Neurosteroids differentially modulate fast and slow interictal discharges in the hippocampal CA3 area

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Abstract

Two types of spontaneous interictal discharge, identified as fast and slow events, can be recorded from the hippocampal CA3 area in rat brain slices during application of 4-aminopyridine (4AP) (50 μ M). Here, we addressed how neurosteroids modulate the occurrence of these interictal events and of the associated high-frequency oscillations (HFOs) (ripples, 80–200 Hz; fast ripples, 250–500 Hz). Under control conditions (i.e. during 4AP application), ripples and fast ripples were detected in 12.3 and 17.5% of fast events, respectively; in contrast, the majority of slow events (> 98%) did not co-occur with HFOs. Application of 0.1, 1 or 5 μ M allotetrahydrodeoxycorticosterone (THDOC) to 4AP-treated slices caused a dose-dependent decrease in the duration of the fast events and an increase in the occurrence of ripples, but not fast ripples; in contrast, the duration of slow events increased. THDOC potentiated the slow events that were recorded during pharmacological blockade of glutamatergic transmission, but had no effect on interictal discharges occurring during GABA_A receptor antagonism. These results demonstrate that potentiation of GABA_A receptor-mediated signaling by THDOC differentially affects slow and fast interictal discharges; these differences may provide insights into how hyperexcitable activity is influenced by neurosteroids.

Keywords

4-aminopyridine; epileptiform synchronisation; fast ripples; ripples; THDOC

Introduction

Excitatory and inhibitory mechanisms contribute to two types of interictal discharge that are recorded in the CA3 area in *in vitro* preparations during application of the K⁺ channel blocker 4-aminopyridine (4AP) (Perreault & Avoli, 1991, 1992; reviewed in Avoli & de Curtis, 2011). The first type consists of brief epileptiform discharges, also termed fast interictal events, that are associated with intracellular paroxysmal depolarizing shifts and are mainly contributed by excitatory synaptic currents (Avoli & de Curtis, 2011); accordingly, these fast events are abolished by ionotropic glutamatergic receptor antagonists (Perreault & Avoli, 1991, 1992). The second type is characterised by long-lasting slow events that are thought to arise from the periodic synchronous activity of GABAergic interneurons, and

continue to occur during application of glutamatergic receptor antagonists but are abolished by GABA_A receptor antagonists (Perreault & Avoli, 1992; Avoli & de Curtis, 2011). These two types of interictal discharge have been correlated with both protective and precipitating roles in the generation of ictal discharges that occur outside the CA3 area (Engel *et al.*, 1990; Avoli & de Curtis, 2011).

In addition to interictal discharges, high-frequency oscillations (HFOs) (80–500 Hz) also represent potential biomarkers of epileptogenicity (Jefferys *et al.*, 2012). HFOs were initially identified as physiological events playing a role in consolidating synaptic plasticity and episodic memory (Axmacher *et al.*, 2006; Buzsaki, 2009). Subsequently, however, HFOs were recorded in the electroencephalograms of epileptic patients and in animal models of epilepsy, and were proposed to be specifically localised to regions of seizure onset (Jacobs *et al.*, 2008, 2010; Engel & da Silva, 2012; Jefferys *et al.*, 2012). Pathological HFOs can be categorised into two frequency bands: ripples (80–200 Hz) and fast ripples (250–500 Hz). HFOs in the ripple band – like those recorded under physiological conditions – are thought to represent population inhibitory postsynaptic potentials generated by principal neurons entrained by synchronously active interneuron networks, whereas those in the fast ripple band (250–500 Hz) presumably reflect the asynchronous firing of abnormally active principal cells, and are independent of inhibitory neurotransmission (Engel *et al.*, 2009; Ibarz *et al.*, 2010; Bragin *et al.*, 2011).

