Cellular/Molecular

Production of 5α-Reduced Neurosteroids Is Developmentally Regulated and Shapes $GABA_A$ Miniature IPSCs in Lamina II of the Spinal Cord

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In lamina II of the spinal dorsal horn, synaptic inhibition mediated by ionotropic $GABA_A$ and glycine receptors contributes to the integration of peripheral nociceptive messages. Whole-cell patch-clamp recordings were performed from lamina II neurons in spinal cord slices to study the properties of miniature IPSCs (mIPSCs) mediated by activation of $GABA_A$ and glycine receptors in immature (<30 d) and adult rats. Blockade of neurosteroidogenesis by 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide (PK11195), an inhibitor of the peripheral benzodiazepine receptor (PBR), or finasteride, which blocks 5α-reductase, accelerated the decay kinetics of $GABA_A$ receptor-mediated mIPSCs in immature, but not in adult animals. Glycine receptor-mediated mIPSCs remained unaffected under these conditions. These results suggest the presence of a tonic production of 5α-reduced neurosteroids in young rats that confers slow decay kinetics to $GABA_A$ mIPSCs. At all of the ages, selective stimulation of PBR by diazepam in the presence of flumazenil prolonged $GABA_A$ mIPSCs in a PK11195- and finasteride-sensitive manner. This condition also increased the proportion of mixed $GABA_A$/glycine mIPSCs in the immature animals and led to the reappearance of mixed $GABA_A$/glycine mIPSCs in the adult. Our results might point to an original mechanism by which the strength of synaptic inhibition can be adjusted locally in the CNS during development and under physiological and/or pathological conditions by controlling the synthesis of endogenous 5α-reduced neurosteroids.

Key words: **pain; nociception; neurosteroids; inhibition; cotransmission; mIPSCs; $GABA_A$; substantia gelatinosa; dorsal horn; benzodiazepine; neurosteroidogenesis; allopregnanolone**

Introduction

In the dorsal horn of spinal cord, synaptic inhibition mediated by $GABA_A$ receptors ($GABA_A$Rs) and glycine receptors (GlyRs) is of fundamental importance to prevent the generation of hyperalgesia or allodynia (Yaksh, 1989; Sherman and Loomis, 1996; Sorkin and Puig, 1996). In lamina II, local excitatory and inhibitory interneurons receive and integrate cutaneous nociceptive messages conveyed by nonmyelinated (type C) primary afferent fibers before transmitting them to supraspinal centers (Millan, 1999). Recently, it has been shown that, although GABA and glycine are coreleased at a subset of lamina I–II inhibitory synapses, postsynaptic codetection by $GABA_A$Rs and GlyRs disappeared around postnatal day 23 (P23), leading to an overall reduction of synaptic inhibition (Chéry and De Koninck, 1999; Keller et al., 2001). This phenomenon could, however, be reversed by increasing the affinity of $GABA_A$Rs with the benzodiazepine diazepam (DZP). Apart from a direct interaction with $GABA_A$Rs [at a binding site referred to as central benzodiazepine receptor (CBR)], most benzodiazepines/endozepines stimulate neurosteroidogenesis by activating peripheral benzodiazepine receptors (PBRs) located on glial/neuronal mitochondrial membranes (Compagnone and Mellon, 2000). Although it is well established that neurosteroids and benzodiazepines can modulate $GABA_A$ receptor function (Harrison et al., 1987; Lambert et al., 1995; Cooper et al., 1999; Mehta and Ticku, 1999; Vicini et al., 2002; Koksma et al., 2003), a role for endogenously produced neurosteroids or endozepines in the acute functional modulation of synaptic transmission has not been clearly reported so far. We addressed this issue by evaluating the consequences of selective activation or blockade of the CBR and PBR on inhibitory synaptic transmission within lamina II of the spinal cord slices from immature and adult rats. Pharmacological manipulation of neurosteroidogenesis allowed us to demonstrate that 5α-reduced neurosteroids are continuously produced during immature stages, but not in adulthood. As a consequence, synaptic inhibition is sustained in the spinal cord of young rats and is mediated by prolonged $GABA_A$R currents as well as by mixed GABA/glycine synaptic currents. In contrast, in the adult, the decay kinetics

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of GABAergic miniature IPSCs (mIPSCs) are much faster, and mixed GABA/glycine mIPSCs are no longer detected. At all of the ages examined, stimulation of neurosteroidogenesis remained possible and led to a significant increase in overall inhibition by prolonging $GABA_A$ receptor-mediated mIPSCs and by unmasking a GABA/glycine cotransmission. Our results suggest an important role of endogenously produced neurosteroids in the modulation of processing of nociceptive information at the spinal cord level via the control of the strength of inhibitory transmission among dorsal horn interneurons.

Materials and Methods

Slice preparation and solutions. All of the procedures were performed in accordance with the guidelines of the European Community on animal experimentation. Transverse slices were prepared from very young (P6–P8), immature (P13–P23), and adult (>P30) Wistar rats as described previously (Keller et al., 2001). The spinal cord was removed and immediately immersed in cold (4°C) sucrose-based artificial CSF (ACSF) containing (in mM): 248 sucrose, 11 glucose, 26 $NaHCO_3$, 2 KCl, 1.25 KH_2PO_4, 2 $CaCl_2$, 2 $MgSO_4$, 2 kynurenic acid (Fluka, Buchs, Switzerland) continuously bubbled with 95% O_2–5% CO_2, pH 7.35 ± 0.05. Transverse slices (250–300 μm thick) were cut from the lumbar segment with a Vibratome (Pelco International, Redding, CA) and stored at room temperature in regular ACSF containing 125 mM of NaCl instead of sucrose and no kynurenic acid. After at least 1 hr, slices were transferred to the recording chamber and continuously perfused with oxygenated ACSF containing 0.5 μM TTX (Latoxan, Rosans, France) and 2 mM kynurenic acid to record mIPSCs. All of the recordings were made at 20–22°C.

Electrophysiological recordings, data acquisition, and analysis. Lamina II neurons were identified under an upright microscope (Axioscope; Zeiss, Oberkochen, Germany) equipped with digital interference contrast optics and an infrared camera (Hamamatsu, Hamamatsu City, Japan). Whole-cell voltage-clamp recordings were obtained with an Axopatch 200B amplifier (Axon Instruments, Union City, CA) and borosilicate glass electrodes with an inner filament (1.2 outer diameter to 0.69 inner diameter; Clark Electromedical Instruments, Pangbourne, UK) filled with a intracellular solution containing (in mM): 130 CsCl, 2 $MgCl_2$, and 10 HEPES, pH 7.3, CsOH.

