

Stiripentol, A Putative Antiepileptic Drug, Enhances the Duration of Opening of GABA_A-Receptor Channels

*†Pascale P. Quilichini, ‡Catherine Chiron, *†Yehezkel Ben-Ari, and *†Henri Gozlan

*INSERM U29-INMED, and †Université de la Méditerranée, Marseille; and ‡INSERM U663- Service de Neurologie et Métabolisme, Hôpital Necker, Paris, France

Summary: *Purpose:* Stiripentol (STP) is currently an efficient drug for add-on therapy in infantile epilepsies because it improves the efficacy of antiepileptic drugs (AEDs) through its potent inhibition of liver cytochromes P450. In addition, STP directly reduces seizures in several animal models of epilepsy, suggesting that it might also have anticonvulsive effects of its own. However, its underlying mechanisms of action are unknown.

Methods: We examined the interactions of STP with γ -aminobutyric acid (GABA) transmission by using patch-clamp methods in CA3 pyramidal neurons in the neonatal rat.

Results: STP markedly increased miniature inhibitory postsynaptic current (mIPSC) decay-time constant in a concentration-dependent manner. The prolongation of mIPSC duration does not result from an interaction with GABA transporters because it persisted in the presence of GAT-1 inhibitors (SKF-89976A and NO-711). An interaction with benzodiazepine or neurosteroid

binding sites also was excluded because STP-mediated increase of decay time was still observed when these sites were initially saturated (by clobazam, zolpidem, or pregnanolone) or blocked (by flumazenil or dehydroepiandrosterone sulfate), respectively. In contrast, saturating barbiturate sites with pentobarbital clearly occluded this effect of STP, suggesting that STP and barbiturates interact at the same locus. This was directly confirmed by using outside-out patches, because STP increased the duration and not the frequency of opening of GABA_A channels.

Conclusions: At clinically relevant concentrations, STP enhances central GABA transmission through a barbiturate-like effect, suggesting that STP should possess an antiepileptic effect by itself. **Key Words:** Stiripentol—Anticonvulsant drugs—GABA transmission—Miniature IPSCs—Single channel—Decay-time constant—GAT-1—Benzodiazepines—Neurosteroids—Barbiturates—Hippocampus—In vitro.

A wide range of antiepileptic drugs (AEDs) is presently available. They are differentiated by their mechanism of action and their clinical efficacy in various epileptic syndromes. However, the adverse effects that AEDs exert directly or through their metabolites (1,2) considerably limit their usefulness, particularly in resistant epilepsies that often require a progressive enhancement of AED concentrations. To prevent or to diminish these adverse effects, a useful strategy might be to combine AEDs with drugs that inhibit their oxidative degradation (3,4). In this context, stiripentol (STP) has been shown to reduce efficiently the metabolic degradation of several cytochromes P450 (CYPs)-sensitive AEDs (5–9). Accordingly, clinical studies have highlighted the usefulness of STP as an adjunctive therapy in pediatric epilepsies, notably in the Dravet syndrome (6,10,11). This striking efficiency of STP raised the possibility that it may have a direct antiepileptic action, although clinical trials failed to demonstrate a specific therapeutic action different from that of AEDs given in associa-

tion (6). In contrast, when administered alone, STP did display anticonvulsant properties in several animal models of epilepsy. STP attenuated seizures induced by pentylenetetrazol (12,13) and partially protected from convulsions induced by electrical stimulation (12). In addition, short- and long-term STP treatments were effective in reducing generalized seizures induced by 4-deoxypyridoxine in monkeys (14). These observations are consistent with a direct antiepileptic action of STP, but the mechanisms by which STP may exert its direct anticonvulsant properties are largely unknown, particularly at the cellular level. Earlier in vitro studies suggested, however, that STP interferes with γ -aminobutyric acid (GABA)ergic transmission (12,15). We have therefore examined the effects of STP on isolated GABA transmission in postnatal rat hippocampal neurons and investigated the underlying mechanisms. We report that at relevant clinical concentrations, STP markedly enhances GABA release and prolongs GABA_A receptor-mediated currents. STP increases the mean open duration of GABA_A receptor-dependent chloride channels by a barbiturate-like mechanism, suggesting that it has potential antiepileptic properties on its own.

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Address correspondence and reprint requests to Dr. H. Gozlan at INSERM U29-INMED, B.P. 13, route de Luminy, 13273 Marseille cedex 09, France. E-mail: gozlan@inmed.univ-mrs.fr

METHODS

Preparation of hippocampal slices

All protocols were designed according to INSERM guidelines for the care and use of animals. Experiments were performed on hippocampal slices taken from Wistar rats between postnatal (P) days 7 and 8. In brief, animals were killed by decapitation, and brains were extracted from the skull and rapidly submerged in oxygenated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (aCSF) at 4°C with the following composition (in mM): NaCl, 126; KCl, 3.5; CaCl₂, 2; MgCl₂, 1.3; NaHCO₃, 25; NaHPO₄, 1.2; and glucose, 10 (pH, 7.3). Hippocampal slices, 500 μm thick, were cut with a McIlwain tissue chopper and left in aCSF at room temperature for ≥1h. Individual slices were transferred to the recording chamber where each slice was fully submerged and superfused with oxygenated aCSF at 32 ± 1°C, at a flow rate of 3.0 ± 0.2 ml/min. Drugs were applied through the perfusion.

Drugs

NBQX(2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoline);D-APV(2-amino-5-phosphonovaleric acid); TTX(tetrodotoxin);and SKF-89976A [*N*-(4,4-diphenyl-3-butenyl)-3-piperidine carboxylic acid]were supplied byTocris Ltd (U.K.).Pentobarbital[5-ethyl-5-(1'-methylbutyl)barbituric acid];zolpidem [*N,N*,6-trimethyl-2-(4-methylphenyl)imidazo(1,2a)pyridine-3-acetamidehemitartrate];flumazenil[8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5α][1,4]benzodiazepine-3-carboxylic acid ethylester];NO-7111(2-[[[(diphenylmethyl-ene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid);-pregnanolone(5β-hydroxy-3α-pregnan-20-one);and DHEAS (dehydroepiandrosterone sulfate)were purchased from Sigma-Aldrich(France). Clobazam and stiripentol {4,4-dimethyl-1-[3,4-(methylenedioxy)-phenyl]-1-penten-3-ol} were generous gifts from Sanofi-Aventis (France) and Biocodex Laboratories (Montrouge, France), respectively. Drugs were first dissolved in water or in DMSO and then diluted in aCSF. The final concentration of DMSO (0.1%) has no effect on recorded signals.

Whole-cell recordings

Experiments were generally performed in the CA₃ area of the hippocampus. Blind whole-cell recordings of pyramidal cells were performed in voltage-clamp mode. Borosilicate recording electrodes (5–7 MΩ) were filled with an internal solution with the following composition (in mM): CsCl, 140; NaCl, 10; HEPES, 10; EGTA, 1; CaCl₂, 0.1; MgATP, 2; GTP, 0.5, to which biocytin (5 mg/ml) was added. The final solution was adjusted to pH 7.3and 270–280 mOsm. Recordings were performed by using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.), sampled at 10 kHz (Digidata 1200; Axon Instruments). Isolated GABAergic

events were obtained in the presence of NBQX (10 μM) and D-APV (40 μM) to block AMPA/kainate and NMDA receptors-mediated currents, respectively. Miniature GABAergic inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of tetrodotoxin (TTX, 1 μM) and glutamate antagonists. Data were recorded at a holding potential of –70 mV. Drugs were superfused for 30 min because their effects were stabilized after 15 min, allowing their analysis during the last 15 min. Occlusion experiments were performed on the same cell: control (CTR) values were first determined during ≥15 min. The reference drug was then applied alone for 30 min to the same neuron. Finally, a combination of the drug and STP was further applied to the same neuron for an additional period of 30 min. The modification induced by the reference drug was first compared with the CTR value and then with that obtained in the presence of STP. Evoked IPSCs (e-IPSCs) were obtained by using a bipolar twisted nichrome electrode (15–60 V; 30–60 μs), placed in the hilus.