Pregnane neurosteroids are potent positive allosteric modulators of GABA_A receptor-mediated signaling, and play an important role in modulating hippocampal excitability (Mienville & Vicini, 1989; Baulieu & Robel, 1990; Hosie *et al.*, 2006; Reddy & Rogawski, 2012). Enzymes involved in the synthesis of these neurosteroids are strongly expressed in the CA3 area (Agís-Balboa *et al.*, 2006), and the dysregulation of neurosteroids, either an increase or decrease, can have a profound effect on hyperexcitable brain networks. To date, Salazar *et al.* (2003) have demonstrated that anticonvulsant neurosteroids such as allopregnanolone can reduce epileptiform bursting induced by either 4AP or picrotoxin (PTX) in hippocampal slices. On the basis of this evidence, along with the presence of two distinct types of interictal activity with differing GABAergic and glutamatergic contributions in the CA3 area, we sought to determine how allotetrahydrodeoxycorticosterone (THDOC) can differentially modulate fast and slow events. Furthermore, as ripples and fast ripples should have distinct underlying mechanisms, and neurosteroids modulate GABA_A receptor-mediated signaling, we expected that THDOC could also lead to selective changes in ripple and fast ripple occurrence. Thus, our study could contribute to understanding how neurosteroids differentially influence glutamatergic and GABAergic circuits in the CA3 area.

Materials and methods

In vitro preparation

Horizontal brain slices with a thickness of 450 µm were obtained from male Sprague-Dawley rats weighing 250–275 g (Charles River Laboratories, Saint Constant, QC, Canada). Rats were decapitated following anesthesia with isoflurane (Baxter Corporation, Mississauga, ON, Canada), and their brains were then quickly removed and chilled for 3 min

in ice-cold artificial cerebrospinal fluid (ACSF) with the following composition: 124 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 1.25 mM KH₂PO₄, 26 mM NaHCO₃, and 10 mM D-glucose. The ACSF was continuously bubbled with an O₂/CO₂ (95/5%) gas mixture to maintain the pH at 7.4. Slices were obtained with a Vibratome (VT1000S; Leica, Concord, ON, Canada) and directly transferred to an interface chamber, where they were laid between warm (31–33 °C) ACSF (pH 7.4, 305 mOsm/kg) and humidified gas (O₂/CO₂, 95%/5%). Brain slices were allowed to recover for at least 1 h before initiation of continuous bath application of 4AP (50 µM) at a flow rate of 2 mL/min. All pharmaceuticals were bath-applied and obtained from either Sigma-Aldrich Canada (Oakville, ON, Canada) or Tocris Bioscience (Ellisville, MO, USA). The care and handling of animals was in compliance with the guidelines of the Canadian Council on Animal Care, and all procedures were approved by the McGill University Animal Care Committee.

To assess changes over time in spontaneous 4AP-induced epileptiform activity, the CA3 area was continuously recorded during 4AP perfusion for a period of up to 2 h. THDOC (Sigma-Aldrich) was applied to slices at concentrations of 0.1, 1 or 5 µM in separate experiments 20 min following the appearance of 4AP-induced spontaneous interictal discharges. THDOC data were analysed following THDOC perfusion for a period of ~20 min. The glutamatergic antagonists [2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX); Sigma-Aldrich] and [3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP); Tocris Bioscience] were applied 20 min following the onset of 4AP-induced spontaneous interictal activity. PTX (Sigma-Aldrich) was applied 20 min following the onset of 4AP-induced spontaneous interictal activity, for a period of 15 min; THDOC was also bath-applied to brain slices. The benzodiazepine diazepam (CDMV, Saint-Hyacinthe, QC, Canada) was bath-applied 20 min following the onset of 4AP-induced spontaneous events, and recordings were analysed 10 min following diazepam application.

Field potential recordings

Field potential recordings were obtained with ACSF-filled glass pipettes (1B150F-4; World Precision Instruments, Sarasota, FL, USA; tip diameter, < 10 µm; resistance, 5–10 MΩ) that were pulled with a Sutter P-97 puller (Sutter, Novato, CA, USA). A recording electrode was placed in the stratum pyramidale or stratum radiatum of the CA3 area. Signals were fed to an AI 401 amplifier and a Cyber-Amp 380 (Molecular Devices, Silicon Valley CA, USA), and then digitised with a Digidata 1322A (Molecular Devices). Signals were sampled at 5 kHz and cut off at 1 kHz.

