictal and nonictal states were discriminated on the basis of the ECoG. Ictal states were identified by the occurrence of high-voltage SWDs (300–1,000 μV; 7–11 cycles/s). The first and last spike-and-wave complex, in which the size of the spike was at least 3 times the peak-to-peak amplitude of the baseline ECoG, were considered the beginning and the end of a seizure, respectively. Nonictal states corresponded to wakefulness identified by a low-amplitude and desynchronized ECoG with a sustained EMG activity. Only periods of typical stationary ECoG lasting >10 s were considered for further analysis. Discharge rate and pattern of SNpr neurons were analyzed offline by the Spike 2 analysis software. Mean discharge rates were calculated during ictal and interictal states (pre- and postictal) and compared by using Wilcoxon's test for paired data.

The positions of the spike-and-waves and action potentials within the original file containing the rough data were encoded into separate event channels by using the memory buffer function of Spike2. The discharge patterns of identified units were analyzed by using interspike interval histograms (ISHs) and autocorrelograms (ACs). ISHs were built with 500 bins of 1 ms, and asymmetry indexes were compared by using Wilcoxon's test (paired data) (19). The asymmetry index was defined as the ratio of the mode (i.e., the most frequent interspike interval) to the mean interspike interval (i.e., the reciprocal of the mean firing rate) and calculated to assess the regularity of the discharge pattern (20). An asymmetry index close to 1 reveals a relatively regular discharge pattern, whereas the more this index differs from unity, the more irregular is the spike train. ACs were computed by using the event correlation function of Spike 2 (width, 2 s; bin size, 10 ms) either during or between seizures. The same function was used to investigate the relation between unit activity and the ECoG and between activities of different units (cross-correlograms, CCs). Autocorrelation and cross-correlation analysis was used to detect oscillatory neuronal activity of single neurons and synchronization between two neurons, respectively.

All data are expressed as mean ± SEM, and the significance level for all statistical analysis was set at $p < 0.05$.

RESULTS

Of the four implanted rats, 12 recordings with multi-unit activity within the SNpr were obtained. Figure 1A shows an example of an original recording with the ECoG and the four channels of the tetrode. The spike waveform of SNpr neurons was typically biphasic (~1-ms duration). After spike sorting, 20 single units were isolated from the 12 multi-unit recordings. These neurons were located at the same rostrocaudal coordinate (+3.7 mm from lambda, according to ref. 14) throughout the whole mediolateral and dorsoventral extent of the SNpr (Fig. 1B). During interictal periods (pre- and postictal), the single SNpr units showed different patterns of background firing, irrespective of their topographic location. Eight SNpr neurons were identified as regular-firing cells (asymmetry index, 0.774 ± 0.053; firing rate, 49.0 ± 5.8 spike/s) and 12 as irregular-firing cells (asymmetry index, 0.096 ± 0.021; firing rate, 17.3 ± 3.9 spike/s).

Changes in the firing rate

Changes in the firing rate of 19 of the 20 recorded neurons were observed during ictal periods. The firing rate of these SNpr neurons increased during ictal periods (Fig. 1C). Figure 1D illustrates the average firing rates for 19 neurons (only one of 20 neurons changed neither its firing rate nor its firing pattern between ictal and interictal periods). For each neuron, data collected from five seizures (mean duration, 24.7 ± 2.9 s) were pooled, irrespective of their firing mode (no difference in the changes of the firing rate was observed between regular and irregular neurons). At the onset of a seizure, a sudden and marked increase of the firing rate was observed. This increase reached up to +81% of the basal preictal firing rate and usually lasted for 1–2 s. After this initial increase, the firing rate decreased to a plateau level at about +40% of the basal interictal firing rate, which was maintained throughout the seizure. Before the seizure stopped, the firing rate decreased rapidly to +12% during the last second of the seizure. As soon as the seizure stopped, the neurons resumed their preictal