Membrane currents were filtered (DC; 5 kHz; Bessel filter of the amplifier) and stored on videotape after digitization with a pulse-code modulator (40 kHz; Sony, Tokyo, Japan). For off-line analysis, current traces were filtered at 2 kHz (eight-pole Bessel filter; 9002; Frequency Devices) and digitized at 4 kHz on an Intel (Santa Clara, CA) Pentium-based personal computer. Data were acquired using the Strathclyde electrophysiology software CDR [courtesy of Dr. J. Dempster (University of Strathclyde, Glasgow, UK)] and analyzed with a custom software developed and kindly provided by Dr. Yves De Koninck (University of Laval, Québec, Québec, Canada). Mean values of the amplitudes, 10–90% rise times, decay time constants, and frequency of occurrence of mIPSCs were compared between groups using Student's *t* tests with a confidence interval of 0.95. Decay time constants of mIPSCs were fitted using a nonlinear least square methods, and goodness of fit was evaluated on the basis of fitting subsets of points drawn from the entire set of data points, from evaluation of the reduced χ^2 values, and the change in the *F* values calculated from the sum of squared differences from the fitted line. The Kolmogorov–Smirnov test was used to compare the cumulative distributions. Distributions were considered different when $p < 0.01$. All of the numerical results are expressed as mean ± SEM.

Drug application. Selective blockade of glycine or $GABA_A$ receptors was achieved by adding strychnine hydrochloride (1 μM; Sigma, St. Louis, MO) or bicuculline methiodide (10 μM; Sigma), respectively. The effects of diazepam (Sigma) and allopregnanolone (AP) (5α-pregnan-3α-ol-20-one; Sigma) were determined after steady-state applications lasting at least 30 min. For experiments requiring >1 hr of drug exposure, slices were preincubated in ACSF containing the substance to be tested [e.g., 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide (PK11195) (Tocris; Bioblock, Illkirch, France), flumazenil (FLU) (gift of Roche, Basel, Switzerland), diazepam (Sigma), or finasteride (FIN) (Sigma)]. In such experiments, the controls consisted of sister slices incubated under the same conditions but in the absence of the pharmacological agents. The $GABA_A$ receptor agonist isoguvacine (Peninsula Laboratories, San Carlos, CA) was applied locally by pressure ejection (duration, 100 msec) through a borosilicate glass pipette.

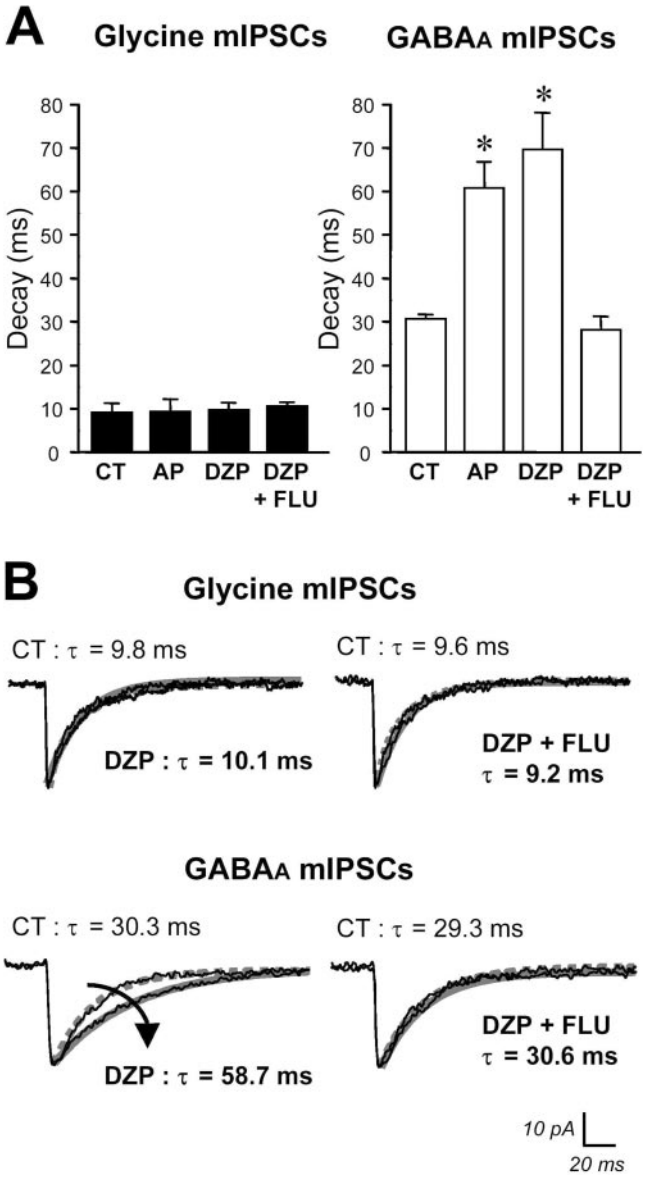

Figure 1. Allopregnanolone and diazepam prolong $GABA_AR$ mIPSCs without affecting GlyR mIPSCs. Pharmacologically isolated GlyR and $GABA_AR$ mIPSCs were recorded at a holding potential of −60 mV from immature lamina II neurons (<P23) in the presence of 10 μM bicuculline or 1 μM strychnine, respectively. *A*, Histograms illustrate the mean value ± SEM of the decay time constant for GlyR (left histogram; black bars) and $GABA_A$ mIPSCs (right histogram; white bars) fitted with a monoexponential function under control conditions (CT) ($n = 10$) and after application of AP (100 nM; $n = 5$), DZP (1 μM; $n = 7$), or DZP in the presence of the CBR antagonist flumazenil (DZP+FLU) (10 μM). Asterisks indicate statistically significant difference (*t* test; $p < 0.05$) with respect to control. *B*, Traces representing averages of 10 isolated glycine (top traces) and $GABA_A$ mIPSCs (bottom traces) recorded under control conditions and after application of DZP (1 μM; left traces) or DZP plus 10 μM FLU (DZP+FLU) (right traces). The results of the monoexponential fits are shown as gray lines superimposed on the original traces (black lines). τ indicates the value of the mean decay time constant of mIPSCs for each experimental condition.

Table 1. Characteristics of $GABA_A$R mIPSCs recorded in immature (<P23) lamina II neurons after various pharmacological treatments

	Control	FLU (10 μM)	PK (10 μM)	FIN (50 μM)	DZP (1 μM)	AP (100 nM)	DZP + FLU <1 hr	DZP + FLU >1 hr	DZP + FLU + PK
RT (msec)	0.71 ± 0.04	0.74 ± 0.08	0.53 ± 0.11	0.82 ± 0.14	0.87 ± 0.09	0.72 ± 0.20	0.71 ± 0.12	0.87 ± 0.07	0.63 ± 0.05
Amp. (pA)	−27.0 ± 1.7	−30.1 ± 2.8	−27.8 ± 6.2	−29.2 ± 2.4	−30.4 ± 3.2	−26.5 ± 4.1	−32.38 ± 6.4	−28.5 ± 4.5	−27.8 ± 1.6
τ (msec)	30.8 ± 0.9	30.8 ± 1.5	**22.8 ± 1.8**	**23.1 ± 2.4**	**69.7 ± 8.4**	**60.9 ± 5.9**	25.6 ± 2.5	**41.3 ± 1.7**	29.5 ± 1.5
n	48	11	7	7	6	5	5	4	10

The table gives the mean values of the main parameters of $GABA_A$R mIPSCs: 10–90% rise time (RT), peak amplitude (Amp.), and monoexponential decay time constant (τ). Values in bold are significantly different from the control (Student's *t* test; $p < 0.05$). PK, PK11195.

