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Epileptiform activity in rat spinal dorsal horn in vitro has common features with neuropathic pain

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Abstract

Neuropathic pain and epileptic seizures bear several similarities, among them is the response to anticonvulsant drugs. It has therefore been hypothesized that epileptiform activity of nociceptive spinal dorsal horn neurons may contribute to paroxysmal forms of neuropathic pain. We used patch-clamp and field potential recordings from young rat spinal cord slices to test if nociceptive dorsal horn structures are indeed able to sustain epileptiform activity. Application of the convulsant 4-aminopyridine (100 μM) evoked epileptiform activity that was most pronounced in superficial dorsal horn and involved nociceptive lamina I neurons with a projection to the brain. The epileptiform activity was dependent on fast excitatory and inhibitory synaptic transmission through ionotropic glutamate receptors and $GABA_A$ receptors. During epileptiform activity, previously silent polysynaptic pathways from primary afferent C-fibers to superficial dorsal horn neurons were opened. Stimulation of primary afferents at Aδ- and C-fiber intensity interfered with the epileptiform rhythm, suggesting that both affect the same dorsal horn structures. Similar to neuropathic pain, spinal dorsal horn epileptiform activity was much less reduced by classical analgesics than by anticonvulsant agents.

Keywords: Nociception; Seizure; Anticonvulsant drugs; Gabapentin; 4-Aminopyridine; Spino-parabrachial neurons

1. Introduction

Paroxysmal forms of neuropathic pain have common features with epileptic seizures. Both can be triggered by banal stimuli, have a stereotyped course and a refractory period after an attack where no new attack can be evoked. If not adequately treated, both may end up in a status, i.e. a series of attacks without complete recovery between the attacks. These observations have led to the successful attempt to relieve neuropathic pain by anticonvulsant drugs (Swerdlow, 1984). Part of the pain relief by anticonvulsant drugs probably relies on the attenuation of the ectopic activity that arises in injured peripheral nerves (Devor, 1994). However, neuropathic pain also has a central component as rhizotomy is not reliably effective in deafferentation pain (Pagni et al., 1993). Spinal dorsal horn neurons in cats and humans show rhythmic hyperactivity under conditions of neuropathic pain (Loeser and Ward, 1967; Loeser et al., 1968). Application of convulsants to the spinal trigeminal nucleus of rats evoked a tic-like pain syndrome similar to trigeminal neuralgia (Sakai et al., 1979). It is therefore tempting to hypothesize that the combination of deafferentation and ectopic activity that reaches the dorsal horn from an injured peripheral nerve kindles an epileptiform focus in dorsal horn that becomes autonomous after a certain time. It is not known, however, if nociceptive dorsal horn structures are indeed able to sustain epileptiform activity and if this activity is transmitted to the brain. Here, we used a spinal cord-dorsal root slice preparation and an established pharmacologic model of epilepsy to address this basic question and to investigate the interaction between primary afferent stimulation in the noxious range and spinal dorsal horn epileptiform activity. In addition, we compared the effects of classical analgesics and anticonvulsants on spinal dorsal horn epileptiform activity.

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2. Materials and methods

2.1. Preparation of spinal cord slices

The lumbar spinal cord was removed from 18- to 28-day-old Sprague–Dawley rats under deep ether anesthesia. The rats were then killed by an overdose of ether. Transverse slices, 500 μm thick, some of which had an attached dorsal root, were cut on a microslicer (DTK-1000, Dosaka, Kyoto, Japan). In some instances, 200 μm thick transverse slices or 500 μm thick parasagittal slices were made. Only parasagittal slices from middle third of the dorsal horn (cut at the level of the dorsal root entry) were used. Slices were stored in an incubation solution (in mM: NaCl 95, KCl 1.8, KH_2PO_4 1.2, $CaCl_2$ 0.5, $MgSO_4$ 7, $NaHCO_3$ 26, glucose 15, sucrose 50, oxygenated with 95% O_2, 5% CO_2; pH 7.4, measured osmolarity 310–320 mOsmol). For recording, one slice was transferred to the recording chamber where it was superfused by recording solution (identical to the incubation solution except for, in mM: NaCl 127, $CaCl_2$ 2.4, $MgSO_4$ 1.3 and sucrose 0) at 3 ml/min at room temperature (20–24 °C).

2.2. Patch-clamp recording

Dorsal horn neurons were visualized with Dodt-infrared optics and recorded in the whole-cell patch-clamp configuration with glass pipettes (2–6 MΩ) filled with internal solution (in mM: potassium gluconate 120, KCl 20, $MgCl_2$ 2, Na_2ATP 2, NaGTP 0.5, HEPES 20, EGTA 0.5, pH 7.28 with KOH, measured osmolarity 300 mOsmol) as described elsewhere (Ruscheweyh and Sandkühler, 2002). Voltage- and current-clamp recordings were made using an Axopatch 200B amplifier and the pCLAMP 8 acquisition software (Axon Instruments). Signals were low-pass filtered at 2–5 kHz, amplified fivefold, sampled at 5–10 kHz and analysed offline using pCLAMP 8 and the Mini-Analysis software (Synaptosoft). Series resistance was usually between 5–25 MΩ. No correction for the liquid junction potential was made. The locations of the neurons were assessed by visually inspecting the positions of the pipette tips under a 4× objective.

2.3. Recording and analysis of epileptiform activity

Where not stated otherwise, neurons in the medial two-thirds (medial to the lateral border of the dorsal root entry zone) of the superficial dorsal horn (laminae I and II) were selected for recording. Epileptiform activity was evoked by adding 4-aminopyridine (4-AP, at 100 μM unless indicated otherwise) to the bath solution and recorded in voltage-clamp mode at −70 mV for ≥15 min. Epileptiform activity was quantitatively assessed by calculating the fast Fourier transform (FFT) of consecutive 32.8-s-segments before and after the application of 4-AP. Epileptiform activity was considered to be present when two or more consecutive 32.8-s-segments exhibited a significant peak between 0.5 and 2 Hz in the FFT using the leave-three-out method (Zhao-Guo, 1988) implemented by a C++ program kindly provided by M. Eichler, Department of Applied Mathematics, Heidelberg University, Germany. To obtain a measure of the intensity and rhythmicity of the epileptiform activity independent of the size of the postsynaptic currents impinging on the recorded neuron, the normalized power of the epileptiform activity at a specific 32.8-s-segment was calculated by dividing the square root of the FFT peak power by the mean peak amplitude of epileptiform activity in the segment. The mean peak amplitude of epileptiform activity in a segment was the mean of the negative peak current amplitudes of its four 8.2-s-subsegments. The latency to the onset of epileptiform activity after application of 4-AP was the time to the first segment where significant epileptiform activity was detected. The frequency of the epileptiform activity was defined as the frequency associated with the highest normalized power during the first five segments showing significant epileptiform activity. The normalized power was taken from the same segment. The duration of the epileptiform activity was determined as the number of segments displaying significant epileptiform activity during the 20 segments following the onset of epileptiform activity, multiplied by 32.8 s. Therefore, the maximum possible duration was 656 s.