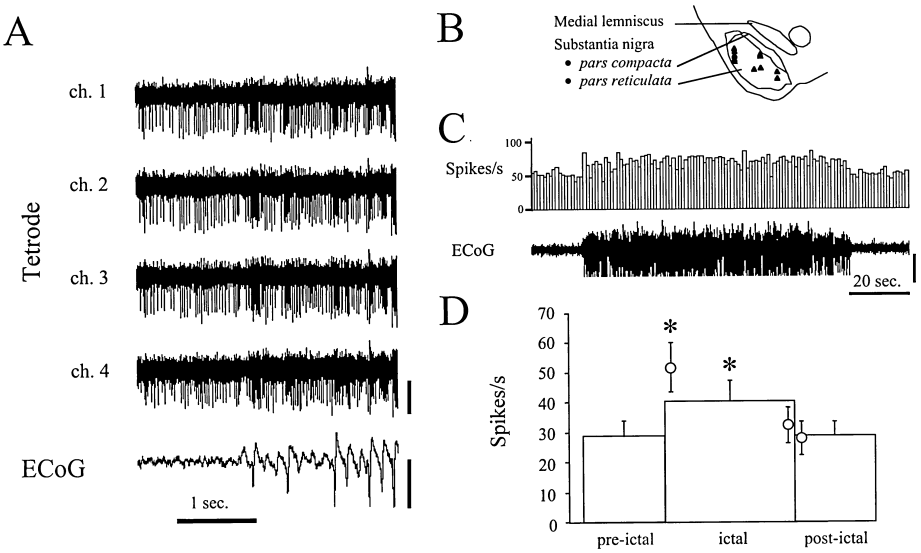

FIG. 1. Extracellular activities of substantia nigra pars reticulata (SNpr) neurons during spike-and-wave discharges (SWDs). **A:** Original recording with electrocorticography (ECoG) and the four channels of the tetrode. Calibration bars indicate 500 μV for ECoG recordings and 200 μV for the unit activity. **B:** Histologic reconstruction of the 12 tetrode recording sites (black triangles). **C:** Changes in the firing rate for a typical single-unit regularly firing SNpr neuron. **D:** Average firing rates of 19 neurons that changed their firing rate during SWDs. The white dots correspond to the mean discharge rate during the first second and last second of the ictal period and the first second during the postictal period (*$p < 0.05$ vs. preictal period, Wilcoxon test).

firing frequency, shortly after a transient decrease in firing (–3%; Fig. 1D). In four neurons, transient silences in neuronal activities lasting for ~500 ms were observed at the end of some seizures. For the same neuron, these transient silences were not constant from one seizure to another.

Changes in the firing mode

Figure 2 illustrates in more detail the changes of the firing mode of a SNpr neuron during and between seizures. The onset of the seizure was associated with a sudden reorganization of the firing mode, which persisted throughout the seizure (Fig. 2A). As soon as the first spike-and-wave complex appeared on the ECoG, the SNpr neurons started to fire in bursts of action potentials. This firing pattern was maintained throughout the seizure and ended at or just before the termination of the SWD. Once the seizure stopped, the neurons resumed their preictal firing mode, and the bursting activity was no longer observed. Figure 2B illustrates the changes for a given "regular firing" neuron, which displayed a slight increase in the asymetry index during the ictal state. However, data for both regular and irregular cells showed that such a trend for an increased asymmetry index during the ictal state was not statistically significant [0.849 ± 0.100 ($p = 0.401$) and 0.100 ± 0.023 ($p = 0.530$), respectively]. Applied to all the recorded neurons, the ictal autocorrelation function displayed the shape of an oscillation with a frequency of 7.65 ± 0.12 Hz, which corresponds precisely to the frequency of the SWD in the ECoG (7.66 ± 0.08 Hz from the ictal ECoG interspike interval histograms). The modulation depths for both the regular and irregular firing cells were significantly increased during the ictal state (interictal, 0.456 ± 0.087; ictal, 1.338 ± 0.269; $p = 0.0002$), indicating an increased correlation between spikes and SWDs. This reorganization of the firing mode into bursting activities during ictal periods was observed in 14 neurons concomitant with an increased firing rate. Only one of 20 neurons changed neither its firing rate nor its firing pattern between ictal and interictal periods (mean firing rate, 55.3 Hz), as ascertained by its flat AC (not shown). The propensity for cells to display bursting activities was not correlated with their basal interictal regular or irregular firing pattern (three nonbursting cells were identified in each group).