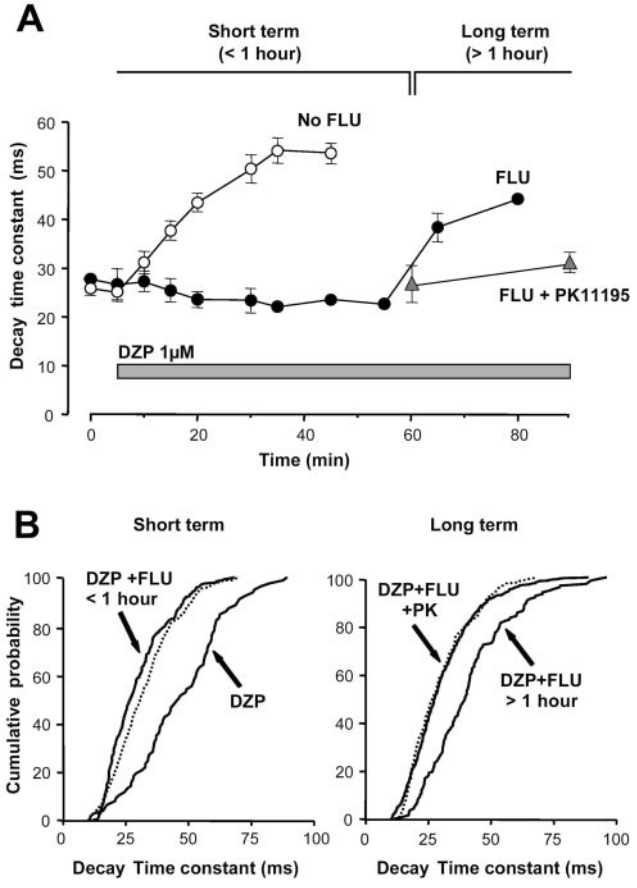

Figure 2. Flumazenil antagonizes the short-term effect of diazepam, whereas PK11195 antagonizes the delayed prolongation of $GABA_A$R mIPSCs. *A*, Pharmacologically isolated $GABA_A$R mIPSCs (strychnine; 1 μM) were recorded at a holding potential of −60 mV. Effect of DZP (1 μM) on mean decay time constant as function of time of application (in minutes) in immature (<P23) lamina II neurons under different conditions: absence of flumazenil (No FLU) (open circles), preincubation of slices with FLU (10 μM; filled circles), or with both flumazenil and the PBR antagonist PK11195 (FLU+PK11195) (10 μM; filled triangles). Each point represents the mean ± SEM value of the decay time constant of mIPSCs sampled over periods of 5 min during superfusion of DZP (horizontal gray bar), except in the case of FLU+PK11195, for which we sampled the values of mIPSC decay time constants after steady-state incubation for 60–90 min. *B*, Cumulative distribution of $GABA_A$R mIPSCs decay time constants pooled from five immature neurons (<P23) and recorded after different pharmacological treatments. The dotted line illustrates the distribution under control conditions. Left panel, Short-term application (<1 hr) of DZP shifted the distribution to the right (Kolmogorov–Smirnov test; $p < 0.01$), an effect blocked by flumazenil. Right panel, Long-term application of DZP and FLU (>1 hr) increased the duration of mIPSCs (rightward shift of the distribution; Kolmogorov–Smirnov test; $p < 0.01$). This effect was blocked by PK11195 (10 μM).

Results

Diazepam and allopregnanolone are potent positive modulators of synaptic $GABA_A$Rs expressed by immature (<P23) lamina II neurons

In immature lamina II neurons, inhibitory synaptic transmission is complex and involves a significant proportion of GABA/glycine cotransmission (Chéry and De Koninck, 1999; Keller et al., 2001). Therefore, we first recorded pharmacologically isolated glycine receptor-mediated miniature IPSCs (GlyR mIPSCs) or $GABA_A$ receptor-mediated miniature IPSCs ($GABA_A$R mIPSCs), and characterized their modulation by the benzodiazepine DZP (1 μM) and the neurosteroid AP (100 nM).

$GABA_A$R and GlyR mIPSCs occurred at a low frequency [$GABA_A$R mIPSCs, 1.34 ± 0.12 Hz ($n = 9$); GlyR mIPSCs, 1.35 ± 0.08 Hz ($n = 9$)] and exhibited amplitudes ranging from 8 to 100 pA [mean peak amplitude, $GABA_A$R mIPSCs, 33.2 ± 2.4 pA ($n = 9$); GlyR mIPSCs, 33.3 ± 6.2 pA ($n = 9$)]. The decaying phase of pharmacologically isolated $GABA_A$R and GlyR mIPSCs could be fitted by a monoexponential function. Decay time constants were of 30.8 ± 0.9 and 11.7 ± 1.5 msec for $GABA_A$R ($n = 12$) and GlyR ($n = 5$) mIPSCs, respectively.

Figure 1 summarizes the effects of DZP and AP on the decay kinetics of GlyR and $GABA_A$R mIPSCs. Exogenous application of DZP (1 μM) or of AP (100 nM) increased the decay time constant of $GABA_A$R mIPSCs [DZP, 69.7 ± 8.4 msec ($n = 6$); AP, 60.9 ± 5.9 msec ($n = 5$); *t* test; $p < 0.05$ in both conditions) without affecting their mean amplitude or frequency of occurrence. Superfusion of FLU (10 μM), a potent antagonist of the benzodiazepine site on $GABA_A$Rs, fully antagonized the rapid modulatory effect of diazepam on $GABA_A$R mIPSC mean decay kinetics (Fig. 1*A*). Application of FLU alone had no significant effect on $GABA_A$R mIPSCs (*t* test; $p > 0.05$; $n = 11$) (Table 1). GlyR mIPSC kinetics was not affected by DZP or AP (*t* test; $p < 0.05$) (Fig. 1).