Outside-out recordings

Borosilicate recording electrodes (8–10 MΩ) were filled with an internal solution with the following composition (in mM): CsCl, 140; HEPES, 10; BAPTA, 5; MgCl₂, 2; MgATP, 2; GTP, 0.5; pH, 7.3; 270–280 mOsm. CA₃ pyramidal cells were blindly patch-clamped at 22°C. Outside-out patches were then excised and removed from the slice. Drugs were applied for 5 min through the perfusion at a flow rate of 6 ml/min. Data were recorded at a holding potential of –50 mV.

Data analysis

The series resistance (15–40 MΩ) was determined on line in each experiment by a fitting analysis of the transient currents in response to a 5-mV pulse by using Acquis 5.0 software (G. Sadoc, Orsay, France). Cells were discarded when their series resistances varied by >20% during the course of the experiment. From the digitized records, spontaneous, evoked, and mIPSCs were identified by using threshold-based event detection and analyzed by using MiniAnalysis software (Synaptosoft, Decatur, GA, U.S.A.). They were generally individually inspected and selected before pooling. Frequency and mean amplitudes were determined over a 5-min period; 200–300 consecutive events were selected and averaged to determine 10–90% rise time and 10–90% decay-time constants. IPSCs with slow-onset kinetics and traces with multiple events preventing a clear observation of the 10–90% decay segment were not included in the analysis. Decay-time constants (τ) of IPSCs (individual and averaged) were best fitted by using a single exponential function

$$I = I_{\max} \times \exp(-t/\tau)$$

because no significant reduction of the standard deviation was generally observed when data were fitted with

two exponentials. Digitized outside-out recordings were also analyzed by using MiniAnalysis. Results were expressed as mean values \pm SEM of (n) independent experiments performed on different animals. Paired *t* tests were performed for significance determination of mean values; the Kolmogorov–Smirnov test was used for comparison of distributions. A *p* value <0.05 indicated a significant difference between mean values.

Cell-type identification

After recording, each slice was processed for morphologic identification of the patched cell. All treatments were performed in 0.12 M phosphate buffer. Slices were fixed overnight at 4°C in the buffer containing 4% fresh paraformaldehyde and then rinsed. They were cryoprotected in a sucrose (20%) solution for ≥ 24 h and then quickly frozen on ice. Slices were incubated 30 min with H₂O₂ (1%) to neutralize endogenous peroxidases, washed again, and incubated overnight in the avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Peroxidase kit; Biovalley, France) containing 0.3% Triton X-100. Slices were then rinsed for 1 h and incubated 10–15 min in a 3,3'-diaminobenzidine/H₂O₂ solution. Neurons were visualized, and only pyramidal cells were selected for analysis.

Stiripentol concentrations

When epilepsy patients were coadministered STP and a conventional AED, the mean plasma concentration of STP varied between 4 and 22 $\mu\text{g/ml}$ (6,16,17), corresponding to a concentration range of 30–100 μM . When STP was administered alone in animal models of epilepsy, higher plasma values were reported. The maximal anticonvulsive effect obtained with a single administration of STP to rats given a pentylenetetrazol infusion was correlated with a plasma concentration of STP of ~ 120 $\mu\text{g/ml}$ (13). This value corresponds to ~ 500 μM . To cover this large range of concentrations, STP was used in this study between 30 and 300 μM . Concentrations of STP also have been determined in the rat brain and were found to be comparable to those determined in the plasma (13).

RESULTS

Effect of stiripentol on isolated GABAergic transmission

The effects of STP on isolated GABAergic transmission were examined in the hippocampus, in the presence of TTX (1 μM) to block action potential-dependent release and glutamate antagonists (NBQX, 10 μM , and D-APV, 40 μM) to block AMPA/kainate and NMDA receptors, respectively. In CA3 pyramidal cells, STP (100 μM) did not modify the amplitude ($p = 0.4$; $n = 10$; Fig. 1A and B) of miniature GABA_A receptor-mediated currents (mIPSCs) but significantly decreased the mean of interevent intervals (Fig. 1C), indicating an increase in

the frequency of mIPSCs ($166 \pm 9\%$ of CTR; $p = 0.002$; Table 1). Examination of mIPSCs kinetics indicated that STP also markedly increased the mIPSC decay-time constant ($190 \pm 11\%$; $p = 0.001$; Fig. 1B and Table 1) but not the rise time ($p = 0.7$; Table 1). Bicuculline (10 μM) completely blocked mIPSCs (not shown), indicating that these synaptic events were mediated by GABA_A receptors. These effects were concentration dependent (Table 1) because STP (30–300 μM) gradually increased duration (Fig. 1D) and frequency (Fig. 1E) of mIPSCs without modification of their amplitude and rise time. Similar effects of STP (100 μM) on mIPSC duration and frequency were observed in CA1 pyramidal cells ($186 \pm 15\%$ and $149 \pm 11\%$; $n = 4$) and granular cells ($168 \pm 17\%$ and $137 \pm 13\%$; $n = 4$). These results indicate that at clinically relevant concentrations (see Methods), STP clearly enhances GABAergic transmission in the hippocampus.

Mechanism of stiripentol-induced prolongation of decay-time constant

The decay-time constant of mIPSCs can be modulated by pre- or postsynaptic mechanisms. We examined these two possibilities by using reference drugs that are known to inhibit GABA uptake or to increase mIPSC duration by a modulation of GABA_A-receptor responses. A saturating concentration of reference drugs [GABA transporter inhibitors, benzodiazepines (BZDs), neurosteroids, or barbiturates] was first used to obtain the maximal response. STP was then applied together with the reference drug to the same cell. An occlusion of the effect indicates that STP and the reference drug act at the same locus, whereas a synergetic effect of STP rather indicates that STP and the reference drug have distinct sites of action.

The effects of STP also were challenged in the presence of antagonists when available.

GABA transporter-inhibitors do not prevent STP-induced increase in mIPSC duration

We first examined the maximal effects of two efficient GABA transporter-1 (GAT-1) inhibitors on the decay-time constant of mIPSCs: SKF-89976A (18,19) and NO-711 (19,20). SKF-89976A interacts with GAT-1 and blocks GABA influx and efflux with an IC₅₀ value in the range of 0.1–1 μM (19,21,22). Therefore we used a concentration of 50 μM to saturate GAT-1 sites. Application of SKF-89976A (50 μM ; $n = 6$) did not modify the decay-time constant (13.6 ± 0.6 ms; $p = 0.8$) or frequency (1.2 ± 0.2 Hz; $p = 0.5$) of mIPSCs (Fig. 2A), in agreement with a previous report (23). In the presence of SKF-89976A, STP (100 μM ; $n = 6$) still increased the decay-time constant (24.5 ± 1.8 ms; $180 \pm 8\%$; $p = 0.003$ vs. SKF-89976A) and frequency (1.9 ± 0.3 Hz; $153 \pm 2\%$; $p = 0.002$, vs. SKF-89976A; Fig. 2A) of mIPSCs. These values are similar to those obtained with STP in the absence SKF-89976A (see Table 1). The amplitude ($p = 0.8$) and rise time