The correlation between units and cortical activities was supported by the cross-correlation function, from which a periodicity of 7.68 ± 0.08 Hz was obtained (Fig. 2B). The bursts generally appeared to follow the spike component of the spike-and-wave complexes (see Fig. 2A and third burst in the first upper trace in Fig. 3A2 for representative examples).

Synchronization of SNpr neurons

Combining the tetrode resources with spike sorting, seven of the 12 multi-unit activity recordings allowed discrimination between six pairs and one triad of neighboring SNpr units. In addition to similar properties of firing, these recordings showed that the generally independent firing of neighboring SNpr cells in the absence of SWDs (Fig. 3A1) became highly correlated (Fig. 3A3) and tightly time-locked to the spike-and-wave complexes during ictal periods (Fig. 3A2). During an SWD, the priority in bursting activity of a neuron over another (Fig. 3A2) was sometimes reversed, which was reminiscent of a phase displacement (not shown). Such neurons that started to fire in bursts (Fig. 3A2, upper trace) also were able to display transient lack of bursting activity (Fig. 3A4, upper trace). The triple-unit recording showed that although two cells displayed similar changes of their firing properties (units a and c), the properties of the third one (unit b) was not affected by the occurrence of the seizures (Fig. 3B).

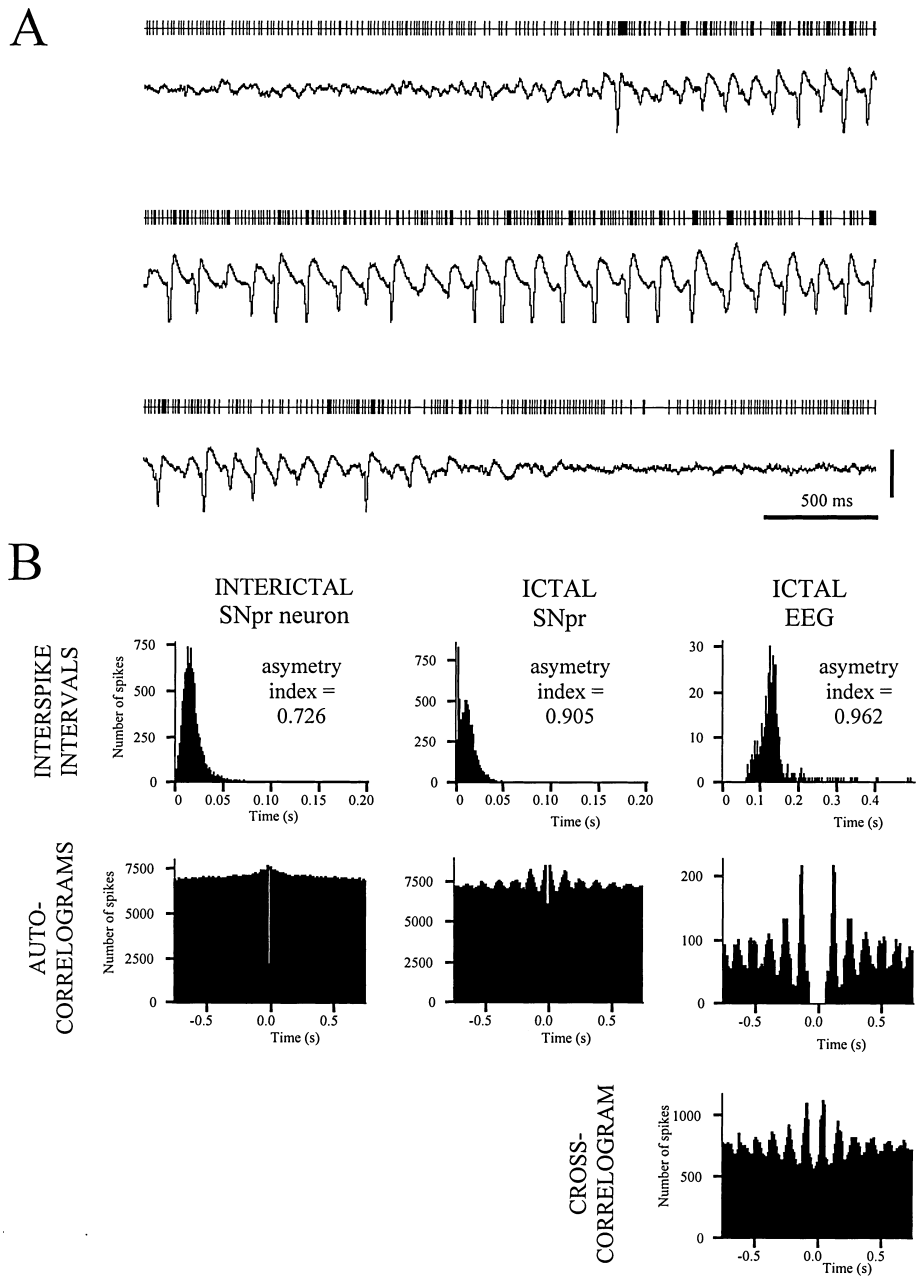

FIG. 2. Correlated activities during spike-and-wave discharges (SWDs). **A:** Changes in the firing mode of a regular-firing substantia nigra pars reticulata (SNpr) neuron during ictal periods. Calibration bar, 500 μV for electrocorticography (ECoG) recording. **B:** *Interval histograms*: high frequency of action potentials within bursts during the ictal period (301 $\pm$ 76 Hz). The asymmetry index for this