Detection and analysis of high-frequency oscillatory events

The same method as in Salami *et al.* (2012) was used to analyse HFOs co-occurring with interictal discharges, and will be described here. Time periods containing interictal discharges recorded from the CA3 area were extracted and analysed offline with MATLAB (The Mathworks, Natick, MA, USA). Recordings were first bandpass-filtered in the 80–200-Hz and 250–500-Hz frequency ranges with a finite impulse response filter. The 200–250-Hz frequency band was omitted in order to prevent oversampling of HFOs because of the properties of the filters. Zero-phase digital filtering was used to avoid phase distortion. Each recording was then normalised by use of a 10-s artefact-free period. To be considered as

HFO candidates, oscillatory events in the 80–200-Hz and 250–500-Hz frequency ranges had to show four consecutive peaks at three standard deviations above the mean as determined by the reference period. Overlapping events, which may be caused by the filtering of sharp spikes (Bénar *et al.*, 2010), were excluded from the analysis. The Morlet wavelet transform was used to verify events detected by bandpass analysis. HFO duration was measured as the time difference between the first peak and last peak of the oscillation in a given event. The number of cycles per event was considered to be the number of positive peaks in a given HFO. HFO amplitude was measured in the raw trace, and reported as the average amplitude of the oscillations occurring in each ripple or fast ripple.

Statistical analysis

Offline analysis of the duration and interval of occurrence of fast and slow events was performed with CLAMPFIT 8.2 (Molecular Devices). Fast and slow events were identified by visual analysis based on the duration and morphology of the field event (Fig. 1A). Slow events were longer-lasting and less ‘sharp’ than their fast counterparts. In both cases, we measured the duration as the time period between the first deflection of the discharge from baseline and its return to baseline. Frequency was measured as the rate of onset of discharges occurring over a period of 100–200 s. Amplitude was measured from peak to peak. All visual analyses were conducted blind to the pharmacological manipulations of the slice.

Distributions were tested for skewness and kurtosis. Data were analysed with either two-way ANOVA followed by Tukey’s multiple comparisons test, or with Student’s *t*-test. HFO occurrence was analysed with the non-parametric Mann–Whitney rank sum test followed by the Bonferroni correction for multiple comparisons, as values were not normally distributed. Throughout the text, *n* indicates the number of slices studied, unless otherwise specified. Results are expressed as mean $\pm$ standard error of the mean, and were considered to be significantly different at $P < 0.05$.

Results

Characteristics of interictal activity and HFOs

As reported in previous studies (Voskuyl & Albus, 1985; Perreault & Avoli, 1991, 1992; Bruckner & Heinemann, 2000), 4AP (50 μ M) induced in the CA3 areas two types of interictal discharge, which were classified as either fast or slow interictal events (Fig. 1A). On average, fast events lasted for 121.2 ± 11.5 ms, and slow events lasted for 1428.0 ± 127 ms ($n = 15$ slices, 657 fast events and 157 slow events; Student’s *t*-test, $P = 0$) (Fig. 1B). Fast events occurred at a higher rate (1.8 ± 0.3 Hz; $n = 15$ slices) than slow events (0.02 ± 0.004 Hz; $n = 15$ slices) (Student’s *t*-test, $P = 0.000013$) (Fig. 1B). To verify our classification, we applied *k*-mean cluster analysis, and identified two distinct groups (Fig. 1C). As found in previous experiments performed in the hippocampal slice preparation (Voskuyl & Albus, 1985; Perreault & Avoli, 1991), fast event polarity was inverse with respect to slow event polarity. As illustrated in Fig. 2, properties of fast and slow events, such as duration, frequency, and amplitude, remained constant for up to 90 min following the onset of epileptiform activity during continuous bath application of 4AP.

Fast events were more likely to be associated with HFOs than slow events ($F_{1,59} = 30$, $P = 0.0004$; multiple comparison test, $P < 0.05$; $n = 15$ slices). The coincidence of fast ripples and fast events occurred at a higher rate (17.5% of discharges) than the coincidence of ripples and fast events (12.3% of discharges) (multiple comparison test, $P < 0.05$) (Fig. 1D). Under control conditions, ripple duration was significantly higher than fast ripple duration, i.e. 29.4 ± 1.1 ms and 11.9 ± 0.8 ms, respectively (Student's t -test, $P = 0.000000014$; $n = 18$ slices). On average, ripples had 4.5 ± 0.2 oscillations per event, and fast ripples had 4.7 ± 0.2 oscillations per event. Ripple and fast ripple amplitudes were comparable, i.e. 245.8 ± 40.1 μ V and 238.4 ± 37.2 μ V, respectively. Both ripple occurrence and fast ripple occurrence associated with slow events were low ($1.5\% \pm 0.1\%$, for both HFO types; $n = 15$ slices) (Fig. 1D).