Values are given as mean ± SEM. The Mann–Whitney and Wilcoxon rank sum tests were used for statistical comparison. $p < 0.05$ was considered significant.

2.4. Field potential recording

Glass micropipettes (4–8 MΩ) were filled with a NaCl-based solution (in mM: NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.2 with NaOH) and inserted into the medial two-thirds of lamina II. A position of the recording electrode where electrical stimulation of Aδ-fibers in the dorsal root evoked a negative-going field potential was considered adequate for recording sum activity of nociceptive neurons. Signals were recorded with the same equipment as described above, amplified 500-fold, band-pass filtered at 0.1 Hz to 1 kHz and sampled at 10 kHz. Epileptiform activity was then evoked and analysed as described for patch-clamp recordings.

2.5. Primary afferent stimulation

The dorsal root was stimulated through a suction electrode with a constant current stimulator (WPI, Sarasota) at 0.1 ms pulse width. Excitatory postsynaptic currents (EPSCs) were classified according to their latency and threshold to be Aδ- or C-fiber-evoked as described previously (Chen and Sandkühler, 2000). Constant latencies and absence of failures during 10 Hz stimulation (for Aδ-fibers) or 1 Hz stimulation (for C-fibers) were used as criteria for apparently monosynaptic transmission.

Responses were obtained at 30-s-intervals and ≥5 min were allowed for controls. The mean of five EPSC amplitudes before application of 4-AP was compared to the mean of five EPSC amplitudes at 15 min after wash-in of 4-AP to quantify the effect of 4-AP on evoked responses.

To assess the effect of primary afferent stimulation on ongoing epileptiform activity, the inter-discharge intervals of the 13 spontaneous epileptiform discharges preceding the primary afferent stimulation were measured, termed $C = \{c_1, \ldots, c_{12}\}$ and tested for normality using the Kolmogorov–Smirnov test. The interval between the epileptiform discharge preceding primary afferent stimulation and the EPSC evoked by primary afferent stimulation was termed a and the interval between the EPSC and the following spontaneous epileptiform discharge was termed b (see Fig. 7F). a, b, $(a + b)$ and $((a + b)/2)$ were compared to the set of normally distributed inter-discharge intervals C, drawing conclusions according to Table 1. A short a (as defined in Table 1) means that the evoked discharge occurred before the spontaneous epileptiform discharge was expected. If in addition the distance between the two spontaneous epileptiform discharges adjacent to the evoked discharge was longer than expected for an interval between two spontaneous discharges ($a + b$ long) then the primary afferent stimulation interfered with the epileptiform rhythm. A failure was detected if the spontaneous epileptiform discharge expected to occur shortly after the evoked discharge was omitted but the epileptiform rhythm was otherwise undisturbed. A reset of the epileptiform activity was detected if the epileptiform activity was resumed after the evoked discharge as if it had been a spontaneous discharge. Of 122 traces obtained from 11 neurons, 62 had to be discarded from further analysis either because C was not normally distributed or a was not significantly different from C.

Table 1
Scheme to assess the interference of primary afferent stimulation with epileptiform activity

a	$a + b$	b	$(a + b)/2$	Conclusion
n.s.				No conclusion possible
Short	n.s.			No interference cannot be discarded
Short	Long			PAS disturbed the epileptiform rhythm
Short	Long	Long	n.s.	PAS evoked a failure in the epileptiform rhythm
Short	Long	n.s.	Short	PAS evoked a reset of the epileptiform rhythm

a and b are as defined in Section 2.5 (primary afferent stimulation) and Fig. 7. n.s., not significantly different from the set of inter-discharge intervals preceding the afferent stimulation (C as defined in Section 2.5). Short/long, significantly below/above the 95% probability range calculated from the normal distribution (defined by mean and standard deviation) derived from C. PAS, primary afferent stimulation. See Fig. 7 for examples of failure and reset of epileptiform activity evoked by primary afferent stimulation.

2.6. Retrograde labeling of spino-parabrachial neurons

The Sprague–Dawley rats, 18- to 24-day-old, were anaesthetized with a mixture of ketamine and xylazine (9:1, 3.4 ml/kg) and placed in a stereotaxic apparatus. A Hamilton syringe was used to inject 200 nl of 1,1′-didodecyl-3,3,3′,3′,-tetramethylindocarbocyanine perchlorate ($DiIC_{12}(3)$, DiI) (2.5%) into the right parabrachial area. After a 3-day survival period, spinal cord slices were prepared as described above. The brain was removed, cooled to −20 °C and cryostat sections (30 μm thick) of the brainstem were obtained to allow histological verification of the injection site (Fig. 5A). Recordings were made from retrogradely labeled neurons in the medial two-thirds of lamina I detected under fluorescent light (Fig. 5C).

2.7. Drugs

Drugs and their sources were as follows: 4-AP, (−)-bicuculline methiodide (bicuculline), strychnine, bumetanide, clonidine hydrochloride, 5,5 diphenylhydantoin free acid (phenytoin), carbamazepine and sodium valproate were from Sigma (Deisenhofen, Germany). D-2-amino-5-phosphovaleric acid (D-AP5), tetrodotoxin, [D-Ala^2,NMe-Phe^4,Gly^5-ol]-enkephalin (DAMGO) and 6-cyano-7-nitroquinoxinaline-2,3-dione (CNQX) were from Tocris (Bristol, UK). DiI was from Molecular Probes (Leiden, The Netherlands). Gabapentin was a courtesy from Pfizer (Karlsruhe, Germany). Stock solutions were prepared by dissolving the drugs in DMSO (strychnine, bumetanide, phenytoin, CNQX, DiI), ethanol (carbamazepine), acidic buffer (pH 4.8, tetrodotoxin) or distilled water (all others) and stored in aliquots at −20 °C. The final concentrations of DMSO and ethanol in the recording solution were ≤1 and ≤0.1%, respectively, and were shown not to affect epileptiform activity. Wash-in times of drugs before adding 4-AP were ≥20 min (carbamazepine, valproate), ≥30 min (D-AP5, strychnine, phenytoin, clonidine, DAMGO), ≥1 h (bumetanide) and ≥10 min for the other drugs.