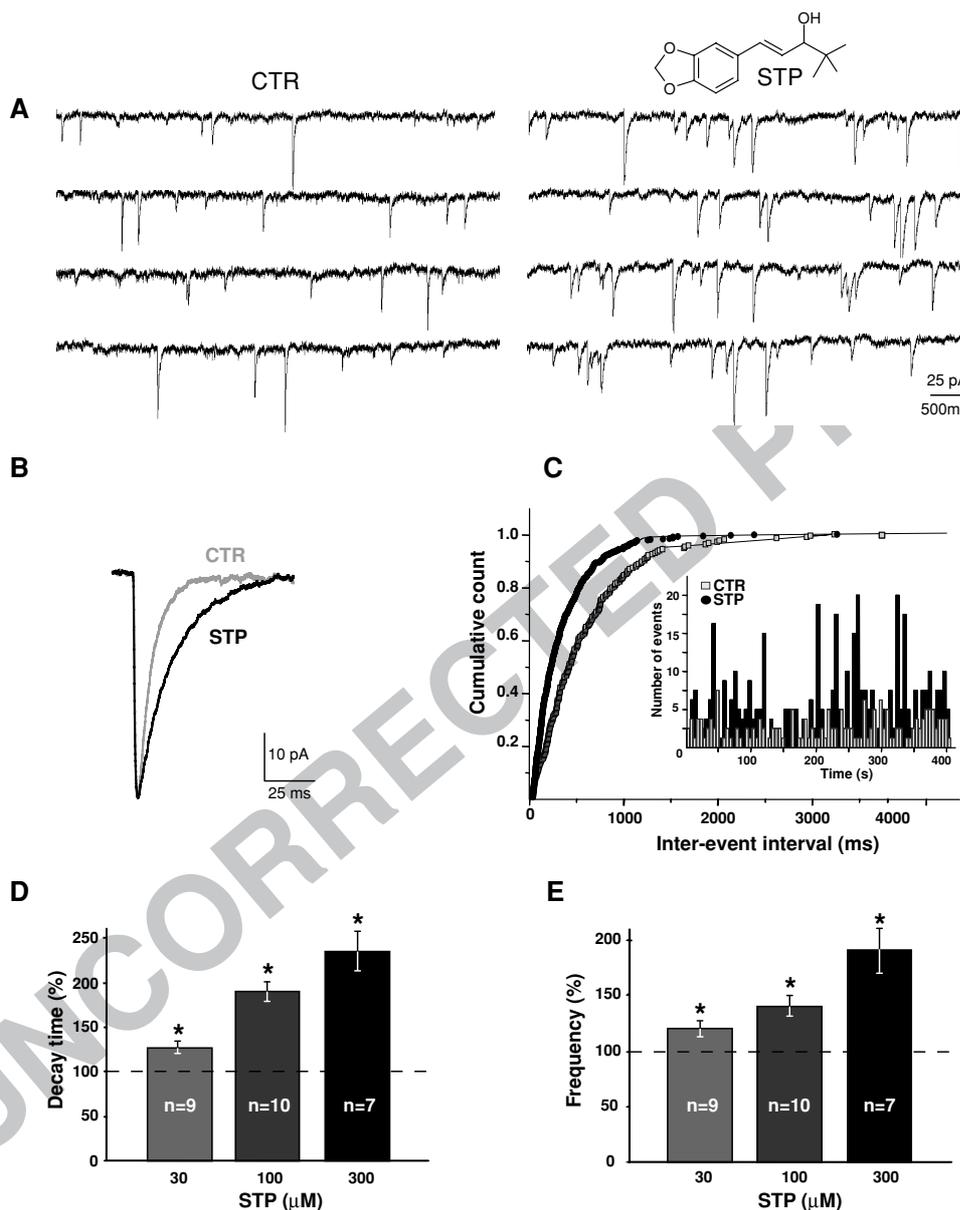


FIG. 1. Stiripentol (STP) dose-dependently enhances duration and frequency of miniature GABAergic inhibitory postsynaptic currents (mIPSCs). Miniature IPSCs were recorded in CA3 pyramidal cells from P8 rats in whole-cell configuration (-70 mV) in the presence of NBQX (10 μ M), D-APV (40 μ M), and tetrodotoxin (TTX; 1 μ M). **A:** Consecutive sweeps of inward currents mediated by γ -aminobutyric acid (GABA) release illustrate mIPSC activity before (CTR) and during superfusion of STP (100 μ M). **B:** Superimposed traces represent the mean of 200–300 events obtained in the same cell before (CTR) and during superfusion of STP (100 μ M). **C:** The distribution of mIPSC interevent intervals was significantly shifted to lower values with STP. **Insert:** The number of events recorded in control (*open bars*) and during STP application (*black bars*). **D:** The decay-time constant of mIPSC (see Table 1) increased from $122 \pm 6\%$ ($p = 0.002$) with STP (30 μ M) to $235 \pm 22\%$ ($p = 0.0001$) with STP (300 μ M). **E:** The frequency of mIPSCs (see Table 1) increased from $120 \pm 5\%$ ($p = 0.008$) with STP (30 μ M) to $194 \pm 19\%$, ($p = 0.002$) with STP (300 μ M). *A significant change between mean \pm SEM values.

($p = 0.9$) of mIPSCs were not affected by STP + SKF-89976A (Fig. 2A).

NO-711 inhibits GAT-1 (19) with an affinity of 0.04 – 0.1 μ M (19,20,21). Consequently, we used a concentration of 3 μ M to saturate GAT-1 sites. Application of NO-711 (3 μ M; $n = 5$) did not significantly modify decay time (14.3 ± 0.8 ms; $p = 0.3$) or frequency (1.6 ± 0.4 Hz; $p = 0.2$) of mIPSCs (Fig. 2B). Other studies also reported that

NO-711 does not affect IPSC characteristics in neonates (24,25). In the presence of NO-711, STP (100 μ M; $n = 5$) still increased mIPSC decay-time constant (31.4 ± 2.5 ms; $205 \pm 16\%$; $p = 0.002$ vs. NO-711) and frequency (2.5 ± 0.5 Hz; $158 \pm 5\%$; $p = 0.03$), whereas amplitude ($p = 0.17$) and rise-time values ($p = 0.16$) remained unchanged compared with NO-711 (Fig. 2B). These results indicate that in neonates, saturating concentrations of GAT-1

TABLE 1. Effects of stiripentol on mIPSCs characteristics

	No.	Frequency (Hz)	Amplitude (pA)	Rise time (ms)	Decay time (ms)
CTR	9	1.0 ± 0.1	34.3 ± 1.5	1.2 ± 0.1	14.4 ± 0.7
STP, 30 μM	9	1.2 ± 0.2 ^a	34.9 ± 2.2	1.2 ± 0.1	17.6 ± 0.9 ^a
CTR	10	0.9 ± 0.1	31.6 ± 2.0	1.2 ± 0.1	13.5 ± 1.1
STP, 100 μM	10	1.5 ± 0.3 ^a	30.6 ± 2.6	1.3 ± 0.1	25.7 ± 1.6 ^a
CTR	7	1.1 ± 0.2	35.5 ± 2.8	1.3 ± 0.1	13.4 ± 1.2
STP, 300 μM	7	2.1 ± 0.5 ^a	34.5 ± 3.9	1.3 ± 0.2	31.5 ± 3.1 ^a

mIPSCs were recorded before (CTR) and 30 min after perfusion of stiripentol (STP) to the same CA3 pyramidal neuron. Frequency, amplitude, rise time, and decay-time constants corresponded to the mean of values determined in *n* independent experiments. For each STP concentration, paired *t* tests were performed. Note that amplitude and rise time are not affected by any STP concentration.

^a*p* < 0.05.

inhibitors did not affect mIPSC duration and did not modify the STP-induced increase of mIPSC duration.

Because GABA-uptake blockers were suggested to be more efficient when GABA release was high (26), we first examined separately the effects of STP and NO-711 on IPSCs evoked by a high-intensity focal stimulation. In these conditions, STP alone (100 μM; *n* = 5) markedly increased the e-IPSC duration (29.5 ± 2.0 ms; 179 ± 9%; *p* = 0.001), whereas NO-711 alone, (3 μM; *n* = 6) exhibited a smaller but significant increase of e-IPSC decay-time constant (20.0 ± 1.9 ms; 123 ± 8%; *p* = 0.023; Fig. 2C). This effect was comparable to that previously reported in neonates (24). In the presence of NO-711, STP (100 μM; *n* = 6) induced a large additional increase of the decay-time constant (33.7 ± 2.5 ms; 208 ± 8%; *p* = 0.0001 vs. NO-711), without modification of the amplitude (*p* = 0.9; Fig. 2C). These results suggest that even when GABA release is high, blocking GATs marginally contributes to the decay-time constant in neonates.