regular-firing cell increases from 0.726 (interictal) to 0.905 (ictal). *Autocorrelograms*: during the ictal period, the frequency was 7.6 Hz (frequency of the SWD in the ECoG); increased modulation depth (interictal, 0.120; ictal, 0.344). *Cross-correlograms*: between-unit and cortical activities showing strong correlation between single-unit spike activity and ECoG signal during the ictal period (modulation depth, 0.763).

In some cases, a second peak in the interspike-interval histograms reflected the spacing between bursts (see unit c in Fig. 3B). No difference was observed between the ictal- and interictal-interval histograms and autocorrelograms for unit b. Cross-correlogram between the ictal ECoG and unit b was almost flat, contrasting with that of units a and c (with a modulation depth $\sim$2 and a mean interspike interval of 0.13) (not shown). Spike activity of units a and c are strongly correlated, whereas unit b is not correlated with either unit a or c. This indicates that the entire network is not synchronized during seizures. This lack of synchrony between all neighboring cells is further supported by the shape of the cross-correlograms (Fig. 3B).

DISCUSSION

Simultaneous recordings of single-unit spike activity in the SNpr and ECoG allowed us to investigate the dynamic changes in the temporal relation between single-unit activity and ECoG, and between single-unit activities during ictal and interictal states in freely moving rats with spontaneous absence seizures. The main findings of this study are (a) that in a genetic model of absence epilepsy, the basal interictal firing mode of SNpr neurons is similar to that described in nonepileptic animals; (b) changes in the firing pattern of SNpr neurons are time-locked to the occurrence of SWDs and follow a typical temporal profile; and (c) pairs of neurons in the SNpr show strong