3. Results

3.1. 4-Aminopyridine (4-AP) evokes epileptiform activity in the spinal dorsal horn network

Superficial dorsal horn neurons were recorded in voltage-clamp mode. Bath application of 4-AP (12.5–100 μM) dose dependently evoked epileptiform activity (Fig. 1A and B, Table 2). Dose dependency was reflected by incidence and latency to onset, not by graded intensity of the epileptiform activity (Table 2). In all subsequent experiments, 4-AP was used at 100 μM. The FFT (Fig. 1G) revealed a highly regular rhythm with a frequency around 1.2 Hz. A single epileptiform discharge consisted of multiple superimposed inward postsynaptic currents and its duration was variable

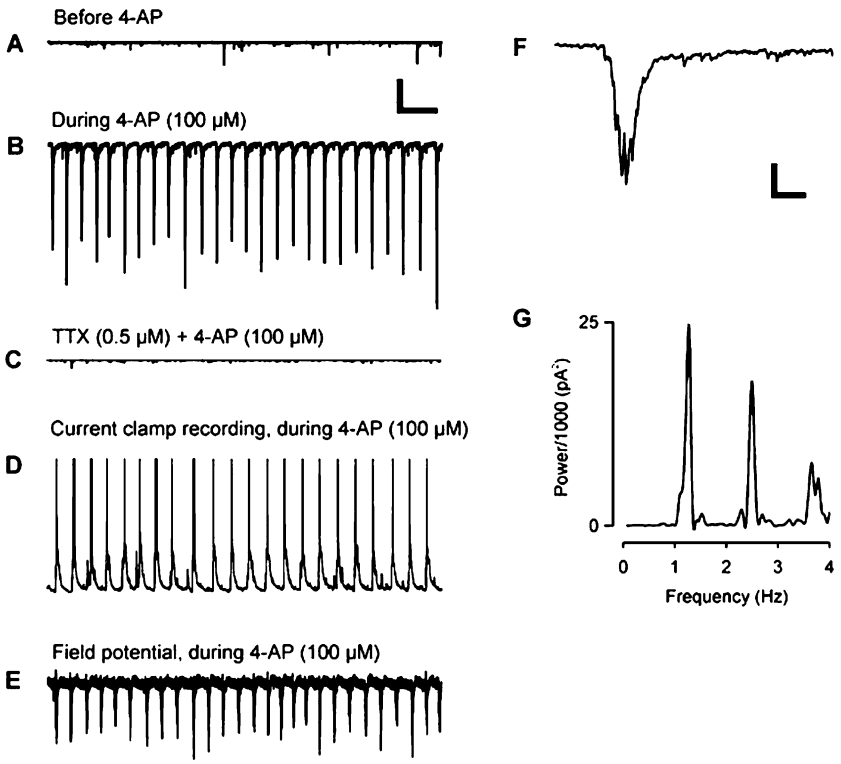

Fig. 1. 4-AP evokes epileptiform activity in superficial spinal dorsal horn. (A–C) Dorsal horn neurons were voltage-clamped at −70 mV. (A) Control trace in normal recording solution. (B) Same neuron 130 s after adding 4-AP to the bath solution, showing rhythmic epileptiform activity. (C) TTX prevented the expression of epileptiform activity. (D) In current-clamp recordings, epileptiform activity was reflected by rhythmically occurring depolarizations that in most neurons led to action potential discharges when the neuron was held at its resting membrane potential which was −67 mV in the illustrated example. Action potentials are truncated at +10 mV. (E) Epileptiform activity after application of 4-AP was also present in field potential recordings from superficial dorsal horn. (F) The amplification of a single epileptiform discharge from (B) illustrates that it is formed by superimposed EPSCs. (G) The FFT of the segment in (B) shows a narrow peak at 1.2 Hz. The subsequent peaks are artifacts inherent to the method. Calibration: A–C, 300 pA/2 s; D, 20 mV/2 s; E, 200 µV/2 s; F, 200 pA/50 ms.

among neurons (100–900 ms, Fig. 1F). Epileptiform activity usually outlasted the recording period, but the normalized power, a measure of the intensity of rhythmic activity, decreased with recording time. When recorded in current-clamp mode at resting membrane potential, epileptiform activity led to rhythmic action potential discharges in most neurons (Fig. 1D). Epileptiform activity was also evident in field potential recordings (Fig. 1E, $n = 4$) where it was still visible 60 min after application of 4-AP. Action potential generation was necessary for epileptiform activity since it could not be evoked in the presence of TTX (0.5 µM, Fig. 1C). The frequency of the epileptiform activity was not affected by holding the recorded neuron at different potentials (−50, −70, −90 and −110 mV, $n = 5$, data not shown). The epileptiform activity was not prevented by cutting off the ventral horns and the contralateral side of the slice (Table 2). While epileptiform activity could be evoked from thinner transversal slices (200 µm), parasagittal slices

Table 2
Dose dependency and effect of different slicing techniques on the 4-AP-evoked epileptiform activity

Concentration of 4-AP (µM)	Experimental condition	No. of slices showing EA/*n*	Latency to onset of EA (s)	Normalized power × 100	Frequency (Hz)	Duration (s)
100	(Control)	10/10	154 ± 14	173 ± 21	1.20 ± 0.07	582 ± 31
50		9/9	211 ± 25*	160 ± 26	1.22 ± 0.07	554 ± 58
25		4/6	312 ± 31**	133 ± 38	1.14 ± 0.03	500 ± 125
12.5		0/5	n.a.	n.a.	n.a.	n.a.
100	200 µm thick slice	5/5	177 ± 27	129 ± 25	1.25 ± 0.11	433 ± 61
100	Parasagittal slice	3/5	109 ± 11	258 ± 61	1.29 ± 0.11	405 ± 126
100	Isolated dorsal horn	5/5	216 ± 32	164 ± 46	1.19 ± 0.13	459 ± 101
100	Lamina I projection neurons	8/10	291 ± 54*	105 ± 21*	1.19 ± 0.07	353 ± 60*

n, number of tested slices; EA, epileptiform activity; n.a., not applicable; *Significantly different from control ($p < 0.05$); **Significantly different from control ($p < 0.01$). Control conditions: recording from a lamina I or II neuron in a 500 µm thick transverse slice bathed in normal recording solution.