Stiripentol does not interfere with benzodiazepine modulatory sites

We then examined the effects of STP in the presence of BZDs. We used clobazam (CLB) and zolpidem (ZPD), which are chemically unrelated and which bind to different GABA_A-receptor alpha subunits. In addition, CLB is the BZD given in association with STP in the Dravet syndrome (6). CLB (27) and ZPD (28,29) were reported to produce their maximal modulation of GABA_A-receptor responses at ~10 μM. In our conditions, CLB (10 μM; *n* = 5) did not modify amplitude (*p* = 0.3), rise time (*p* = 0.7), or frequency (*p* = 0.15), but increased mIPSC decay-time constant (163 ± 5%; *p* = 0.008; Fig. 3A). Coapplication of STP (100 μM; *n* = 5) led to a further significant enhancement of mIPSC decay-time constant (231 ± 5%; *p* = 0.003 vs. CTR; and 141 ± 6%; *p* = 0.024 vs. CLB), without affecting their size (*p* = 0.4) and rise time (*p* = 0.9) (Fig. 3A). Likewise, ZPD (10 μM; *n* = 4), another drug acting at BZD sites but chemically unrelated to CLB, increased the duration of mIPSCs (181 ± 13%; *p* = 0.017; Fig. 3B) in agreement with previous reports (30,31) but not the frequency (1.3 ± 0.1 Hz; *p* = 0.42; Fig.

3B). Given in association with ZPD, STP (100 μM; *n* = 3) still produced a further prolongation of mIPSC decay time (254 ± 4%; *p* = 0.003 vs. CTR, and 155 ± 5%; *p* = 0.012 vs. ZPD; Fig. 3B). The increase in frequency observed after the coadministration of STP and CLB (1.6 ± 0.1 Hz; 133 ± 2%; *p* = 0.004; Fig. 3A) or ZPD (1.9 ± 0.4 Hz; 146 ± 2%; *p* = 0.004; Fig. 3B) is more likely due to STP, as this modification was not observed with CLB or ZPD in the absence of STP (Fig. 3B). Because CLB and ZPD are agonists at BZD sites, we tested whether the effects of STP were also additive with flumazenil (FMZ), which fully antagonizes the effect of BZDs at 10 μM. At this concentration, FMZ did not increase mIPSC duration (+12 ± 7%; *n* = 5; *p* = 0.17) without modification of rise time (Fig. 3C). Previous studies have reported that FMZ had no effect on GABA transmission (32) and did not modify mIPSC decay-time constant in the hippocampus (33). Blocking BZD sites with FMZ did not prevent STP (100 μM; *n* = 5) further increasing mIPSC decay-time constant (258 ± 17%; *p* = 0.00028 vs. FMZ; Fig. 3C). Together these data suggest that STP does not interact with the BZD regulatory sites of the GABA_A receptor.

Stiripentol does not interfere with neurosteroid modulatory sites

Neurosteroids are potent positive allosteric modulators of GABA_A receptors, but their modulatory sites on the GABA_A receptor are still unknown. However, most of them increase mIPSC decay-time constant (34–36). In the CA1 area of the hippocampus, an endogenous 5β-neurosteroid, such as pregnanolone (PRG), was reported to dose-dependently (30 nM to 1 μM) increase mIPSC decay-time constant (+32% to +170%) (37). To examine whether STP interferes with this modulatory site, we first used a high concentration of PRG (10 μM) and then applied PRG together with STP. As shown in Fig. 4A, PRG significantly increased the decay-time constant (270 ± 37%; *p* = 0.0006 vs. CTR; *n* = 11) but the amplitude (*p* = 0.9) and frequency (*p* = 0.8) of mIPSCs were unchanged. In the presence of PRG, STP (100 μM; *n* = 11) did not modify the amplitude (*p* = 0.4) but enhanced the