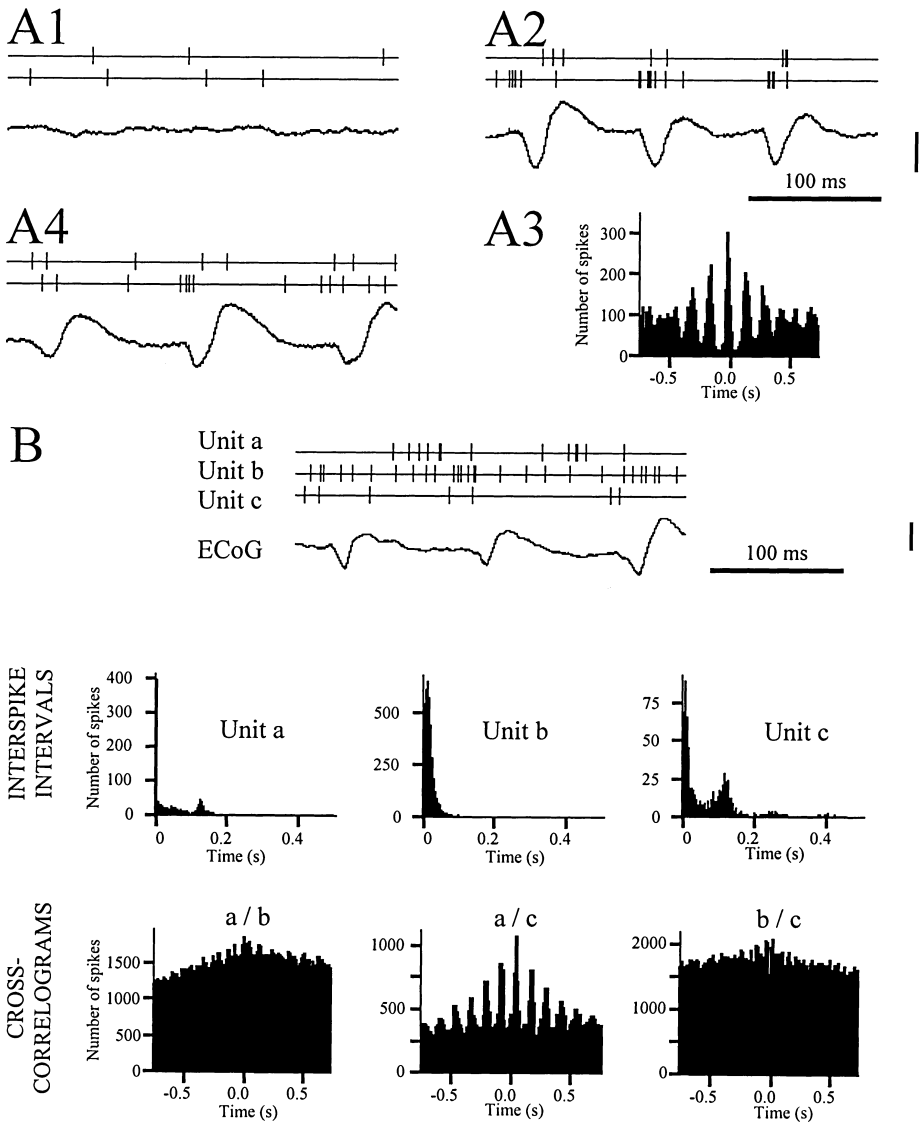

FIG. 3. Firing properties of neighboring substantia nigra pars reticulata (SNpr) neurons during spike-and-wave discharges (SWDs). Calibration bars, 500 μV for electrocorticography (ECoG) recordings. **A:** The generally random firing pattern of neighboring cells during interictal periods (A1, upper two traces) became highly correlated with each other (A3) and tightly time-locked to the spike-and-wave complexes (A2). Lack of bursting activity in a neuron previously firing in bursts (A4, upper trace). **B:** Triple-unit recordings. Units a and c fired in bursts together, whereas unit b was not affected by the occurrence of seizures (see Results section). The second peak in the interspike interval displays the spacing between bursts (unit c).

periodic correlation of spike activity in seizing animals. In the following, the possible basis for an involvement of SNpr neurons in the pathophysiology of absence epilepsy is discussed, taking into consideration the large body of evidence for a role of the SNpr in the control of epileptic seizures (7,21).

During interictal periods, when the animals were in a state of quiet wakefulness, no periodicity in the firing pattern of SNpr neurons and no synchrony between pairs of neurons were observed. However, the possibility remains that cross-correlation between pairs of neurons cannot detect weak neuronal synchronization that might involve a larger population of neurons (22,23). SNpr units in awake unrestrained GAERS rats exhibit at least two characteristic firing patterns that differ in their degree of regularity. The regular and irregular firing patterns observed in the present study are consistent with SNpr activity behaviors reported from experiments in which nonepileptic rats were maintained in either anesthetized or freely moving conditions (17,24–26). This suggests that SNpr neurons in GAERS are not functionally impaired as compared with those in nonepileptic animals. This is further supported by a previous report showing that no persistent interictal changes were observed between SNpr of control and kindled animals with respect to the number of spontaneously active neurons encountered, their firing rate, or their sensitivity to iontophoretically applied excitatory or inhibitory neurotransmitters (27). These data support the view that alterations in the intrinsic properties of these SNpr neurons are unlikely in models of either induced or spontaneous generalized seizures.