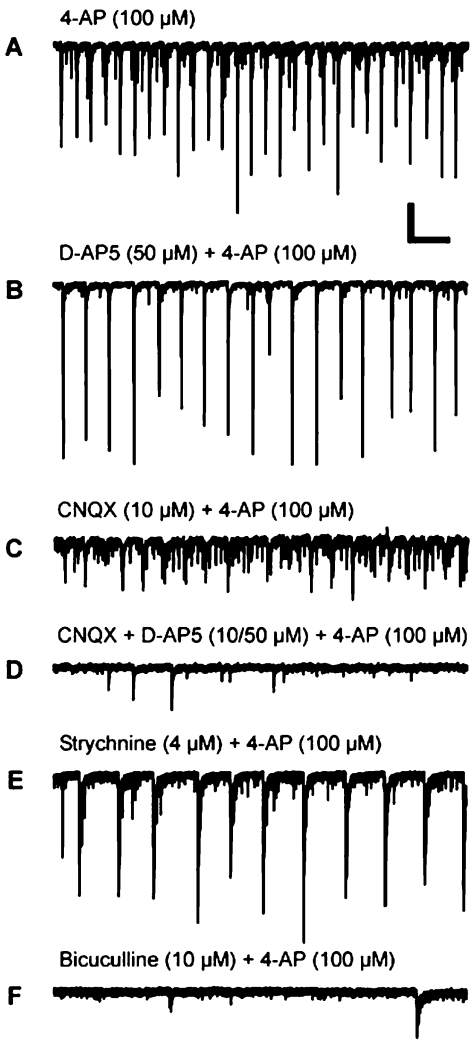

Fig. 2. Epileptiform activity depends on excitatory and inhibitory synaptic transmission. Voltage-clamp recordings at 3–5 min after wash-in of 4-AP in the presence of the indicated drugs are shown. (A) 4-AP-evoked epileptiform activity in the absence of further drugs. (B–D) The NMDA receptor antagonist D-AP5 did not strongly affect epileptiform activity but the AMPA/kainate receptor antagonist CNQX prevented it in 5/6 experiments and markedly reduced its intensity in the remaining experiment that is illustrated here. Epileptiform activity was prevented by combination of both antagonists. (E) The glycine receptor antagonist strychnine reduced the frequency but not the intensity of the epileptiform activity. (F) The $GABA_A$ receptor antagonist bicuculline prevented epileptiform activity. Calibration: A, B, E and F, 100 pA/2 s; C and D, 50 pA/2 s.

failed to show epileptiform activity in two of the five slices tested (Table 2).

3.2. Both excitatory and inhibitory synaptic transmission are necessary for epileptiform activity

The AMPA/kainate receptor antagonist CNQX (10 μM) prevented epileptiform activity in 5/6 experiments and reduced its intensity in the remaining experiment while the NMDA receptor antagonist D-AP5 (50 μM) only reduced the duration of epileptiform activity. Combination of both antagonists prevented epileptiform activity (Fig. 2B–D, Table 3). Blocking inhibitory synaptic transmission through glycine receptors by strychnine (4 μM) reduced the frequency of the epileptiform activity (Fig. 2E, Table 3) and blockade of $GABA_A$ receptors by bicuculline (10 μM) prevented the epileptiform activity (Fig. 2F, Table 3), suggesting a facilitatory action of GABA via $GABA_A$ receptors. To test if a reduced Cl^- gradient sustained by a Na^+–K^+–$2Cl^-$-cotransporter was involved, we blocked the cotransporter by bumetanide (100 μM), but this did not affect epileptiform activity (Table 3).

3.3. Actions of 4-AP on synaptically isolated dorsal horn neurons

In the presence of CNQX (10 μM), bicuculline (10 μM) and strychnine (4 μM), neurons were synaptically isolated and no epileptiform activity occurred. Under these conditions, the effect of 4-AP on membrane properties of dorsal horn neurons was investigated. The membrane resistance was not affected (423 ± 67 MΩ before 4-AP, 424 ± 77 MΩ 8–14 min after 4-AP, $n = 11$) and in only one of the nine neurons, application of 4-AP evoked a transient inward current of about −30 pA. However, the action potential threshold was significantly decreased (−37.9 ± 1.2 mV before 4-AP, −41.0 ± 1.7 mV after 4-AP, $n = 11$, $p < 0.01$), reflecting an increased excitability of the neurons. The action potential width at base was doubled by 4-AP (2.0 ± 0.1 ms before 4-AP, 4.0 ± 0.3 ms after 4-AP, $n = 11$, $p < 0.01$). The firing patterns, obtained by 1-s-long depolarizing current injections of increasing intensity (25–350 pA) and classified as described (Ruscheweyh and Sandkühler, 2002), turned from phasic bursting to tonic firing in four of the 11 neurons, but were otherwise unaffected by 4-AP. However, in four of the 11 neurons after application of 4-AP, action potentials were followed by a second action potential of reduced height that occurred before repolarization was complete. In one case, several small action potentials were riding on a shoulder of the first action potential (Fig. 3). These intrinsic bursts also arose spontaneously and repetitively from baseline in the synaptically isolated neuron (Fig. 3C), suggesting that neurons of this type may develop a pacemaker function under 4-AP. However, the spontaneous bursts in synaptically isolated neurons did not occur rhythmically, suggesting that synchronizing and feedback mechanisms from the surrounding network are necessary for the spinal dorsal horn epileptiform activity. In subsequent experiments, four additional neurons showing intrinsic bursting under 4-AP were found, and all five intrinsically bursting neurons were located at the border between lamina II and III and mediolaterally at the level of the dorsal root entry zone.

Table 3
Contribution of action potential generation and excitatory and inhibitory neurotransmission to the 4-AP-evoked epileptiform activity

Experimental condition	No. of slices showing EA/*n*	Latency to onset of EA (s)	Normalized power × 100	Frequency (Hz)	Duration (s)
Control	10/10	154 ± 14	173 ± 21	1.20 ± 0.07	582 ± 31
TTX (0.5 μM)	0/8	n.a.	n.a.	n.a.	n.a.
CNQX (10 μM)	1/6	262	75	1.13	164
D-AP5 (50 μM)	5/5	190 ± 28	169 ± 24	1.25 ± 0.08	353 ± 102*
CNQX (10 μM) + D-AP5 (50 μM)	0/5	n.a.	n.a.	n.a.	n.a.
Bicuculline (10 μM)	0/5	n.a.	n.a.	n.a.	n.a.
Strychnine (4 μM)	6/6	180 ± 34	159 ± 19	0.93 ± 0.08*	547 ± 51
Bumetanide (100 μM)	7/7	183 ± 10	162 ± 29	1.03 ± 0.05	511 ± 78

n, number of tested slices; EA, epileptiform activity; n.a., not applicable; *Significantly different from control ($p < 0.05$). Drugs were washed-in before application of 4-AP. The concentration of 4-AP was 100 μM. Control conditions: see Table 2.

3.4. Actions of 4-AP on miniature postsynaptic potentials (mPSCs)

Mixed excitatory and inhibitory mPSCs were recorded in the presence of TTX (0.5 μM). 4-AP did not significantly affect the frequency of mPSCs at 3 or 10 min after its application (frequencies obtained from 120 s recordings; prior to 4-AP application: 2.2 ± 0.6 events/s (range 0.1–3.5 events/s), 3 min after 4-AP: 2.3 ± 0.8 events/s, 10 min after 4-AP: 2.0 ± 0.5 events/s, $n = 5$). A slight but significant increase in mPSC amplitude was seen in two of the four neurons at 3 min (to 104 and 112% of control, respectively) but amplitudes were back to baseline values at 10 min after 4-AP (amplitudes sampled from 200–300 mPSCs; prior to 4-AP application: 19.2 ± 2.6 pA (range 16.0–26.8 pA), 3 min after 4-AP: 19.5 ± 2.0 pA, 10 min after 4-AP: 18.0 ± 1.8 pA, $n = 4$).