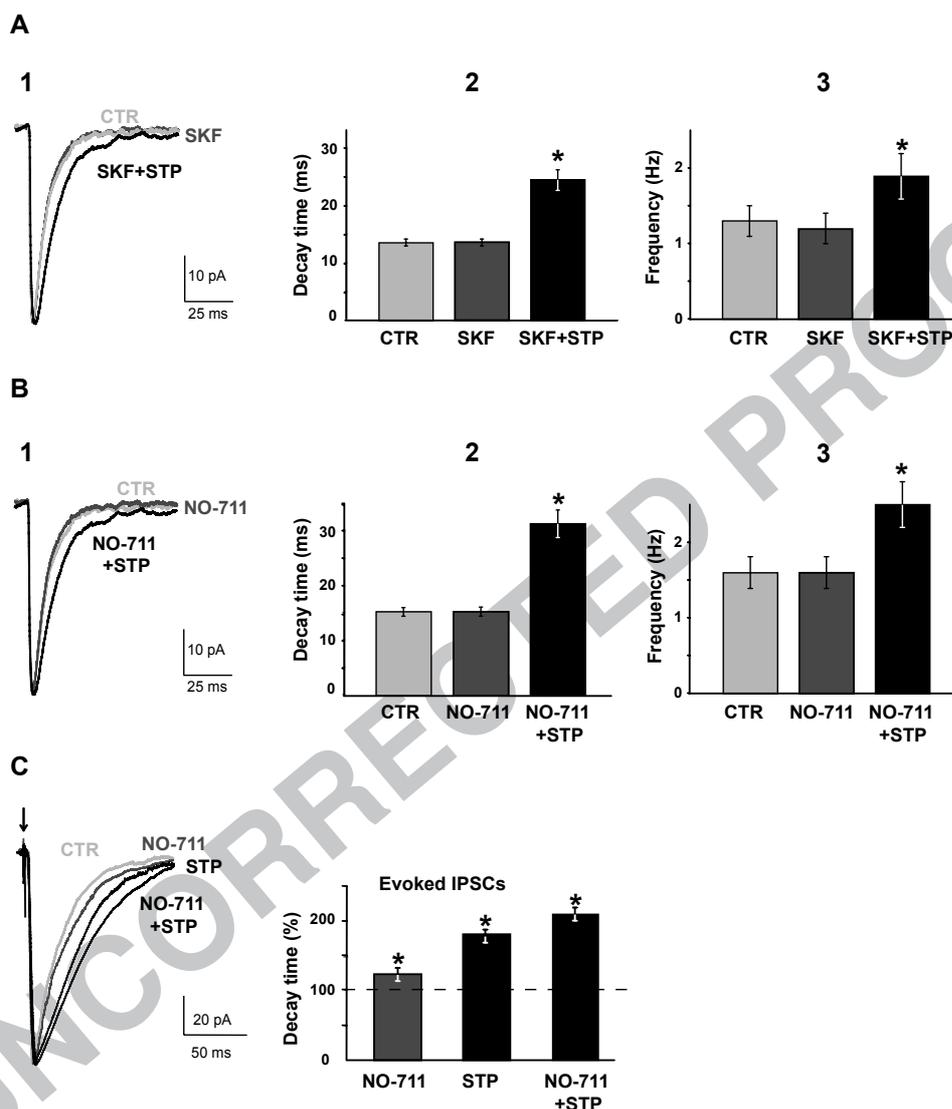


FIG. 2. Stiripentol (STP) does not interact with γ -aminobutyric acid (GABA) transporter-1. **A, B:** Miniature and evoked inhibitory postsynaptic currents (IPSCs) were recorded in CA3 pyramidal cells before (CTR) and during application of saturating concentrations of GAT-1 inhibitors, SKF-89976A (SKF, 50 μ M) or NO-711 (3 μ M). The combination of STP (100 μ M) with either SKF-89976A or NO-711 was then superfused to the same cell. **1:** Average traces of \sim 300 individual mIPSCs obtained in a representative experiment, in control condition (CTR) and during superfusion of GAT-1 inhibitors alone and in combination with STP. **2, 3:** Mean decay-time and frequency values obtained in six (SKF-89976A) and five (NO-711) independent experiments, respectively. **C:** Evoked IPSC responses were recorded in CA3 pyramidal cells with a modified aCSF containing 4 mM Ca^{2+} . *Arrow*, Stimulation artifact. Superimposed traces represent the average of 30 evoked responses obtained in the same neuron, in control condition (CTR), in the presence of either STP (100 μ M) or NO-711 (3 μ M) and in combination of NO-711 (3 μ M) and STP (100 μ M). The histogram represents the mean increases of decay time obtained under perfusion of STP and NO-711 alone and of NO-711 + STP. *Significant increase of decay time compared with control. Intergroup comparisons indicate that the mean values of decay time are significantly different between NO-711 and STP ($p = 0.014$), STP and STP + NO-711 ($p = 0.037$), and NO-711 and STP + NO-711 ($p < 0.001$).

frequency ($159 \pm 14\%$; $p = 0.0001$ vs. PRG) of mIPSCs. In addition, STP significantly further increased the decay-time constant ($536 \pm 65\%$; $p = 0.0003$ vs. CTR and $204 \pm 12\%$; $p = 0.00001$ vs. PRG; Fig. 4A), suggesting that STP and PRG act at two distinct binding sites at the GABA_A-receptor protein. It was not possible to challenge this result by blocking neurosteroid sites, because a drug that antagonizes the effects of 5α - and 5β -neurosteroids is not yet available (34). However, for a number of neuroactive

steroids, introducing a sulfate group at the C-3 position, such as in dehydroepiandrosterone sulfate (DHEAS), reversed neurosteroid modulation of GABA_A receptor responses from positive to negative (38). Although this is certainly not sufficient to consider DHEAS an antagonist, we have examined its putative synergy with STP. In the CA3 area, DHEAS (10 μ M; $n = 6$) decreased the amplitude of mIPSCs ($49 \pm 6\%$) and had no significant effect on decay-time constant ($122 \pm 11\%$; $p = 0.1$; Fig. 4B), in line

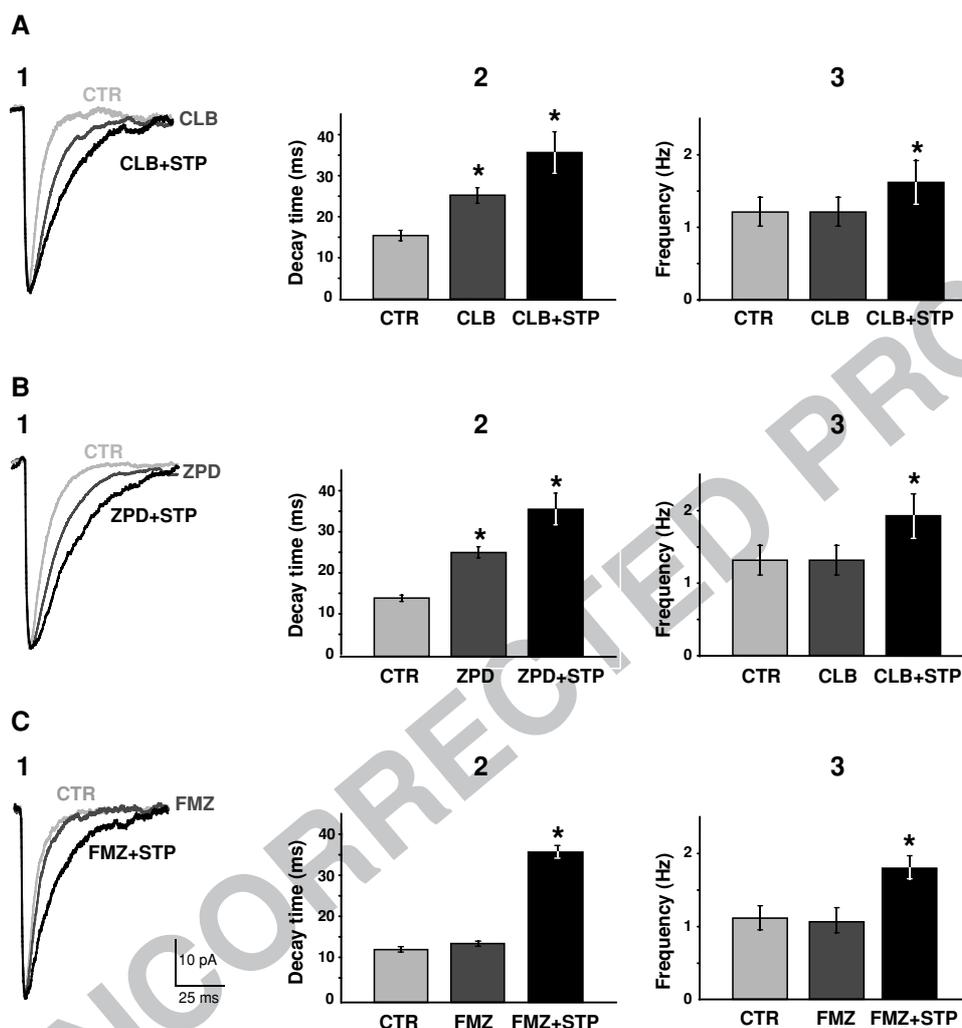


FIG. 3. Stiripentol (STP) does not interact with benzodiazepines sites: A–C: Miniature GABAergic inhibitory postsynaptic currents (IPSCs) were recorded before (CTR) and during application of saturating concentrations of benzodiazepine agonists clobazam (CLB, 10 μM) or zolpidem (ZPD, 10 μM) or antagonist flumazenil (FMZ, 10 μM). STP (100 μM) was then superfused with CLB (A), ZPD (B), or FMZ (C) to the same cell. Superimposed traces represent the average of 200–300 individual mIPSCs obtained in a representative experiment, in control conditions (CTR) and during superfusion of CLB, ZPD, or FMZ alone or in combination with STP. **2, 3:** Mean decay time and frequency values, obtained in five (CLB), four (ZPD) and five (FMZ) independent experiments, respectively.

with a previous report (39). In the presence of DHEAS, STP (100 μM; n = 6) did not modify the amplitude (p = 0.5) but markedly increased the duration (223 ± 19; p = 0.00003 vs. CTR and 185 ± 11%; p = 0.0002 vs. DHEAS; Fig. 4B) of mIPSCs, indicating that STP did not interact with DHEAS-binding sites. Together these results suggest that the effect of STP on mIPSC duration is unlikely to result from an interaction with neurosteroid-binding sites.