Diazepam stimulates neurosteroidogenesis and increases synaptic $GABA_A$R function

As shown above for immature lamina II neurons (Fig. 1, Table 1), DZP (1 μM) rapidly increased the mean decay time constant of $GABA_A$R mIPSCs, and this effect was fully blocked when DZP was applied together with 10 μM FLU ($n = 5$). However, when the application of DZP in the presence of FLU exceeded 1 hr, we observed a progressive and delayed increase in $GABA_A$R mIPSC decay time constants, reaching an average of 41.3 ± 1.7 msec ($n = 4$) after 80 min (Fig. 2, Table 1). This delayed effect of DZP on $GABA_A$ mIPSCs was blocked by the PBR antagonist PK11195 (DZP+FLU+PK, $\tau = 29.5 \pm 1.5$; $n = 10$) and by the 5α-reductase inhibitor finasteride (DZP+FLU+FIN, $\tau = 31.7 \pm 1.6$; $n = 5$). In some cases, we noted that the decay time constant of $GABA_A$ mIPSCs had a tendency to slightly decrease during the initial phase (<1 hr) of superfusion with DZP+FLU (Figs. 1*A*, 2*A*). However, it must be emphasized that this apparent reduction never reached statistical significance (*t* test; $p > 0.05$).

A tonic production of neurosteroids shapes synaptic $GABA_A$R mIPSCs in immature, but not in adult, spinal cord slices

In these experiments, pharmacologically isolated $GABA_A$R mIPSCs were recorded from immature lamina II neurons (<P23) in the presence of strychnine (1 μM).

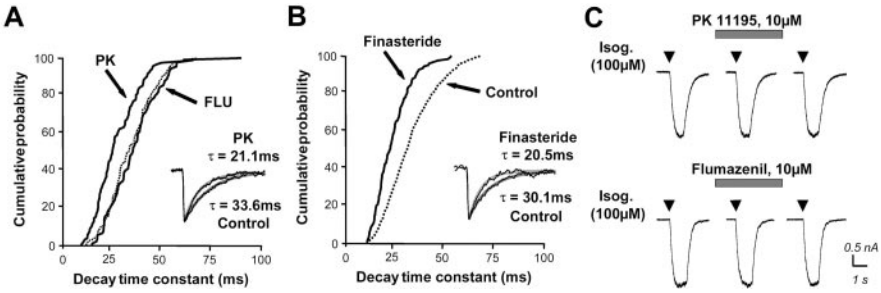

Figure 3. PK11195 and finasteride accelerate $GABA_A$R mIPSCs decay kinetics in neurons from immature animals (<P23). Pharmacologically isolated $GABA_A$R mIPSCs were recorded in the presence of strychnine (1 μM) at a holding potential of −60 mV. The graphs represent the cumulative distributions of $GABA_A$R mIPSCs decay time constants pooled from two immature neurons recorded under control conditions (dotted line), in the presence of the CBR antagonist FLU (10 μM) or the PBR antagonist PK11195 (PK) (10 μM) (*A*), or after long-term incubation (>6 hr) with finasteride (50 μM), an inhibitor of 5α-reductase (*B*). The reduction in mIPSC decay time constant values observed in the presence of finasteride or PK11195 was statistically significant (Kolmogorov–Smirnov test; $p < 0.01$). The insets represent averages of 10 traces of individual mIPSCs recorded under each condition. The deactivation phase of $GABA_A$R mIPSCs was fitted with a monoexponential function shown as the superimposed gray lines. τ represents the value of the decay time constant determined by the fitting procedure. *C*, Whole-cell $GABA_A$ receptor-mediated currents evoked by exogenous 100-msec-lasting pressure applications of 100 μM isoguvacine in the vicinity of the recorded cell during steady-state perfusion of either PK11195 (10 μM) or flumazenil (10 μM).

As shown in Figure 3*A* and Table 1, the properties of $GABA_A$R mIPSCs were unaltered by FLU (10 μM), an antagonist of the CBR. In contrast, incubation of the slices with PK11195 (10 μM), an antagonist of the PBR, significantly reduced the mean decay time constant [control, $\tau = 30.6 \pm 0.9$ msec ($n = 9$); PK11195, $\tau = 22.8 \pm 1.8$ msec ($n = 9$); *t* test; $p < 0.05$], whereas all of the other parameters remained unchanged (Fig. 3*A*, Table 1). Blockade of 5α-reductase, an enzyme involved in the synthesis of 5α-reduced neurosteroids, by FIN (50 μM), significantly reduced the mean decay time constant of $GABA_A$R mIPSCs to 23.1 ± 2.4 msec ($n = 7$), a value comparable with that obtained during a prolonged (>1 hr) incubation with PK11195 (Fig. 3*B*, Table 1) (*t* test; $p < 0.05$). It should be emphasized that whole-cell $GABA_A$ currents, evoked by exogenous application of 100 μM isoguvacine, were unaffected by FLU, PK11195, or FIN (Fig. 3*C*). The lack of significant change in the peak current during bath perfusion of FLU (0.36 ± 1.00%; $n = 5$), PK11195 (0.09 ± 2.57%; $n = 6$), or FIN (0.66 ± 0.88%; $n = 3$) indicated that these substances did not directly modulate the activity of postsynaptic $GABA_A$ receptors. Together, these results suggested that $GABA_A$R mIPSC kinetics in spinal cord slices from immature rats were tonically and positively modulated by endogenous 5α-reduced neurosteroids generated via the tonic activation of the PBR.

The situation was slightly different in lamina II neurons of adult spinal cord preparations. The decay time constants of $GABA_A$R mIPSCs were significantly shorter ($\tau = 18.8 \pm 1.1$ msec; $n = 11$; *t* test; $p < 0.05$) than in young rats ($\tau = 30.8 \pm 0.9$ msec; $n = 48$), whereas all of the other kinetic characteristics were similar to that of mIPSCs recorded in neurons from <P23 animals (Tables 1 and 2). In the adult, $GABA_A$R mIPSCs could still be positively modulated by exogenous application of 1 μM DZP ($\tau = 28.9 \pm 3.0$ msec; $n = 6$; *t* test; $p < 0.05$) or 100 nM AP (AP, $\tau = 35.2 \pm 1.1$ msec; $n = 4$; *t* test; $p < 0.05$), or long incubation (>3 hr) with DZP plus FLU ($\tau = 34.1 \pm 3.6$ msec; $n = 6$; *t* test; $p < 0.05$) (Table 2). In the latter experimental conditions aimed at stimulating neurosteroidogenesis (i.e., DZP+FLU) in adult slices, it should be noted that prolongation of $GABA_A$ mIPSC decays was sensitive to the 5α-reductase inhibitor finasteride (DZP+FLU+FIN, $\tau = 18.5 \pm 2.5$; $n = 6$). The mean decay time constant of the $GABA_A$R mIPSCs was, however, not affected by the steady-state perfusion of PK11195 ($\tau = 22.8 \pm 1.8$ msec; $n = 7$) or FIN ($\tau = 20.5 \pm 0.7$ msec; $n = 3$) alone. Together, these results indicated that, in the adult, synaptic $GABA_A$ receptors were apparently not tonically modulated by endogenously produced neurosteroids under resting conditions, but that such a modulation remained possible after stimulation of PBR.