3.5. Location of epileptiform activity in spinal dorsal horn

Except where otherwise stated, recordings were made from the medial two-thirds of the superficial dorsal horn. To investigate if other regions of the spinal dorsal horn take part in the epileptiform activity, 29 neurons were recorded from different laminae. Epileptiform activity was present at most sites tested (in 26 of the 29 neurons tested), but the latency to onset was shortest and the intensity of epileptiform activity was highest in the medial two-thirds of superficial dorsal horn (Fig. 4). Two of the five neurons in the lateral third and one of the 13 neurons in the middle third of the dorsal horn failed to show significant epileptiform activity. The relatively frequent failure to record epileptiform activity from lateral regions may be inherent to the phenomenon or due to the slicing technique.

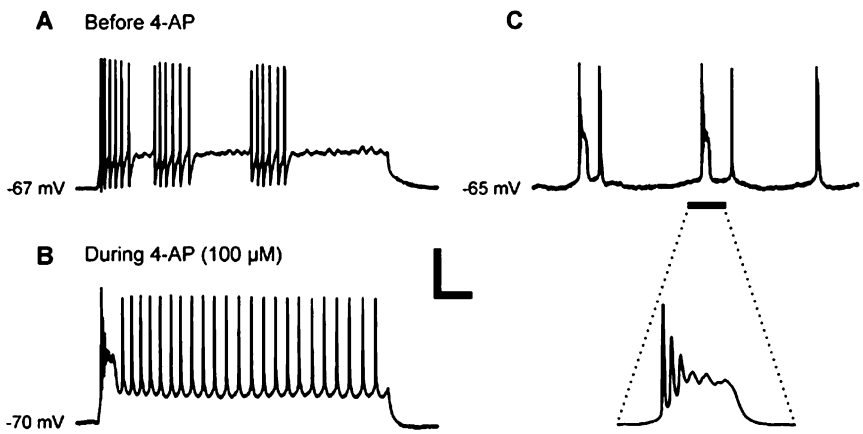

Fig. 3. Intrinsic bursting evoked by 4-AP. Current-clamp recordings from one neuron in the presence of blockers of fast synaptic transmission (CNQX, bicuculline, strychnine) are shown. (A and B) Response to a 75 pA, 1-s current injection before and in the presence of 4-AP. During 4-AP, an intrinsic burst appeared at the beginning of the depolarizing current pulse. (C) Spontaneous generation of intrinsic bursts in the presence of 4-AP from resting membrane potential (−65 mV). Higher temporal resolution shows that an intrinsic burst consisted of several action potentials of decreasing amplitude riding on a slow depolarizing potential. Calibration: A and B, 40 mV/125 ms; C, 40 mV/250 ms and 25 ms (inset).

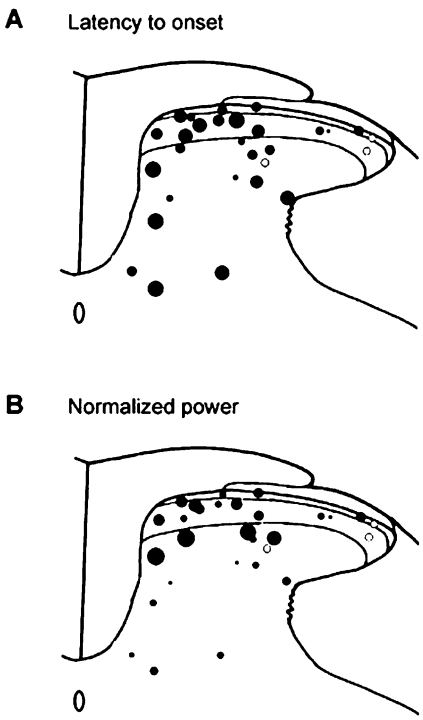

Fig. 4. Location of epileptiform activity in spinal dorsal horn. Circles indicate the location of recorded neurons on an outline of the dorsal horn. Open circles represent neurons that did not show epileptiform activity in response to 4-AP. The diameter of the filled circles linearly decreases with the latency to the onset of epileptiform activity (A) and linearly increases with the normalized power (B).

3.6. Epileptiform activity is transmitted to the brain via nociceptive pathways

Neurons from spinal lamina I that project to the parabrachial area receive input from nociceptive primary afferents (Bester et al., 2000) and express activity-dependent long-term potentiation at synapses with primary afferent C-fibers, a mechanism of central sensitization (Ikeda et al., 2003). Spino-parabrachial neurons were retrogradely labeled with a fluorescent dye and recorded from the medial two-thirds of lamina I (Fig. 5A and C). Of 10 spino-parabrachial neurons examined, eight showed epileptiform activity that was, however, significantly weaker in intensity, later in onset and shorter in duration than in unidentified superficial dorsal horn neurons (Fig. 5B, Table 2).

3.7. Effects of analgesic and anticonvulsant agents on epileptiform activity

μ-Opioid- and α_2-adrenergic agonists are used as spinal analgesics but tend to be ineffective in some forms of neuropathic pain. Here, the μ-opioid receptor agonist DAMGO (10 μM) and the α_2-adrenergic agonist clonidine (10 μM) did not reduce the intensity of epileptiform activity. Its frequency was reduced by clonidine (Fig. 6B and C, Table 4). However, anticonvulsant agents effectively reduced the intensity of epileptiform activity. Phenytoin

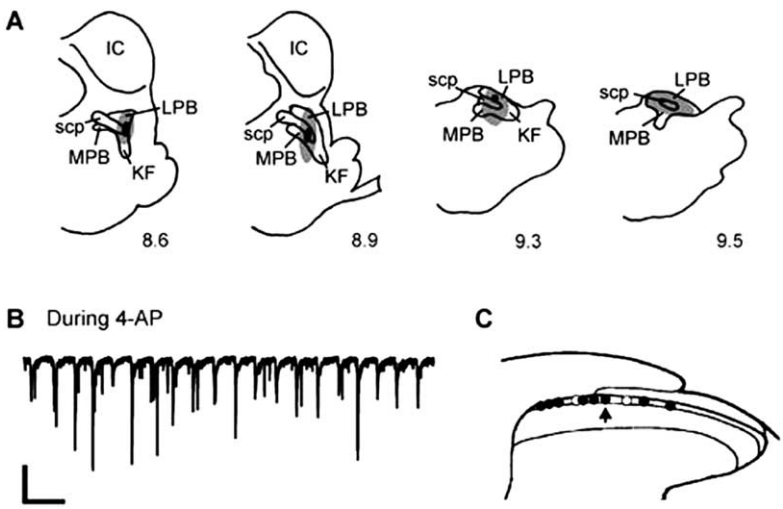

Fig. 5. Epileptiform activity is present in spino-parabrachial projection neurons in lamina I of the spinal cord. (A) Distribution of the fluorescent tracer DiI in the parabrachial area in one representative animal 3 days after injection of DiI. Black indicates the area damaged by the injection, gray indicates the spread of the tracer. IC, inferior colliculus; KF, Kölliker Fuse nucleus; LPB, lateral parabrachial area; MPB, medial parabrachial area; scp, superior cerebellar peduncle. The numbers below each section indicate the distance in mm posterior to bregma according to the atlas of Swanson (1992). (B) Voltage-clamp recording of a spino-parabrachial neuron from the animal shown in (A). 4-AP evoked epileptiform activity that is, however, less regular in rhythm and amplitude than in unidentified superficial dorsal horn neurons (see Figs. 1B, 2A and 5A). (C) Location of the spino-parabrachial neurons recorded. Filled circles indicate neurons that showed epileptiform activity in response to 4-AP, open circles represent neurons that failed to show epileptiform activity in the presence of 4-AP. The arrow indicates the neuron shown in (B). Calibration: B, 100 pA/2 s.