THDOC differentially modulates interictal activity in the CA3 area

We then tested the effect of 0.1, 1 or 5 μ M THDOC (in a series of separate experiments) on the fast and slow events recorded from the CA3 area ($n = 6$ slices for each concentration) (Fig. 3A). Application of THDOC led to a dose-dependent ($F_{3,3504} = 55.60$, $P < 0.00000001$; multiple comparison test, $P < 0.05$) decrease in fast event duration (Fig. 3B). Concomitantly, THDOC application increased slow event duration, with the greatest increase occurring during application of 5 μ M THDOC ($F_{3,1107} = 55.11$, $P < 0.00000001$; multiple comparison test, $P < 0.05$). Analysis of fast event duration at half the maximum amplitude (half-width) showed an increase from an average of 44 ± 4.6 ms in control conditions to 55 ± 4.9 ms following application of 5 μ M THDOC (Student's t -test, $P = 0.00035$; $n = 6$ slices). There was no significant change in the half-width of slow events following application of 5 μ M THDOC (Fig. 3E). In addition, application of 5 μ M THDOC led to a significant reduction in fast event frequency ($F_{3,67} = 5.03$, $P = 0.004$), whereas there was no significant change in slow event frequency at any of the concentrations tested (Fig. 3C). Fast event amplitude was not affected by application of THDOC, but 1 and 5 μ M THDOC significantly increased slow event amplitude ($F_{3,51} = 32.48$, $P < 0.00000001$; multiple comparison test, $P < 0.05$).

THDOC effects on HFO properties

The co-occurrence of ripples with fast events increased only during application of 5 μ M THDOC ($n = 6$ slices; $F_{3,43} = 3.47$; $P = 0.03$), whereas fast ripple levels were not significantly affected by any of the THDOC concentrations tested (Fig. 4A). We also measured the duration, number of oscillations and amplitude of ripples and fast ripples associated with the fast events induced by 4AP during application of 0.1, 1 and 5 μ M THDOC (Fig. 4B–D). The addition of THDOC failed to significantly change either the duration or number of oscillations of ripples and fast ripples ($n = 6$ slices at each concentration). Only at the highest concentration of THDOC tested (i.e. 5 μ M) were there increases in ripple and fast ripple amplitude (ripples, 367.2 ± 42.9 μ V; fast ripples, 477.3 ± 36.5 μ V; $P < 0.05$) as compared with the control (ripples, $F_{3,39} = 6.46$, $P = 0.0019$; fast ripples, $F_{3,39} = 17.38$, $P = 0.000000017$; multiple comparison test, $P < 0.05$). THDOC induced changes in the occurrence of HFOs associated with slow events, because they were virtually absent to begin with.

Network effects of THDOC

To determine whether neurosteroids affect the site of genesis and propagation of interictal events, adjacent electrodes were placed 1 ± 0.5 mm apart to assess the dynamics of interictal firing in the CA3 area before and after application of 5 μ M THDOC (Fig. 5A) (fast events, $n = 118$; slow events, $n = 52$). The differences in delay of onset of interictal events were measured between electrodes a and b and between electrodes a and c. Whereas there was no significant change in delay of onset between 4AP only and THDOC treatment, the variability of the delay was increased following THDOC treatment in fast events (Fig. 5B). The same trend was not observed in slow events (Fig. 5C). Additionally, whereas HFOs showed similar rates of occurrence in each electrode, the co-occurrence of HFOs between the three recorded sites was low during 4AP application. Less than 1% of HFOs occurred simultaneously between adjacent electrodes (a and b or b and c), and almost no HFOs co-occurred between distant electrodes (a and c). Following THDOC treatment, no HFOs were found to co-occur between electrodes.