Neurosteroidogenesis favors GABA/glycine cotransmission in immature and adult rats

As described previously, lamina II neurons of young rats (<P23) recorded in the absence of ionotropic inhibitory amino acid receptor antagonist displayed a significant proportion of mixed $GABA_A$/GlyR mIPSCs characterized by a fast glycine-receptor-mediated and a slow $GABA_A$ receptor-mediated kinetic component (Keller et al., 2001).

In line with this finding, the decay phases of the mixed mIPSCs recorded in the present experiments were fitted by a biexponential function ($n = 51$) with a fast glycine receptor-mediated component ($\tau_1 = 9.2 \pm 0.3$ msec) and a slow $GABA_A$ receptor-mediated component ($\tau_2 = 34.3 \pm 1.2$ msec) (Fig. 4*A*). Exogenous application of DZP (1 μM) or of the neurosteroid AP (100 nM) selectively increased the slow component of mixed $GABA_A$/GlyR mIPSCs [DZP, $\tau_2 = 64.7 \pm 6.3$ msec ($n = 7$); AP, $\tau_2 = 70.6 \pm 7.1$ msec ($n = 5$)]. This effect was similar to that observed in the case of pharmacologically isolated $GABA_A$R mIPSCs described in the previous section (Figs. 1, 4*A*). The fast decaying phase of mixed mIPSCs (i.e., corresponding to the GlyR current component) was unaffected by DZP or AP (Fig. 4*A*). Application of AP did not change the overall frequency of occurrence of mIPSCs (regardless of their nature) during AP application (0.247 ± 0.032 vs 0.251 ± 0.033 Hz; $n = 6$). However, the relative contribution of different mIPSC subtypes was modified by AP. The proportion of $GABA_A$R mIPSCs remained stable (0.131 ± 0.027 vs 0.129 ± 0.027 Hz; $n = 6$) whereas that of mixed mIPSCs

Table 2. Characteristics of $GABA_A$R mIPSCs recorded in adult (>P30) lamina II neurons after various pharmacological treatments

	Control	PK (10 μM)	Finasteride (50 μM)	DZP (1 μM)	AP (100 nM)	DZP + FLU >3 hr
RT (msec)	0.54 ± 0.06	0.52 ± 0.10	0.70 ± 0.9	0.59 ± 0.02	0.67 ± 0.05	0.65 ± 0.14
Amp. (pA)	−28.5 ± 2.6	−27.2 ± 4.0	−26.2 ± 9.6	−31.7 ± 4.4	−28.6 ± 2.7	−27.4 ± 2.9
τ (msec)	18.8 ± 1.1	21.4 ± 1.5	20.5 ± 0.7	**28.9 ± 3.0**	**35.2 ± 1.1**	**34.1 ± 3.6**
n	11	7	3	6	4	6

The table gives the mean values of the main parameters of $GABA_A$R mIPSCs: 10–90% rise time (RT), peak amplitude (Amp.), and monoexponential decay time constant (τ). Values in bold are significantly different from the control (Student's *t* test; $p < 0.05$). PK, PK11195.

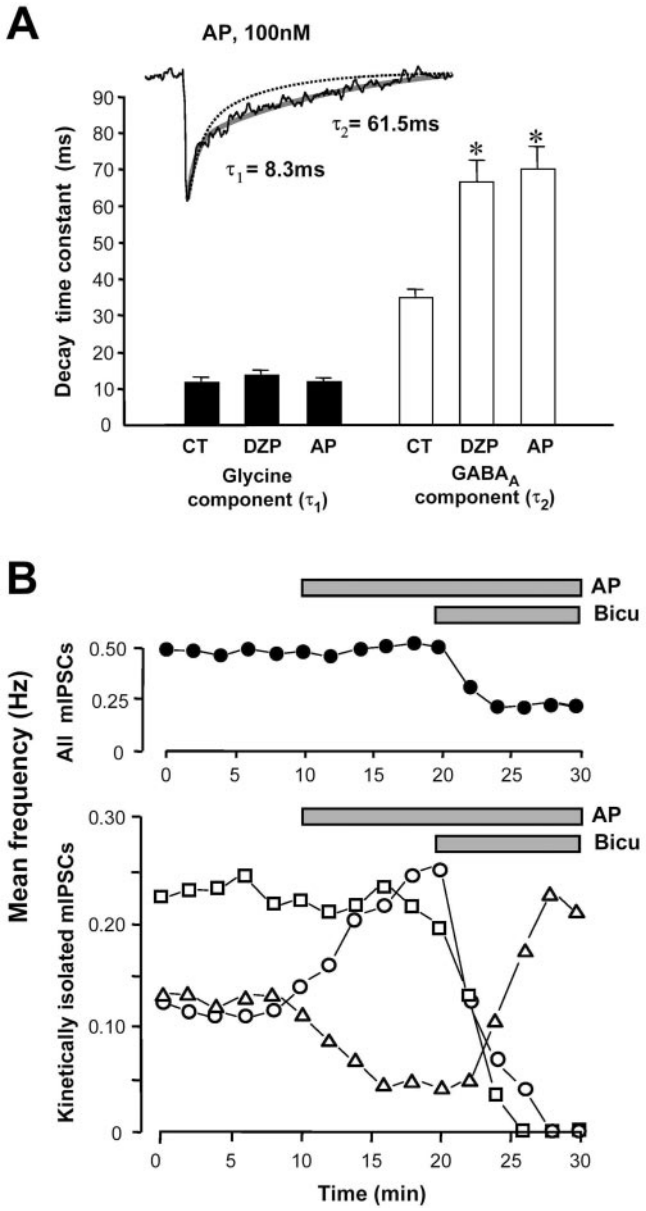

Figure 4. Diazepam and allopregnanolone increase $GABA_A$ mIPSCs and convert some GlyR mIPSCs into mixed $GABA_A$/GlyR mIPSCs. Synaptic mIPSCs were recorded at holding potential of −60 mV in the absence of any ionotropic inhibitory amino acid receptor antagonists in immature neurons (<P23). *A*, The histograms represent the mean (±SEM) decay time constant values of the fast glycine receptor-mediated component (τ_1) (black columns) and the slow $GABA_A$ receptor-mediated component (τ_2) (white columns) of mixed $GABA_A$/GlyR mIPSCs. The trace in inset is a typical example of a mixed $GABA_A$/GlyR mIPSC best fitted with a biexponential function. Compared with the fit obtained in control conditions (dotted black line), superfusion with 100 nM AP prolonged the slowest decay time constant (i.e., the $GABA_A$ component). Asterisks indicate statistically significant differences (*t* test; $p < 0.05$) with respect to control. *B*, The top panel shows the evolution of the frequency of total population of mIPSCs (filled circles). The bottom panel represents the evolution of the relative frequency of occurrence of each mIPSC category (open triangles, GlyR mIPSCs; open squares, $GABA_A$R mIPSCs; open circles, mixed $GABA_A$/GlyR mIPSCs) among the total population as a function of time of AP perfusion in a representative immature neuron (<P23). Each point corresponds to a 2 min sampling period. Horizontal gray bars denote application of AP and bicuculline. The total frequency of mIPSCs remained stable during AP application but decreased in the presence of bicuculline (Bicu) (10 μM). $GABA_A$R mIPSCs frequency was stable during AP application, whereas the proportion of GlyR mIPSCs decreased in parallel with the increase of mixed $GABA_A$/GlyR mIPSCs. In presence of bicuculline, only GlyR mIPSCs were detected, and their frequency corresponded to the sum of the frequencies of GlyR and mixed mIPSCs recorded before the blockade of $GABA_A$R receptors.