Table 4
Antiepileptiform effects of classical analgesics and anticonvulsants

Experimental condition	No. of slices showing EA/*n*	Latency to onset of EA (s)	Normalized power × 100	Frequency (Hz)	Duration (s)
Control	10/10	154 ± 14	173 ± 21	1.20 ± 0.07	582 ± 31
DAMGO (10 μM)	5/5	210 ± 34	207 ± 67	1.18 ± 0.11	499 ± 91
Clonidine (10 μM)	5/5	171 ± 26	225 ± 21	0.85 ± 0.05**	590 ± 45
Carbamazepine (100 μM)	5/5	223 ± 19*	107 ± 17*	1.30 ± 0.09	492 ± 77
Phenytoin (100 μM)	0/5	n.a.	n.a.	n.a.	n.a.
Valproic acid (2 mM)	5/5	208 ± 41	90 ± 15*	1.40 ± 0.10	328 ± 79**
Gabapentin (100 μM)	5/5	137 ± 12	200 ± 32	1.01 ± 0.02	570 ± 25
Gabapentin (10 μM)	5/5	190 ± 12	168 ± 27	0.95 ± 0.05*	564 ± 68
Gabapentin (1 μM)	6/6	202 ± 10*	162 ± 29	1.03 ± 0.05	511 ± 78

n, number of tested slices; EA, epileptiform activity; n.a., not applicable; *Significantly different from control ($p < 0.05$); **Significantly different from control ($p < 0.01$). Drugs were washed-in before application of 4-AP. The concentration of 4-AP was 100 μM. Control conditions: see Table 2.

(100 μM) prevented epileptiform activity and carbamazepine (100 μM) and valproate (2 mM) strongly reduced its intensity (Fig. 6E–G, Table 4). In contrast, gabapentin over a range of concentrations (1–100 μM) only marginally affected epileptiform activity (Fig. 6D, Table 4).

3.8. Interaction between primary afferent-evoked synaptic activity and epileptiform activity

Aδ-fiber-evoked EPSCs were depressed by application of 4-AP to a significantly greater extent than C-fiber-evoked EPSCs (Fig. 7A–C; Aδ-fiber-evoked EPSCs, depression to 29 ± 6% at 15 min after 4-AP, $n = 9$, three monosynaptic, six polysynaptic; C-fiber-evoked EPSCs, depression to 64 ± 9%, $n = 6$, four monosynaptic, two polysynaptic; $p < 0.01$). The degree of the depression was independent of the mono- or polysynaptic nature of the EPSC. Both Aδ- and C-fiber-evoked EPSCs occasionally showed a short-lasting depression during wash-in of 4-AP (Fig. 7B). Stimulation of the dorsal root at C-fiber-intensity but not at Aδ-fiber-intensity in the presence of 4-AP regularly evoked large afterdischarges (Fig. 7D, $n = 12$, threshold for large afterdischarges 2.6 ± 0.6 mA at 0.1 ms pulse width) that were similar in shape and size to the spontaneous epileptiform discharges (Fig. 7E). Large afterdischarges in response to C-fiber stimulation also occurred in the six neurons that received no detectable input from C-fibers before application of 4-AP.

Stimulation of the primary afferents at Aδ- or C-fiber-intensity interfered with the rhythmic epileptiform activity evoked by 4-AP. In 50% of 60 20-s traces from 11 neurons, primary afferent stimulation changed the epileptiform rhythm. While a reset of the epileptiform activity occurred in 18%, failures were seen in 10%. The interference could not be classified in the remaining 22% (Fig. 7F).

4. Discussion

The main result of this study is that nociceptive spinal dorsal horn neurons, including neurons with a projection to the brain, are able to sustain epileptiform activity with a pharmacologic profile similar to that of paroxysmal neuropathic pain.

The 4-AP-model of epilepsy is widely used in forebrain (Aram et al., 1991). 4-AP is also able to evoke rhythmic activity in spinal cord in vivo that can be recorded from the dorsal roots (Dubuc and Rossignol, 1989). It has been known for a long time that spinal motor seizures can be evoked by the application of convulsants to the spinal cord (Lothman and Somjen, 1976). However, only limited evidence gained from dorsal root recordings of neonatal animals exists that the spinal dorsal horn may be able to sustain epileptiform activity on its own (Czéh and Somjen, 1989; Kremer and Lev-Tov, 1998). In the present study, 4-AP dose dependently evoked rhythmic epileptiform activity in nociceptive superficial dorsal horn neurons in vitro. Several lines of evidence indicate that the epileptiform activity arose from the dorsal horn network and involved synchronous activity of many neurons. First, the frequency of the epileptiform activity did not depend on the holding potential, suggesting that it was not generated by intrinsic membrane properties of the recorded neuron. Second, epileptiform activity disappeared when synaptic transmission was blocked. Third, epileptiform activity was also present in field potential recordings that reflect the sum activity of a population of neurons. Fourth, the presence of the ipsilateral superficial dorsal horn was sufficient for the expression of epileptiform activity. This also confirmed that the epileptiform activity recorded in dorsal horn is not due to spread of the rhythmic motor activity that can be readily evoked from the ventral horns (Whelan et al., 2000). Epileptiform activity mainly relied on the extension of the network in the transverse plane since it was not affected by transverse slice thickness but strongly reduced in parasagittal slices. From the distribution shown in Fig. 4 it seems to have a focus in medial superficial dorsal horn but spreads to all regions of the dorsal horn. Most superficial dorsal horn neurons receive input from primary afferent Aδ- or C-fibers and thus are probably nociceptive (Willis and Coggeshall, 1991). Epileptiform activity was also present in neurons