Stiripentol enhances GABA_A receptor-mediated response by a barbiturate-like effect

Barbiturates, and particularly pentobarbital (PTB), greatly increase the duration of mIPSCs in neonatal hippocampal slices (40,41). The concentration of PTB that produces the maximal allosteric effect is difficult to determine because PTB also directly activates chloride chan-

nels in the adult brain (42–45). However, in rat hippocampal neurons, a value of 100 μM is considered to maximally increase GABA_A receptor-mediated response by an allosteric mechanism (42,46). At this concentration, in immature pyramidal neurons, PTB (n = 10) considerably increased the decay-time constant (335 ± 14%; p < 0.0001) but not the frequency of mIPSCs (p = 0.27; Fig. 5B). Coadministration of PTB (100 μM) and STP (100 μM; n = 5) did not significantly change mIPSC decay-time value (341 ± 17%; p = 0.9 vs. PTB). A higher concentration of STP (300 μM) also was not able to modify this value (342 ± 16%; n = 5; p = 0.9 vs. PTB; Fig. 5B), suggesting that its binding site is already saturated. Interestingly, saturation of barbiturate sites occluded the prolongation of mIPSC decay time but not the augmentation of frequency induced by two concentrations of STP (162 ± 16%; p = 0.036

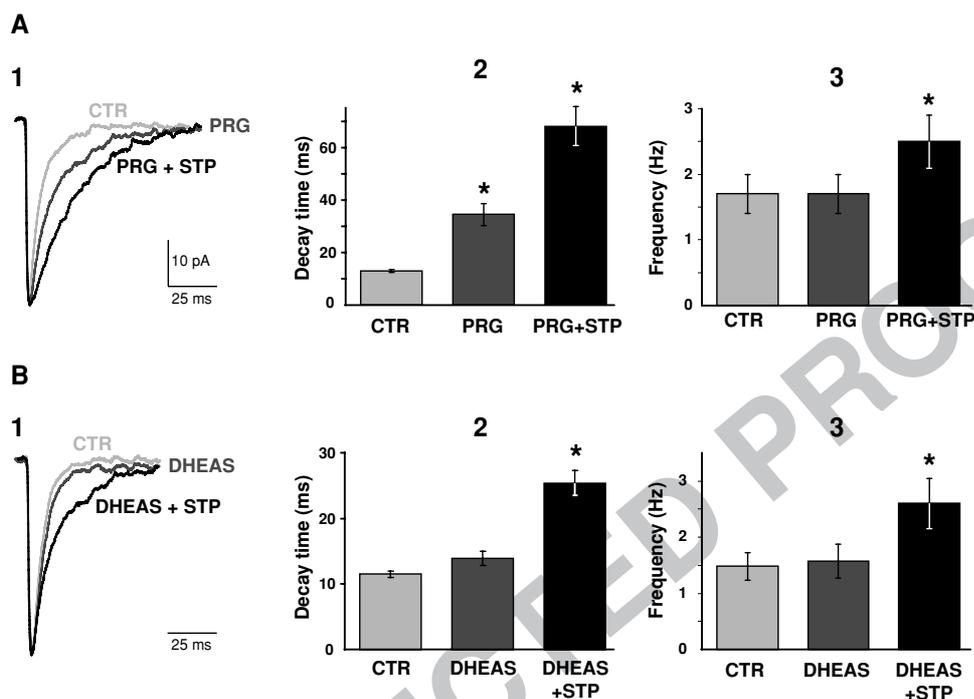


FIG. 4. Stiripentol (STP) does not interact with neurosteroid sites. **A, B:** Miniature IPSCs were recorded before (CTR) and during application of pregnanolone (PRG, 10 μ M), a neurosteroid agonist, or dehydroepiansterone sulfate (DHEAS, 10 μ M), a putative neurosteroid antagonist. STP (100 μ M) was then superfused with PRG (**A**) or DHEAS (**B**) to the same cell. Average traces of 200–300 individual Miniature GABAergic inhibitory postsynaptic currents (mIPSCs) obtained in a representative experiment, in control condition (CTR) and during superfusion of PRG or DHEAS alone or in combination with STP. Note that because DHEAS reduces the amplitude of mIPSCs, the corresponding traces are normalized. **1–3:** histograms represent the mean of decay-time and frequency values, obtained in 11 (PRG) and six (DHEAS) independent experiments.

at 100 μ M and $194 \pm 7\%$; $p = 0.009$, at 300 μ M; Fig. 5B). In addition, we have verified that when barbiturate sites were not saturated, STP does increase mIPSC decay time dose-dependently. Thus a low concentration of PTB (10 μ M) already enhanced decay-time value (17.6 ± 0.9 ms; $p = 0.0001$), and a further significant enhancement was produced by STP (24.9 ± 0.7 ms and 30.0 ± 4.4 ms at 100 and 300 μ M, respectively). Taken together, these results suggest that STP and PTB act on the same modulatory locus on GABA_A receptors. Therefore we decided to verify more directly whether STP interacts with barbiturate modulatory sites of GABA_A receptors by analyzing its effects on single channels.

Stiripentol increases the mean open duration of GABA_A receptor chloride channels

Outside-out patches were excised from CA3 pyramidal cells, and, as expected, no channel opening was observed at -50 mV in the absence of GABA. Application of GABA (2 μ M; $n = 5$) elicited brief bursts of channel openings and closings (Fig. 6A) that reversed ~ 0 mV and were blocked by bicuculline (10 μ M, not shown). These chloride channels were characterized by a mean frequency of 14 ± 2 Hz and a mean amplitude of 1.7 ± 0.1 pA, corresponding to a mean conductance of 29.9 ± 1.1 pS. No rundown was observed during perfusion of GABA. Coap-

plication of STP (100 μ M) and GABA (2 μ M) also induced channel openings and closings (Fig. 6A) that were fully blocked by bicuculline (10 μ M). STP did not affect the mean frequency (14.1 ± 1.8 Hz; $p = 0.11$; $n = 5$; Fig. 6B), amplitude (1.6 ± 0.1 pA; $p = 0.75$), or conductance (31.4 ± 1.3 pS; $p = 0.63$) of chloride channels. In contrast, the duration of channel openings was increased (Fig. 6C). The mean open duration of GABA_A channels elicited by GABA alone was 7.3 ± 0.7 ms, and this value was significantly increased in the presence of STP (9.9 ± 0.5 ms; $p = 0.018$; Fig. 6C), an effect that is compatible with a barbiturate-like mechanism. However, because PTB displays a direct effect on chloride channels, independent of its allosteric modulation of GABA_A receptors, we investigated whether STP also displays such a direct effect in the absence of GABA. We first applied GABA (2 μ M; $n = 7$) to outside-out patches and observed the same pattern of open and closed states as mentioned earlier (Fig. 6D). No spontaneous opening was detected when GABA was completely washed out. Application of STP alone (100 μ M, $n = 3$; or 300 μ M, $n = 4$) for 10 min did not elicit any channel opening (Fig. 6D), indicating that STP did not gate GABA-independent chloride channels and that it did not behave as a GABA_A-receptor agonist. To verify that the absence of an event was not the result of channel rundown or experimental artifacts, GABA (2 μ M,