THDOC modulates pharmacologically isolated slow field events

As reported in previous studies (see for review Avoli & de Curtis, 2011), pharmacologically isolated slow field events continued to occur in the presence of 4AP during blockade of ionotropic glutamatergic transmission with bath application of 10 μ M CPP and 10 μ M NBQX. CPP bath-applied 20 min after the appearance of 4AP-induced synchronous activity led to a significant reduction in the duration of slow events ($n = 6$ slices; $F_{3,715} = 23.57$; $P < 0.00000001$; multiple comparison test, $P < 0.05$) (Fig. 6A and B). NBQX was then applied in addition to CPP, and fast events were abolished. Slow events were significantly reduced in duration (multiple comparison test, $P < 0.05$) and increased in frequency ($n = 6$ slices; $F_{3,35} = 3.15$, $P = 0.043$; multiple comparison test, $P < 0.05$) as compared with the 4AP control, but not as compared with application of CPP and 4AP only.

The effect of 5 μ M THDOC (the highest concentration previously applied; Fig. 3) was then tested on these isolated slow events (Fig. 6A). Bathing slices treated with CPP and NBQX with 5 μ M THDOC led to an increase in slow event duration ($n = 5$, multiple comparison test, $P < 0.05$) (Fig. 6B–D). THDOC at 5 μ M also reduced the frequency of isolated slow events, and increased their amplitude ($F_{3,195} = 5.35$, $P = 0.0016$; multiple comparison test, $P < 0.05$) (Fig. 6D). CPP-mediated blockade of *N*-methyl-D-aspartate receptors also led to a significant reduction in fast ripple occurrence in association with fast events as compared with 4AP control conditions ($n = 6$, $F_{3,35} = 4.97$, $P = 0.008$; multiple comparison test, $P < 0.05$) (Fig. 6E). There were no significant effects of CPP, NBQX or THDOC on the occurrence of ripples or fast ripples associated with slow events, probably because their frequency of occurrence was almost negligible to begin with (Fig. 6E).

To better understand the role of modulation of GABA_A receptor-mediated activity in 4AP-induced epileptiform activity, we also assessed the effect of 150 nM diazepam on fast and slow interictal activity. The reported EC₅₀ of diazepam in the hippocampus is between 100 nM and 150 nM (Aguayo *et al.*, 1994; Kapur & MacDonald, 1996). Overall, the effect of 150 nM diazepam was relatively modest. The duration, amplitude and frequency of fast events were not significantly modulated. The duration and amplitude of slow events also

remained unchanged; however, their frequency of occurrence significantly increased (Student's *t*-test, $P = 0.0256$; $n = 5$ slices). In two separate experiments, we tested whether a high concentration of diazepam was sufficient to abolish interictal events occurring in the CA3 area. In the presence of 50 μM 4AP, interictal events persisted even during the application of 70 μM diazepam over a period of at least 30 min.

Effects of THDOC following blockade of GABAergic transmission

In five slices, we established the effects of high concentrations of the GABA_A receptor antagonist PTX (100 μM) on 4AP-induced interictal activity. PTX application abolished slow events and produced high-amplitude (5.02 ± 0.81 mV) rhythmic events of duration 1.6 ± 0.1 ms, occurring at an average frequency of 0.06 ± 0.02 Hz (Fig. 7A–D). Additionally, fast ripple occurrence increased during PTX application ($F_{2,44} = 7.30$, $P = 0.0028$; multiple comparison test, $P < 0.05$), but ripple occurrence remained unchanged. Application of 5 μM THDOC failed to significantly modulate the duration, frequency and amplitude of the PTX-induced epileptiform activity; however, the occurrence of fast ripples was decreased (multiple comparison test, $P < 0.05$).

Discussion

Our main findings can be summarised as follows. First, fast interictal events recorded from the CA3 area during bath application of 4AP are more likely to be associated with fast ripples than ripples, whereas slow interictal events show almost no association with HFOs. Second, THDOC decreases the duration and frequency of fast events, but increases the duration and amplitude of slow events. Third, pharmacologically isolated slow events are sensitive THDOC. Finally, the occurrence of ripples does not increase with the addition of THDOC following PTX application. It should be emphasised that these effects are not time-dependent, as the duration, amplitude and frequency of the 4AP-induced epileptiform activity remains constant over time, suggesting that they are related to the pharmacological effects of THDOC.