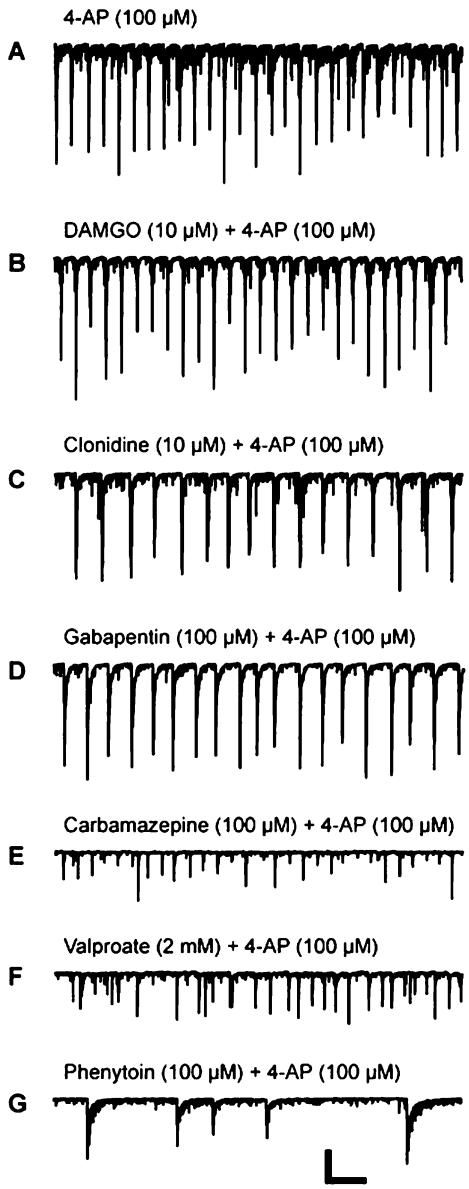

Fig. 6. Effects of analgesic and anticonvulsant drugs on epileptiform activity in spinal dorsal horn. Voltage-clamp recordings during bath application of 4-AP in the presence of the indicated drugs are shown. 4-AP-evoked epileptiform activity in the absence of further drugs (A). Epileptiform activity was not affected by the μ-opioid receptor agonist DAMGO (B) and the anticonvulsant gabapentin (D). The α_2-adrenergic agonist clonidine reduced the frequency but not the intensity of the epileptiform activity (C). In contrast, epileptiform activity was strongly reduced by the anticonvulsants carbamazepine (E) and valproate (F) and prevented by the anticonvulsant phenytoin (G). Calibration: A, 75 pA/2 s, B–G, 150 pA/2 s.

with a projection to the parabrachial area. These neurons belong to the group of NK1-receptor-expressing lamina I neurons that are necessary for the development of neuropathic pain (Nichols et al., 1999) and susceptible to synaptic plasticity (Ikeda et al., 2003). When compared to cortical models of epilepsy, the spinal dorsal horn epileptiform activity described here was similar in shape and duration to interictal, not ictal discharges. Models of interictal activity are widely used as models of epilepsy in the brain, but the meaning of interictal activity for behavioral seizures is controversially discussed (de Curtis and Avanzini, 2001). The significance of this distinction for epileptiform activity in spinal dorsal horn is not clear. To our knowledge, epileptiform activity similar to cortical ictal discharges has not been observed in spinal dorsal horn.

Like other types of epileptiform activity (Gean, 1990), spinal dorsal horn epileptiform activity was dependent on excitatory synaptic transmission via AMPA/kainate and NMDA receptors. It is more difficult to understand why the usually inhibitory transmission at $GABA_A$ receptors was also necessary in the present and previous studies (Aram et al., 1991). GABA has depolarizing actions at neurons that express a $Na^+-K^+-2Cl^-$-cotransporter that reduces the Cl^- gradient (Jang et al., 2001), but blocking the cotransporter had no effect in the present study. Depolarizing effects of GABA under epileptiform activity or tetanic stimulation may also be mediated by predominance of HCO_3^- efflux over Cl^- influx through $GABA_A$ receptors or by extracellular K^+ accumulation (Staley et al., 1995; Kaila et al., 1997). Postinhibitory rebound is another depolarizing GABA action (Olsen and Avoli, 1997) and is present in spinal dorsal horn neurons (Ruscheweyh and Sandkühler, 2002).

It is not known exactly by which mechanisms 4-AP induces epileptiform activity. Apart from its K^+-channel-blocking properties, it affects neuronal Ca^{2+} homeostasis (Aronson, 1992; Grimaldi et al., 2001). Action-potential-evoked and -independent transmitter release increases under 4-AP (Dolezal and Tucek, 1983). Here, the analysis of mPSCs showed that action-potential-independent transmitter release on dorsal horn neurons is not affected by 4-AP. However, the action-potential-evoked transmitter release is probably amplified due to the widened action potentials. In addition, neuronal excitability was increased since 4-AP lowered the action potential threshold of dorsal horn neurons. In a subpopulation of dorsal horn neurons, 4-AP evoked intrinsic bursting. Intrinsically bursting neurons seem to initiate epileptiform activity in neocortex (Connors, 1984) and their number is increased under conditions of acute or chronic epilepsy (Church and Baimbridge, 1991; Sanabria et al., 2001).

Anticonvulsant drugs are successfully used to relieve lancinating pain attacks (Swerdlow, 1984). They attenuate the ectopic activity in injured peripheral nerves (Devor, 1994), and this has often been viewed as their major mechanism of action in neuropathic pain. Our results show that phenytoin, valproate and carbamazepine are also active at the spinal cord level. The attenuation of hypersynchronous activity in spinal dorsal horn might contribute to the analgesic action of antiepileptic drugs in neuropathic pain. Phenytoin, carbamazepine and to some extent also valproate cause a use-dependent block of fast Na^+ channels, thereby preventing burst firing (White, 1999; Löscher, 1999). Gabapentin leaves Na^+ channels mostly unaffected but binds to the $\alpha_2\delta$ subunit of high threshold voltage-gated Ca^{2+} channels (Taylor et al., 1998). The $\alpha_2\delta$ subunit in