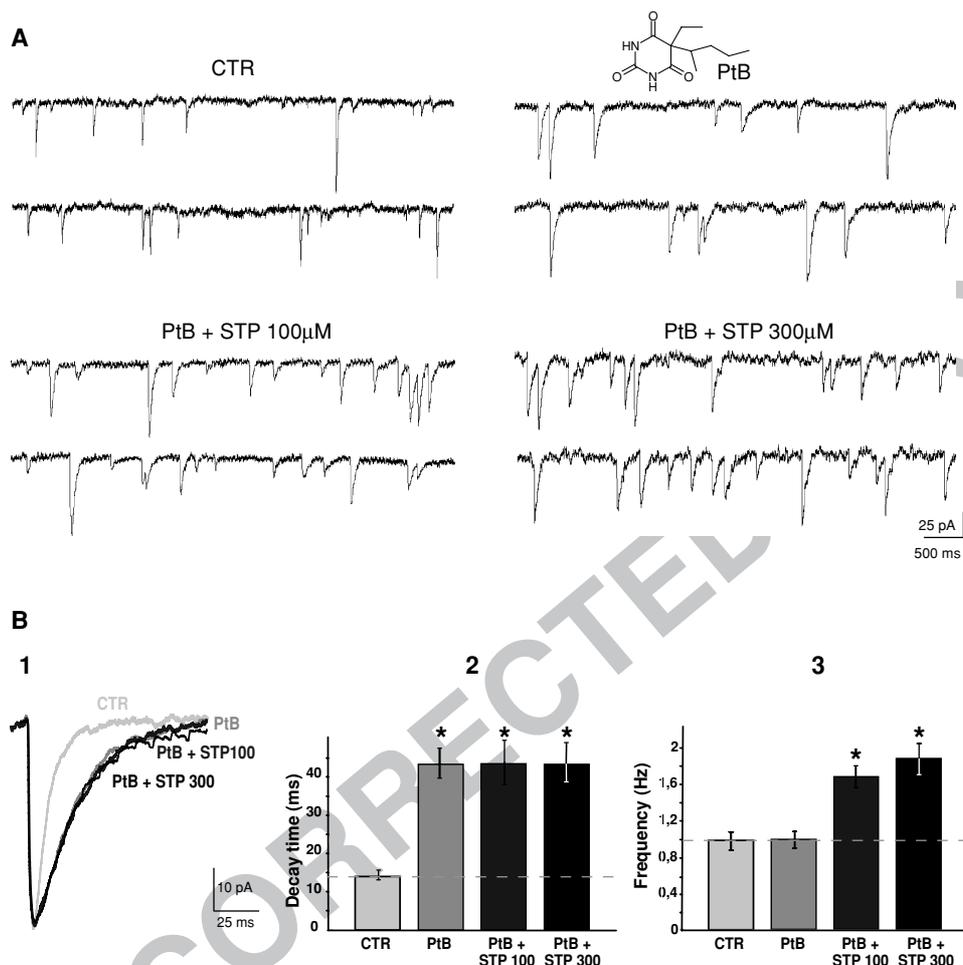


FIG. 5. Stiripentol (STP) interacts with the barbiturate modulatory site. **A:** Consecutive sweeps of miniature GABAergic inhibitory postsynaptic currents (mIPSC) illustrating the activity before (CTR) and during superfusion of pentobarbital (PTB; 100 μ M) alone or in combination with STP at either 100 or 300 μ M. Note the large increase in mIPSC duration. **B: 1:** Superimposed traces represent the average of \sim 200 individual mIPSCs obtained in a representative experiment, in control conditions (CTR) and during superfusion of PTB alone or in combination with STP (100 and 300 μ M). **2:** Mean values of decay time pooled from 10 independent experiments, indicating that the effects of PTB were not further enhanced by STP (100 μ M and 300 μ M). **3:** The histogram shows that the mean values of mIPSC frequency were not affected by PTB but were largely increased by the two concentrations of STP.

$n = 7$) was applied after the washout of STP, and again the same pattern of channel opening and closing was observed (Fig. 6D). From these experiments, we conclude that STP does not directly activate chloride channels in the absence of GABA but increases the mean open duration of GABA-activated chloride channels.

DISCUSSION

The present study provides direct evidence that STP enhances GABA_A receptor-mediated transmission in the immature hippocampus, because this drug increases the frequency and lengthens the decay-time constant of mIPSCs. These properties, observed at clinically relevant concentrations (6,16,17) should confer to STP a potential antiepileptic profile. In this study, we paid attention to the mechanism of STP-induced increase of the decay-

time constant of miniature GABA_A receptor-mediated currents.

Mechanism of STP-induced duration of mIPSCs

One important characteristic of STP to consider first is that this drug is devoid of GABA_A-agonist properties. This is clearly supported by its inability to elicit GABA_A channel openings in outside-out patches and is consistent with the observation that the amplitude of mIPSCs was not affected by STP. This property is in line with previous binding studies indicating that STP does not displace [³H]GABA from its binding sites (12). In this context, the potentiation of mIPSC duration by STP could involve either a presynaptic locus or a postsynaptic modulation of GABA_A-receptor responses.

Blockade of presynaptic GABA transporters (GATs) with SKF-89976A or NO-711 enhances mIPSC duration in adult rats (19,47). In contrast, in neonates, we found

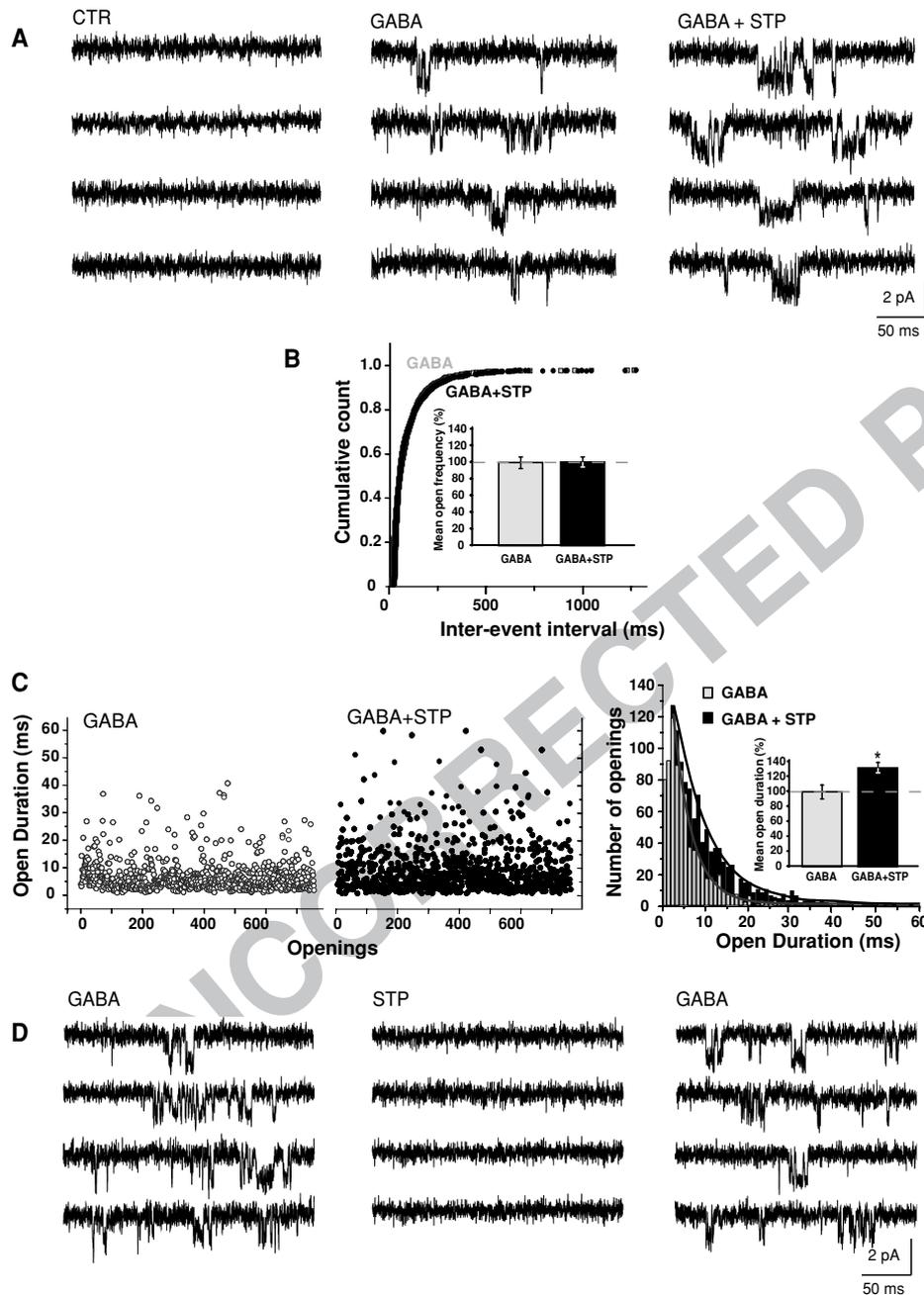


FIG. 6. Effects of stiripentol (STP) on single chloride channels. Outside-out patches were obtained from the soma of CA3 pyramidal cells. STP increases the open duration of chloride channels in the presence of γ -aminobutyric acid (GABA). **A:** Patches were superfused with aCSF (CTR), GABA (2 μ M), or GABA + STP (100 μ M). Recordings were performed at -50 mV, and representative events occurring during 4 min are shown in each condition. Note that currents were observed only in the presence of GABA and that no significant rundown was observed over a recorded period of 4 min. **B:** The distribution of interevent intervals and the histogram of mean open frequency (insert) illustrates that STP did not change the frequency of channel openings. **C:** Open duration of events obtained first with GABA (2 μ M) and then with GABA (2 μ M) + STP (100 μ M). The distribution of open durations was best fitted with a single exponential function, and the time constant was significantly higher with STP (8.0 ± 0.3 ms; $p = 0.001$) than with GABA alone (5.3 ± 0.3 ms). The inserted histogram represents the mean open duration pooled from five independent experiments. **D:** STP does not open GABA channels in the absence of GABA: Patches were first superfused with GABA (2 μ M) for 4 min and washed. STP (300 μ M) was then applied alone to the same patch for an additional period of 4 min and washed, followed by a second application of GABA.