increased from 0.061 ± 0.005 to 0.090 ± 0.01 Hz ($n = 6$), and that of GlyR mIPSCs decreased in a symmetrical manner (from 0.055 ± 0.017 to 0.033 ± 0.017 Hz; $n = 6$). As a consequence, the contribution of mixed $GABA_A$/GlyR mIPSCs to the overall mIPSCs population increased from 26.8 ± 3.1 to 40.2 ± 4.7% ($n = 6$). Application of bicuculline (10 μM) reduced the frequency of synaptic events. This phenomenon could be completely accounted for by the suppression of $GABA_A$R mIPSCs and of the slow component of mixed $GABA_A$/GlyR mIPSCs. As a consequence, mixed mIPSCs appeared to be converted into GlyR mIPSCs. Indeed, the frequency of GlyR mIPSCs remaining in the presence of bicuculline corresponded to the sum of that of mixed mIPSCs and GlyR mIPSCs recorded in the absence of bicuculline.

The effect of AP on the proportion of mixed mIPSCs was mimicked by DZP. After 15–30 min of exogenous application of DZP or AP (Fig. 5*A*), the fractional contribution of mixed $GABA_A$/GlyR mIPSCs (with respect to control) to the overall population of mIPSCs was strongly increased [DZP, from 22.1 ± 1.4 to 30.5 ± 2.5% ($n = 7$); *t* test; $p < 0.05$; AP, from 22.1 ± 1.4 to 35.0 ± 6.2% ($n = 6$); *t* test; $p < 0.05$]. Perfusion of flumazenil alone did not significantly affect the proportion of mixed events (22.0 ± 3.9%; $n = 8$; *t* test; $p > 0.05$). Neither did acute and short-time (<1 hr) applications of DZP in the presence of flumazenil (22.9 ± 3.3%; $n = 16$; *t* test; $p > 0.05$) or of DZP in the presence of flumazenil plus PK11195 (19.8 ± 2.7%; $n = 3$; *t* test; $p > 0.05$). However, stimulation of neurosteroidogenesis by long treatment (>1 hr) with DZP in the presence of flumazenil (Fig. 5*A*) increased the fractional contribution of mixed mIPSCs to 27.5 ± 2.7% ($n = 4$; *t* test; $p < 0.05$). Accordingly, pharmacological treatments designed to inhibit the production of 5α-reduced neurosteroids dramatically decreased the proportion of mixed mIPSCs recorded [FLU+PK, 13.2 ± 1.27% ($n = 6$); *t* test; $p < 0.05$; PK11195, 9.9 ± 2.6% ($n = 4$); *t* test; $p < 0.05$; finasteride, 8.9 ± 1.0% ($n = 3$); *t* test; $p < 0.05$]. These results indicate that endogenous neurosteroids potentiate $GABA_A$ receptor function and thereby favor the occurrence of mixed $GABA_A$/GlyR mIPSCs.

In adult lamina II neurons, mixed $GABA_A$/GlyR mIPSCs were not detected (Fig. 5*B*), although these neurons ($n = 14$) displayed $GABA_A$R and GlyR mIPSCs in an equivalent proportion. Application of DZP or AP led to the reappearance of mixed $GABA_A$/GlyR mIPSCs, which represented respectively 25.6 ± 6.0% ($n = 3$) and 16.3 ± 7.3% ($n = 7$) of all of the mIPSCs (Fig. 5*B*). Similarly, after long incubation with DZP plus FLU (>3 hr), the duration of $GABA_A$R-mediated current components was increased, and mixed mIPSCs represented 16.0 ± 5.3% ($n = 6$) of the total population of mIPSCs (Fig. 5*B*). The mean value of their slow $GABA_A$ receptor-mediated component was 35.0 ± 1.1 msec ($n = 6$), which was close to that of isolated $GABA_A$R mIPSCs recorded under the same experimental conditions. In slices treated with finasteride for at least 6 hr, mixed $GABA_A$/GlyR mIPSCs were never detected.

Developmental regulation of GABA/glycine cotransmission by neurosteroidogenesis

Figure 6 summarizes the role of neurosteroidogenesis in the shaping of mIPSCs during postnatal maturation of inhibitory synaptic transmission in lamina II.

The proportion of $GABA_A$R mIPSCs increased with age to reach 50% of overall mIPSCs in the adult. This was accompanied by a marked decrease in the mean time constant of their decaying phase. Time constant values similar to that observed in P > 30 could be reached in slices from P6 and P15 animals after incubation with finasteride for 3 hr (Fig. 6*A1*, Table 2). Contribution of GlyR mIPSCs to the total number of mIPSCs increased from

~30% at P6 to 50% in the adult, but the decay time constant of GlyR mIPSCs remained stable over the period examined. GlyR mIPSCs kinetics was unaffected by finasteride treatment at any age examined, although the relative proportion of GlyR mIPSCs increased in P6 and P15 animals to reach values close to that observed in the adult (Fig. 6*A2*). At P6, mixed $GABA_A$/GlyR mIPSCs represented 40% of the events recorded. This proportion decreased to 30% at P15, and no mixed currents were detected in the adult. At immature stages (P6 and P15), the decay time constant of the fast component of mixed mIPSCs remained stable as noted for GlyR mIPSCs, whereas that of the slow component decreased as observed for $GABA_A$ mIPSCs (Fig. 6*B*). At P6 and P15, finasteride treatment strongly reduced the proportion of mixed mIPSCs to ~10% as well as the mean decay time constant of the slow component of mixed mIPSCs. This effect was similar to that observed for $GABA_A$R mIPSCs.