Neurosteroidal modulation of interictal activity

THDOC application led to differential changes in the field properties of fast and slow interictal events generated in the CA3 area during 4AP application. Fast interictal events, which are known to be abolished by ionotropic glutamatergic receptor antagonists (see for review Avoli & de Curtis, 2011), became shorter in duration and occurred at a reduced frequency as compared with the control following THDOC application, possibly because of the potentiation of GABAergic conductances by THDOC in pyramidal cells. In contrast, slow interictal events had longer durations and higher amplitudes following THDOC treatment, and a similar trend was observed following the blockade of glutamatergic signaling. Slow field events can be recorded in all limbic areas following the application of 4AP (Avoli & de Curtis, 2011), and are mainly attributed to GABA_A receptor-mediated postsynaptic currents generated by pyramidal cells in response to GABA released from interneurons (Perreault & Avoli, 1991, 1992; Avoli *et al.*, 1996, 2002).

Enzymes involved in the synthesis of neurosteroids such as THDOC and allopregnanolone are strongly expressed in CA3 pyramidal cells (Agís-Balboa *et al.*, 2006), the same cells that fire action potential bursts during fast interictal events (Perreault & Avoli, 1991, 1992); therefore, endogenous neurosteroids should regulate the excitability of pyramidal neurons in the CA3 area in an autocrine fashion. Neurosteroids are also synthesised in glial cells (Jung-Testas *et al.*, 1989; Robel & Baulieu, 1994), so they can also control the tone of a given region through a paracrine mechanism (Lambert *et al.*, 2003), influencing not only principal neurons but also interneurons.

THDOC has the ability to potentiate both tonic and phasic GABA_A receptor-mediated currents (Wohlfarth *et al.*, 2002); therefore, by hyperpolarizing the membrane potential or by shunting of excitatory inputs, THDOC can reduce cell firing and possibly the potential for the neuronal networks to maintain epileptiform activity over time (Farrant & Nusser, 2005). This mechanism may account for what appears to be the reduction in epileptiform activity in the CA3 area following the application of THDOC to 4AP-treated slices.

In line with the evidence indicating that neurosteroids modulate GABA_A receptor-mediated currents, the non-competitive GABA_A receptor antagonist PTX (Sivilotti & Nistri, 1991) effectively abolished the effect of THDOC on the duration, amplitude and frequency of epileptiform events. It is important to note that, in the *in vitro* study conducted in the CA3 area by Salazar *et al.* (2003), it was found that the neurosteroid allopregnanolone produces concentration-dependent suppression of epileptiform bursting induced by application of either PTX (100 μ M) or 4AP (55 μ M). The differences between the aforementioned study and our study may be attributable to the effect of neurosteroids on 4AP alone and PTX alone, as compared with 4AP and PTX together. Salazar *et al.* (2003) also investigated the effects of high concentrations of allopregnanolone and pregnanolone (10–90 μ M), whereas we used lower concentrations (0.1–5 μ M) of THDOC. Those GABA_A receptor channels that remain open in the presence of 100 μ M PTX maintain normal kinetics (Sivilotti & Nistri, 1991), but very high concentrations of neurosteroids may be required to modulate these GABA receptors. These contrasts highlight the differences between more physiological and therapeutically beneficial concentrations of neurosteroids and the roles that they play in controlling brain excitability.

Both THDOC and diazepam (at the EC₅₀) increased the frequency of occurrence of slow events occurring in the CA3 area; however, diazepam did not significantly modulate their amplitude and duration. Moreover, diazepam failed to significantly modulate fast interictal events occurring in the CA3 area. The differences observed between the effects of diazepam and THDOC may be accounted for by differences in the effective concentration. Whereas diazepam was applied at the EC₅₀, THDOC was applied at higher concentrations.

Interictal events in the CA3 area were only minimally modulated by diazepam at the EC₅₀, and events persisted even in the presence of 70 μ M diazepam. The persistence of interictal events may reflect the mechanism by which 4AP enhances epileptiform activity. Under the application of 4AP, the release of both excitatory and inhibitory neurotransmitters is increased, whereas GABA_A receptors can mediate long-lasting depolarizing potentials, which may still maintain a degree of inhibition through a shunting mechanism (Avoli & de

Curtis, 2011). In this sense, increasing GABAergic transmission may not be sufficient to abolish 4AP-induced epileptiform activity unless very high concentrations are applied, as demonstrated by Salazar *et al.* (2003). Why, then, do interictal events persist whereas ictal events recorded in the piriform cortex can be abolished (50% of slices) over time following the application of 5 μ M THDOC (Herrington *et al.*, 2014)? This difference may illustrate the importance of shunting inhibition provided by GABA_A receptor-mediated currents in sustaining ictal event occurrence as compared with interictal event occurrence.