that the irreversible inhibition of these transporters does not modify the mIPSC decay-time constant. Furthermore, blocking GATs does not prevent STP from markedly enhancing mIPSC duration, but it is, however, difficult to accept the possibility of an interaction between STP and GATs, because they do not seem to display any functionality in these conditions. The absence of modulation of mIPSC duration by GAT-1 inhibitors is probably the consequence of the large extracellular space in neonate (48) that prevented the accumulation of GABA in the vicinity of GATs, as suggested previously (24). GATs are, however, functional at this developmental stage, because when

GABA release was dramatically increased by a strong electrical stimulation, NO-711 did enhance e-IPSC duration; however, it is less than STP alone. In these conditions of high GABA release, STP associated to NO-711 further induced a supplementary increase of e-IPSC duration, suggesting that most of the effects of STP on e-IPSC decay time are unlikely to be mediated through a GAT1 mechanism. The contribution of glial GAT-3 to an STP-induced increase in mIPSC duration has not been investigated, but inhibiting this transporter with β -alanine does not increase the duration of GABA events during the first postnatal week (24).

We then examined whether STP increases mIPSC decay-time constant by acting at GABA_A-modulatory sites. The possibility that STP acts on BZD-binding sites was excluded because the effects of STP were not prevented when BZD-binding sites were blocked by flumazenil. In addition, STP acts in synergy with two chemically different types of BZD agonists (CLB and ZPD) further to enhance the maximal modulation of mIPSC duration produced by saturating concentrations of these two drugs. Moreover, in outside-out experiments, STP increased the duration of channel openings and not the frequency, as expected for a BZD-like modulation. This conclusion also is in agreement with *in vitro* studies indicating that STP does not displace [³H]flunitrazepam from BZD-binding sites in cortical tissues (12).

The duration of mIPSCs also is known to be increased by neurosteroids (34,35). Accordingly, we found that pregnanolone, an endogenous neurosteroid, prolongs the duration of mIPSCs in immature CA3 pyramidal cells. However, the effects of STP on mIPSC duration were not occluded by a high concentration of pregnanolone. In addition, DHEAS, a putative antagonist of the positive GABA_A receptor-mediated responses induced by several neurosteroids, was unable to occlude the increase of mIPSC decay time associated with STP. It also is unlikely that STP interacts with a putative neurosteroid modulatory site because neurosteroids affect the frequency of channel opening and generally increase the size of mIPSCs (34), two properties that were not shared with STP. Furthermore, pregnanolone was found to be more efficient at increasing the decay-time constant of mIPSCs in pyramidal than in granular cells (37), whereas STP increases similarly the duration of mIPSCs in the two types of cells.

Our results suggest that the barbiturate site is the best candidate for a postsynaptic target of STP. This GABA_A-receptor modulatory site is functional in neonates because PTB increases mIPSC duration, as previously reported (40,41). STP dose-dependently amplified inframaximal potentiation of mIPSC decay time by PTB. In contrast, when barbiturate sites were saturated, the effect of STP on mIPSC duration was completely prevented, even when the highest concentration of STP was used. A barbiturate antagonist would have been useful to confirm this observation. However, convulsant barbiturates that have been evaluated on GABA transmission were unable to antagonize the positive modulation of barbiturates on the duration of GABA_A receptor-mediated currents (49), suggesting that their convulsant properties are unlikely to be associated with an antagonism at GABA_A barbiturate-binding sites. Nevertheless, the occlusion of STP effects by PTB is not due to experimental artifacts because the presynaptic effect of STP (*i.e.*, the increase in mIPSC frequency) was still present. The possibility that STP could trigger a cascade of intracellular processes leading to an increase in the mIPSC decay-time constant also is unlikely because

in outside-out patches, STP directly modulates GABA_A-channel kinetics in the presence of GABA. This increase more likely stems from a modulation of GABA_A-receptor channels because STP increases their open duration and not their open frequency, in keeping with a barbiturate-like interaction (50). This interaction of STP with barbiturate-binding sites is surprising because the chemical structure of STP (Fig. 1) has no obvious analogies with that of barbiturates (Fig. 5). However, the structural determinants required for modulating the open duration of the GABA_A receptor are still unknown, mainly because the only drugs reported to modulate this parameter are directly derived from PTB (51). STP may therefore represent the prototype of a new chemical family acting on this modulatory site. Variations of its three-dimensional structure may lead to more potent derivatives and therefore may contribute to a better knowledge of the pharmacophore associated with this modulatory site.

STP-induced increase in mIPSC frequency

The increase of mIPSC frequency is probably related to a presynaptic action of STP, because it is generally accepted that the frequency of miniature currents is dependent on the release probability of neurotransmitters (52). The locus of the presynaptic interaction of STP was not investigated in this study. However, an interaction of STP with GABA transporters is unlikely, because GABA-uptake inhibitors did not affect mIPSC frequency. The presynaptic effect of STP may be related to its capacity to inhibit GABA-transaminase (GABA-T, EC 2.6.1.19), the enzyme responsible for the degradation of GABA (15). Indeed, blocking GABA-T activity by chemically different drugs invariably increased the amount of GABA in human and rat brain regions (53–56). Likewise, a single *in vivo* administration of STP increases GABA in the mouse brain (12). Whether STP also inhibits GABA-T in neonates remains to be established, and therefore further experiments are required to elucidate the mechanisms of STP-induced GABA release.

STP as a new antiepileptic drug per se?

STP is presently an efficient drug for add-on therapy in Dravet syndrome (6,10,11), a refractory infantile epilepsy syndrome, but its mechanism of action is not completely resolved. The peripheral action of STP is certainly involved because STP inhibits the degradation of several CYP-sensitive AEDs and increases their plasma concentration. Therefore for a similar therapeutic effect, the concentration of AEDs can be reduced (57). However, methodologic constraints have prevented testing of proper central antiepileptic properties of STP in clinical trials (58). In the present study, we showed that STP enhances GABA transmission and therefore increases the charge transfer associated with GABA events. Drugs that display such properties are usually considered AEDs (59,60). STP increases GABA_A receptor-mediated transmission

at concentrations similar to those found in the plasma of patients, suggesting that part of its clinical efficacy in infantile epilepsy might be related to its central effects associated with a barbiturate-like effect. Supporting this idea, STP administered alone was efficient in blocking different types of seizures induced in vivo in monkeys (14) and in rodents (12,13,61). Therefore the anticonvulsant effects of STP observed in animal models are probably associated with its enhancement of GABA transmission, and we suggest that this central property also contributes to its efficiency when associated with other AEDs. Interestingly, STP modulates GABA_A responses as PTB does, but it does not gate GABA_A receptors in the absence of GABA, whereas PTB directly activates chloride channels by a mechanism independent of GABA_A-receptor modulation (43,44). This difference from PTB suggests that STP should not display the sedative side effects associated with a direct activation of chloride channels by barbiturates (62).

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