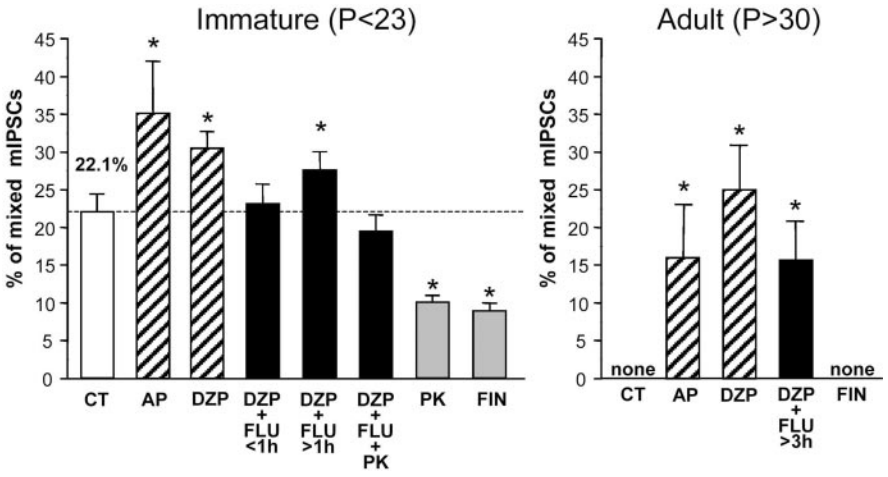

Figure 5. Pharmacological treatments aimed at increasing or inhibiting neurosteroidogenesis affect mixed $GABA_A$/glycine mIPSC synaptic transmission. Neurons were recorded in the absence of any inhibitory amino acid receptor antagonists at a holding potential of −60 mV. Histograms give the percentage of mixed $GABA_A$/GlyR mIPSCs recorded among the overall population of mIPSCs in immature (<P23) (left histogram) and adult neurons (>P30) (right histogram). AP (100 nM) and DZP (1 μM) were applied by superfusion. Other treatments consisted of short-time (<1 hr) and/or prolonged (>1 hr) incubation of slices with FLU (10 μM), FIN (50 μM), PK11195 (PK) (10 μM), diazepam plus flumazenil (DZP + FLU), and diazepam plus flumazenil plus PK11195 (DZP + FLU + PK). A significant increase in the proportion of mixed $GABA_A$/glycine mIPSCs was noted in the presence of AP, DZP, and DZP + FLU (>1 hr) in immature slices. Conversely, a large reduction was noted with PK11195 or finasteride, two inhibitors of neurosteroidogenesis. After 30 d of postnatal life (>P30), no mixed events were detected. However, such mixed events reappeared in the presence of AP, DZP, or DZP + FLU (>3 hr). Incubation with finasteride (>6 hr) never revealed mixed mIPSCs. Asterisks represent statistically significant differences (*t* test; $p < 0.05$).

Discussion

The present study indicates a role for endogenously produced 5α-reduced neurosteroids in the functional modulation of inhibitory synapses. Moreover, our results on inhibitory synaptic transmission in lamina II of the spinal cord suggest that the acceleration of $GABA_A$R mIPSCs and the disappearance of mixed $GABA_A$/GlyR mIPSCs (Keller et al., 2001) observed between immature and adult developmental stages were primarily associated with a reduction in the endogenous and tonic production of 5α-reduced neurosteroids. Although a tonic neurosteroidogenesis was not detected in adult animals, activation of neurosteroid synthesis by stimulation of PBR remained possible and led to an increase in synaptic $GABA_A$R-mediated transmission and to the reappearance of mixed $GABA_A$/GlyR mIPSCs, a phenomenon that might have important physiological consequences.

A developmentally regulated acceleration of $GABA_A$R synaptic currents has been described in various structures of the CNS (Brickley et al., 1996; Draguhn and Heinemann, 1996; Tia et al., 1996; Brussaard et al., 1997; Hollrigel and Soltesz, 1997; Pouzat and Hestrin, 1997; Dunning et al., 1999; Okada et al., 2000) including the spinal cord (Keller et al., 2001). In supraspinal regions of the CNS, there is clear evidence that changes in the decay kinetics of GABAergic mIPSCs, as well as changes in the biophysical and/or pharmacological properties of postsynaptic $GABA_A$Rs (Cherubini and Conti, 2001) are correlated with newly expressed α1-containing (Okada et al., 2000; Vicini et al., 2001) or α6-containing (Tia et al., 1996) $GABA_A$Rs in adult synapses. This is supported by studies of recombinant $GABA_A$Rs (Verdoorn, 1994; Gingrich et al., 1995; Lavoie et al., 1997; McClellan and Twyman, 1999) and has been recently confirmed in the cerebellum of α1-deficient mice (Vicini et al., 2001). However, similar developmental changes in the decay kinetics of GABAergic mIPSCs occur at GABAergic synapses in lamina II of the spinal cord (Ma et al., 1993; Bohlhalter et al., 1996; Keller et al., 2001) that express exclusively α2, α3, β3, and γ2 subunits throughout postnatal development (Poulter et al., 1992; Paysan and Fritschy, 1998). Therefore, one possible explanation of the changes in mIPSC kinetics of lamina II neurons could be that this phenomenon involves a change in the properties of synaptic $GABA_A$Rs mediated by endogenous modulators such as endozepines or neurosteroids rather than a change in $GABA_A$R subunit composition.

In our experiments, the blockade of the benzodiazepine site of $GABA_A$ receptors by the specific antagonist flumazenil did not significantly affect the kinetics of $GABA_A$ mIPSCs, although, in a few cases, the application of DZP together with FLU for durations of <1 hr tended to accelerate mIPSC kinetics. This effect, which never reached statistical significance, (1) might point to the possible role of endozepines, which might not have been fully revealed under our experimental conditions, or (2) might indicate a complex effect of FLU in the presence of DZP. Additional experiments will be required to clearly distinguish between these possibilities. In contrast, during the early postnatal period (<P23), the occurrence of slowly deactivating $GABA_A$R mIPSCs and of mixed $GABA_A$/GlyR mIPSCs in lamina II seems to be clearly correlated with the tonic presence of significant extracellular levels of 5α-reduced neurosteroids, because inhibition of the metabolism of 5α-reduced neurosteroids by finasteride (Concas et al., 1998) conferred to mIPSCs an adult-like phenotype. PBR is implicated in this phenomenon, because its blockade had effects similar to that of finasteride. In the adult, no such tonic neurosteroidogenesis could be detected, although neurosteroid production was still inducible via PBR stimulation with diazepam. Stimulation of neurosteroidogenesis obtained by coapplication of DZP + FLU resulted in a delayed (>1 hr) but apparently sudden increase in the duration of $GABA_A$R mIPSCs. Several phenomena might account for this observation. The precise cellular and subcellular distributions of the key neurosteroidogenic enzymes in the dorsal horn spinal cord are still unknown.