Neurosteroidal modulation of HFOs

Our findings show that ripples and fast ripples are differently associated with fast and slow interictal discharges. Both ripples and fast ripples were more likely to co-occur with fast events than with slow events; in addition, fast events were more likely to be associated with fast ripples, supporting the hypothesis that principal neuron firing is involved in the generation of fast ripples (Engel *et al.*, 2009; Ibarz *et al.*, 2010; Bragin *et al.*, 2011). In line with this view, when we blocked GABAergic signaling with PTX, there was an increase in fast ripple occurrence but no change in ripple occurrence. Finally, in the presence of glutamatergic signaling blockers (CPP and NBQX), both ripple occurrence and fast ripple occurrence were virtually negligible. These findings reinforce the hypothesis that the interplay between interneuronal and principal neuron firing plays an important role in the maintenance of ripple and fast ripple activity (Jefferys *et al.*, 2012).

The application of THDOC to 4AP-treated slices led to an increase in ripple occurrence that coincided with a decrease in interictal frequency, but without a concomitant change in fast ripple occurrence. It is known that THDOC leads to an increase in GABA_A receptor-mediated activity (Reddy & Rogawski, 2012); thus, the increase in ripple occurrence but not fast ripple occurrence further supports the hypothesis that ripples may reflect the activity of synchronous GABAergic interneuronal activity entraining a population of principal neurons (Buzsáki *et al.*, 1992; Ylinen *et al.*, 1995; Zijlmans *et al.*, 2009; Demont-Guignard *et al.*, 2012; Wendling *et al.*, 2012). When THDOC was applied to slices treated with glutamatergic antagonists, HFO occurrence remained low. Interestingly, when 5 μ M THDOC was applied to slices treated with 4AP and PTX, fast ripple occurrence decreased to control levels, whereas parameters such as event duration, frequency and amplitude of epileptiform activity remained unaffected. This may be related to changes induced by THDOC within local networks that can only be detected at the level of HFOs.

The duration of ripples and fast ripples, along with the number of oscillations per event, remained constant between the control condition (i.e. in the presence of 4AP only) and the application of THDOC. Whether changes in the duration and the number of oscillations per HFO can be observed with lower detection thresholds remains uncertain, but it seems that the most important parameter is the shift from higher-frequency bands to lower-frequency bands (fast ripples to ripples) with the application of THDOC. Ripple amplitude and fast ripple amplitude were also increased following the application of 5 μ M THDOC, which may reflect increased recruitment of synchronised pyramidal cells during interictal events regardless of whether or not this is mediated through interneurons; however, this hypothesis needs to be confirmed in future studies.

Concluding remarks

Our study demonstrates that neurosteroids can modulate both fast and slow interictal discharges, as well as HFOs associated with fast events recorded in the CA3 area. Although fast and slow discharges rely predominantly on different glutamatergic and GABAergic contributions, they are both modulated by the neurosteroid THDOC. We propose that the way in which these events are modulated can provide further insights into how GABAergic mechanisms contribute to the control of epileptiform synchronisation.

Acknowledgments

This study was supported by the Canadian Institutes of Health Research (CIHR grants 8109, 74609). R. Herrington is supported by a Savoy Foundation Studentship. We thank Ms P. Salami and Mr H. Yvers for helping with data analysis and recording procedures.

Abbreviations

4AP	4-aminopyridine
ACSF	artificial cerebrospinal fluid
CPP	3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid
HFO	high-frequency oscillation
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
PTX	picrotoxin
THDOC	allotetrahydrodeoxycorticosterone

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Fig. 1.