During the occurrence of an SWD, SNpr neurons increase their firing rate (95% of the cases) and generally fire in bursts (70% of the cases). Both initially increased and decreased firing rates below baseline have been reported during interictal spikes induced by topical

application of penicillin to the sensorimotor cortex in urethane-anesthetized rats (3). The prevalence of cells with initial inhibitory responses was observed mainly in the caudal SNpr (3). Although our data were obtained from a different animal model with long-lasting ECoG manifestations instead of isolated single spikes, the lack of initial decreased firing rate in our study also could account for the small number of recorded cells and their restrained location within the middle third of the SNpr.

The increased spike frequency in SNpr neurons during SWDs was concomitant with an important reorganization of the firing mode. At the beginning of the absence seizure, the firing rate increased significantly, and the SNpr neurons started firing in bursts of action potentials. The underlying alterations responsible for the burst-firing mode in SNpr neurons are likely to be due to an alteration in the activity entering the SNpr during SWD. Bursts were highly correlated to SWDs in the ECoG. The sustained burst-firing mode is likely to enhance the synaptic outputs from SNpr neurons in GAERS during seizures. Bursts recorded from thalamocortical cells—leading structures in the generation of SWDs—have been shown to precede the spike component in GAERS (28,29). Along with data obtained from amygdala-kindled rats (4), this supports that the SWD activity in GAERS is propagated from the thalamocortical circuit to the SNpr (30). This is further supported by the observation that bursts seem mainly to follow the spike component of the spike-and-wave complex, although this points warrants further analyses. Thus the changes in the firing pattern of SNpr neurons appear time-locked to the occurrence of SWDs and follow a typical temporal profile.

Before the complete end of the seizure, the firing rate decreased progressively, and the burst-firing pattern ended at or before the end of the SWD. A similar time course in SNpr bursting activities has been reported in amygdala-kindled seizures (5). The progressive decrease in the firing rate below baseline levels along with the transient silences in neuronal activities sometimes observed at the end of some SWD are reminiscent to the decreased firing rates reported by Kaniff et al. (3). In line with pharmacologic data showing that inhibition of SNpr neurons has antiepileptic effects in GAERS (8,31), the time course in changes accompanying the end of SWDs suggests that the decreased SNpr activity could be part of endogenous inhibitory mechanisms participating in the interruption of an ongoing seizure. Nevertheless, the possibility also remains that it could reflect a general trend for decreased amplitude and/or slowing of spike-and-waves at the end of SWDs.

Our data obtained from pairs or triads of neurons support some degree of synchronization of SNpr neurons during SWDs. Discharge synchronization between pairs of neurons in the SNpr appears limited to oscillatory activity in seizing animals and suggests a common rhythmic drive to neurons pairs rather than to direct interaction between pairs of neurons (23). The synchronized bursting of populations of SNpr neurons certainly enables this structure to exert a powerful influence on its targets during seizures.

In summary, this study shows that the basal interictal firing mode of SNpr neurons in freely moving absence epilepsy–prone rats is similar to that described in nonepileptic animals. Most of the changes in SNpr firing that occur during SWDs appear to reflect mainly changes in the activity of the thalamocortical circuit. However, several changes could account for a role of the SNpr in the modulation of seizures. In particular, a decreased activity of the SNpr neurons sometimes occurs before the end of the seizure. Changes reminiscent of phase displacement as well as transient silencing of neuronal activity—all of them likely to disrupt the synchrony processes—suggest that altogether, they could contribute to endogenous nigral control mechanisms involved in the interruption of an ongoing seizure.

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