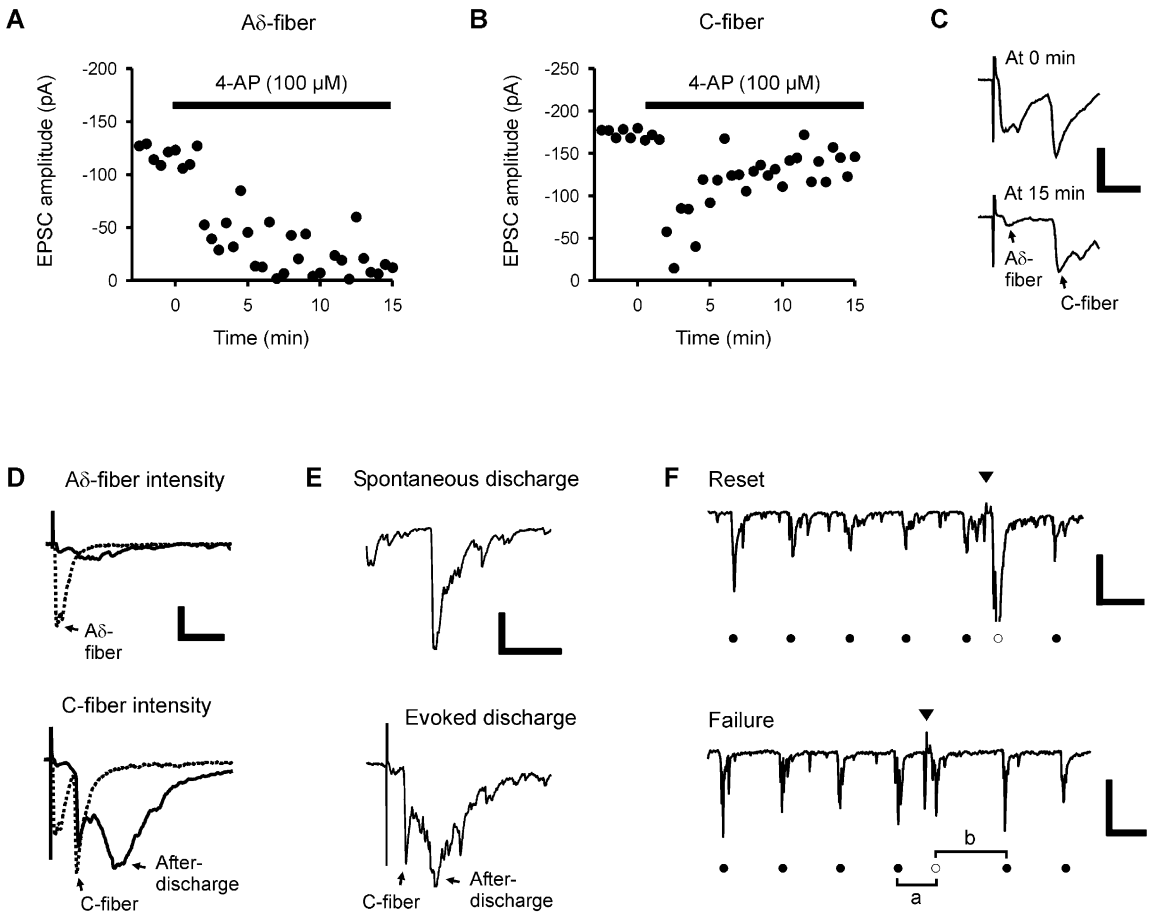

Fig. 7. Interaction between primary afferent-evoked and epileptiform synaptic activity. (A–C) Epileptiform activity depressed Aδ-fiber-evoked EPSCs more strongly than C-fiber-evoked EPSCs. (A and B) Examples of the time course of the depression. (C) Original EPSC traces before and after application of 4-AP. In this neuron, monosynaptic Aδ- and C-fiber-evoked EPSCs were recorded simultaneously. (D) C-but not Aδ-fiber-intensity dorsal root stimulation evoked a large afterdischarge in the presence of 4-AP. Dotted lines: mean curves of four consecutive traces before application of 4-AP. Solid lines: mean curves of 14 consecutive traces ≥15 min after application of 4-AP. (E) Spontaneous epileptiform discharges were similar in shape and size to evoked afterdischarges. Original traces recorded 20 min after application of 4-AP are shown. (F) Primary afferent stimulation interfered with the epileptiform rhythm. Original voltage-clamp traces illustrate that primary afferent stimulation can evoke resets and failures in ongoing epileptiform activity. Arrowhead: stimulation antifact. Filled circles: spontaneous epileptiform discharges. Open circles: EPSCs evoked by primary afferent stimulation. *a* and *b* indicate the intervals between the adjacent circles as defined in Section 2.5. (A–E) and the upper trace in (F) are from the same neuron. Calibration: C, 100 pA/20 ms; D, 50 pA/50 ms; E, 50 pA/100 ms; F, 50 pA/500 ms.

dorsal root ganglia is strongly upregulated after peripheral nerve injury, while the spinal dorsal horn expresses another isoform that is only modestly upregulated (Luo et al., 2001). It is not known to which isoform gabapentin binds preferentially but these data suggest that it has a mainly peripheral mechanism of action in neuropathic pain. Consistently, gabapentin did not strongly affect epileptiform activity in spinal dorsal horn in the present study.

In contrast to the anticonvulsants, and similar to their poor action in some forms of neuropathic pain, neither the μ-opioid agonist DAMGO nor the α_2-adrenergic agonist clonidine blocked spinal dorsal horn epileptiform activity. μ-Opioid agonists exert part of their inhibiting action by opening K^+ channels (Ingram, 2000) while 4-AP is a K^+ channel blocker. Therefore, DAMGO may not be able to express its full inhibiting potential under 4-AP.

EPSCs evoked by stimulation of primary afferent Aδ- and C-fibers were depressed by 4-AP. One possible explanation is that 4-AP increases primary afferent depolarization, a mechanism of presynaptic inhibition in spinal cord (Jankowska et al., 1982). A late, C-fiber-evoked afterdischarge appeared in the presence of 4-AP, also in neurons that did not show C-fiber-evoked EPSCs under control conditions. This can be interpreted as the opening of previously silent polysynaptic pathways, thereby increasing convergent input from C-fiber afferents, including nociceptive nerve fibers. This would probably enlarge receptive fields. 4-AP is known to increase receptive fields of dorsal horn neurons in vivo (Semba et al., 1985). Enlargement of receptive fields of nociceptive superficial dorsal horn neurons has been found after peripheral inflammation (Hylden et al., 1989), strong noxious stimulation (Laird and Cervero, 1989) and in models of neuropathic pain (Tabo et al., 1999) and has been attributed to central sensitization. Thus, epileptiform activity may be a mechanism of central sensitization in addition to loss of inhibition (Ibuki et al.,

1997), synaptic long-term potentiation (Ikeda et al., 2003; Sandkühler, 2000) and synaptic reorganization (Woolf et al., 1992). Prolonged afterdischarges of dorsal horn neurons to noxious stimulation has been reported in neuropathic rats (Laird and Bennett, 1993). 4-AP-induced afterdischarges have also been reported in primary afferent fibers (Kocsis et al., 1983). However, they were observed in A-, not C-fibers and cannot account for the appearance of C-fiber-evoked afterdischarges in neurons without previous C-fiber-input. The afterdischarges evoked in the presence of 4-AP by stimulation of C-fibers often looked very similar to the epileptiform discharges that occurred spontaneously in the same neuron. In addition, evoked responses interfered with the epileptiform rhythm, showing that both involve the same dorsal horn structures.

In conclusion, the 4-AP-model of epileptiform activity in spinal dorsal horn shares several features with neuropathic pain. We propose that the combination of deafferentation and ectopic hyperactivity in injured peripheral nerves may kindle an epileptiform focus in spinal dorsal horn. This hypothesis will need further investigation using animal models of neuropathic pain. If it proves true, the differential action of some anticonvulsants on peripheral ectopic activity and/or the epileptiform focus itself would provide a rationale for combining different anticonvulsant drugs in the treatment of neuropathic pain.

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