Interictal activity induced by 4AP bath application. (A) Epileptiform discharges recorded from the CA3 area of an intact brain slice following the application of 50 μ M 4AP. Top – wavelet transform analysis was used to confirm the frequency components of the HFOs. The traces show the wideband recording along with filtered and normalised traces used for the detection of HFOs. The expanded trace on the left shows a slow interictal event without any corresponding HFO. Highlighted in black is an example of an overlapping event, which was omitted from the HFO analysis. The expanded traces on the right show two fast interictal events with a corresponding ripple and fast ripple, respectively. (B) Bar graphs showing the respective durations and frequencies of fast and slow events. (C) The mean frequencies and durations of fast (black) and slow (gray) events from 16 experiments are plotted on the scatter plot. The green crosses show the centers of the fast and slow clusters calculated by k -means clustering analysis. (D) Bar graph showing the occurrence of ripples and fast ripples associated with fast and slow events. Fast events were more likely to co-occur with fast ripples, and were more likely to be associated with HFOs in general than were slow events.

* $P < 0.05$.

Fig. 2.

4AP-induced epileptiform activity is maintained over time. (A) Example of a field recording of CA3 interictal activity that lasted for 90 min. Portions of the recording, indicated by the dashed lines, are expanded to show that the activity remains consistent over time. Bath application of 4AP began 20 min before the onset of the recording. (C and D) Graphs show the durations, frequencies and amplitudes of fast (B) and slow (C) interictal activity over time. Times are calculated from the 'onset' of 4AP application.

Fig. 3.

THDOC modulates interictal activity in the CA3 area. (A) Three different sets of experiments showing the effects of 0.1, 1 and 5 μM THDOC on fast and slow interictal events in the CA3 area. Fast events indicated below the dots are expanded on the right. (B) Summary of the effects of THDOC on the durations of fast and slow events. Note that, at the highest concentrations tested, THDOC reduced the duration of fast events and increased the duration of slow events recorded in the field ($P < 0.05$). (C) Summary of the effects of THDOC on the frequencies of occurrence of fast and slow events. Note that THDOC significantly reduced the frequency of fast events at 1 and 5 μM ($P < 0.05$), but had no significant effect on slow events. (D) Summary of the effects of THDOC on the amplitudes of fast and slow events. Note that THDOC failed to induce any changes in the amplitude of fast events, but increased the amplitude of slow events at 1 and 5 μM ($P < 0.05$). (E) Diagram showing the changes in half-width (blue) and amplitude (green) in slices treated with 5 μM THDOC as compared with the control (* $P < 0.05$).

Fig. 4.

Properties of HFOs following THDOC treatment. (A) Graphs showing the occurrence of HFOs under control conditions and during THDOC application. Note that ripple occurrence remained constant at the concentrations of THDOC tested; however, fast ripple occurrence increased with the highest concentration of THDOC. (B–D) Graphs showing the durations, cycle numbers and amplitudes of ripples and fast ripples associated with fast interictal events following THDOC treatment. Note that only 5 μM THDOC induced significant increases in the amplitudes of ripples and fast ripples (* $P < 0.05$).

Fig. 5.

Network effects of THDOC. (A) Recordings from three adjacent electrodes placed within the CA3 area. (B) Graphs showing the delay between electrodes a and b and between electrodes a and c before and after treatment with THDOC in fast events in two sets of experiments. (C) Analysis of delay between a and b and between a and c in slow events for two sets of experiments.

Fig. 6.

THDOC modulates isolated slow events. (A) Field potential recordings of 4AP-induced interictal activity following CPP, NBQX and THDOC treatment in the CA3 area. (B) Summary of the durations of fast and slow events during application of CPP, NBQX, and THDOC (5 μ M); note that fast events were abolished by NBQX treatment. (C) Summary of the frequencies of occurrence of fast and slow events under the different pharmacological conditions. (D) Summary of the changes in amplitudes of fast and slow events. (E) Ripple and fast ripple occurrence associated with fast (left) and slow (right) events. Note that ripple and fast ripple occurrence associated with slow events remained low throughout the pharmacological manipulations. * $P < 0.05$.

Fig. 7. GABA_A receptor-mediated activity blockade. (A) Field potential recordings of 4AP-induced epileptiform activity in the CA3 area during PTX and THDOC treatment. The event indicated under the dot is expanded on the right. (B–D) Graphs summarising the durations, frequencies and amplitudes of the epileptiform events occurring in slices treated with PTX and THDOC. Note that THDOC failed to significantly affect the epileptiform activity generated by PTX-treated slices. (E) Application of PTX increased fast ripple occurrence (gray) but did not affect ripple occurrence (black). * $P < 0.05$.