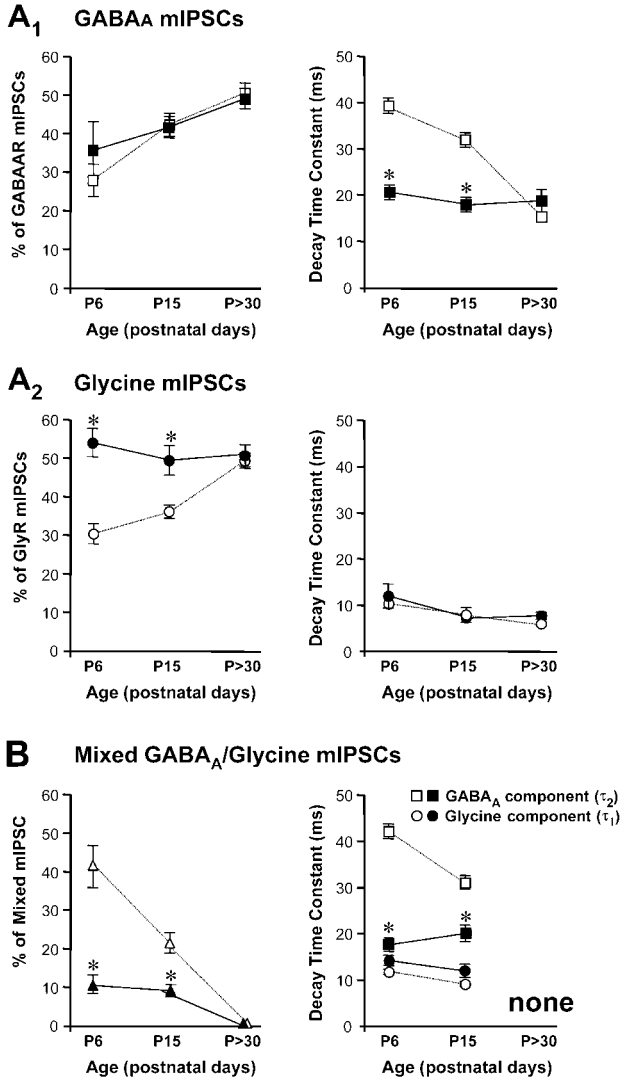

Figure 6. Developmental changes in synaptic inhibition expressed as changes in decay time constant and fraction of total $GABA_A$, glycine, and mixed mIPSCs. Evolution of the proportion (left panels) and mean decay time constant (right panels) of monoexponential $GABA_A$R mIPSCs (*A1*), GlyR mIPSCs (*A2*), and biexponential mixed $GABA_A$/GlyR mIPSCs (*B*) in lamina II neurons of spinal cord slices from 6-d-old (P6), 15-d-old (P15), and adult (>P30) rats. Open symbols represent the values obtained from neurons recorded under control condition (i.e., without any pharmacological treatment). Filled symbols apply to slices that have been preincubated for at least 6 hr with finasteride (50 μM). Asterisks indicate statistically significant differences (*t* test; $p < 0.05$) with respect to control.

In addition, the enzymatic process leading to the synthesis of 5α-reduced neurosteroids is slow, and the site of production might be at some distance from the synaptic cleft. These points might account for the long delay between stimulation of PBR and the observed effect on mIPSCs. In addition, despite a constant speed of production of neurosteroids, their effect on mIPSC kinetics might be detectable only after having crossed a threshold concentration. In summary, a relatively long time might be required to reach this threshold, but once it is reached, the modulation of mIPSC kinetics might reflect the actual speed of production of neurosteroids. Because the expression of 5α-reductase appears to be stable throughout postnatal development (Melcangi et al., 1998; Compagnone and Mellon, 2000; Lephart et al., 2001), the observed developmental changes in tonic neurosteroidogenesis are likely to involve a plastic mechanism upstream of 5α-reductase possibly in relation with the expression of PBR and/or of its endogenous ligands and/or a downregulation of the activity of 5α-reductase in the adult.

Little is known about the neurosteroidogenic activity of the spinal cord (Pomata et al., 2000), and only a single study has reported a high activity of 5α-reductase (Celotti et al., 1997) in this structure of the CNS. In this respect, the cellular origin of neurosteroids is an important issue in our experiments. Neurosteroidogenesis has been first described to occur in glial cells of the central and peripheral nervous system (Compagnone and Mellon, 2000), but recently, some key enzymes have also been localized to neurons (Zwain and Yen, 1999; Follesa et al., 2000; Tsutsui et al., 2000; Patte-Mensah et al., 2003). Stimulation of PBR was shown to increase the concentration of pregnenolone, progesterone, and related steroids in brain tissue and in the plasma (Korneyev et al., 1993; Zwain and Yen, 1999; Serra et al., 2001). Here, we clearly show that stimulation of the PBR induces a modulation of synaptic $GABA_A$R function that is independent of the activation of central benzodiazepine sites on $GABA_A$Rs, because it persisted in the presence of flumazenil, an antagonist of the CBR. Our results indicate that activation of the PBR lead to the production of 5α-reduced neurosteroids at a concentration sufficient to potentiate the function of synaptic $GABA_A$ receptors. These results are in good agreement with our previous *in vitro* study (Schlichter et al., 2000), realized on cultured hypothalamic neurons, which showed that the nonbenzodiazepine anxiolytic etifoxine increased $GABA_A$ receptor-mediated synaptic transmission in part by stimulation of the activity of PBR. The hypothesis of a neosynthesis of neurosteroid is further supported by the long delay (>1 hr) required to observe a modulation when DZP was perfused in the presence of the CBR antagonist FLU.

The superficial layers of the dorsal horn spinal cord play a crucial role in the initial processing of peripheral nociceptive information (Millan, 1999). In the adult spinal cord, antagonists of GlyR or $GABA_A$R induce hyperexcitability of dorsal horn neurons and behavioral states characteristic of hyperalgesia and allodynia (Yaksh, 1989; Sherman and Loomis, 1996; Sorkin and Puig, 1996; Ishikawa et al., 2000). As shown in our experiments, the adult spinal cord has preserved a neurosteroidogenic potential and endogenously produced 5α-reduced neurosteroids are able to efficiently increase the level of synaptic inhibition. It is therefore tempting to speculate that neurosteroids, if produced in physiological/pathological states, might increase the efficacy of inhibitory synaptic transmission and could act as a compensatory antinociceptive local control. Until now, no demonstration of such a role has been reported, although some indications may support this hypothesis. First, fluctuations in the concentrations of 5α-reduced neurosteroids in the brain and plasma have been shown to occur under different physiological (pregnancy, stress) and pathological (depression/anxiety) situations (Uzunova et al., 1998; Dong et al., 1999; Strohle et al., 1999; Reddy and Rogawski, 2002). Second, an antinociceptive effect of allopregnanolone to aversive thermal stimuli has been reported in rodents (Kavaliers and Wiebe, 1987; Frye and Duncan, 1994). Although it seems that $GABA_A$Rs are involved in this process, it is not possible to clearly discriminate between spinal versus supraspinal effects of 5α-reduced neurosteroids in these experiments, because neurosteroids were injected in the peripheral circulation or in the CSF.

In conclusion, we show that spinal inhibition mediated by GABAergic synapses or mixed GABA/glycine cosynapses is controlled by endogenous 5α-reduced neurosteroids. A tonic production of such 5α-reduced neurosteroids was detected in immature animals and then progressively decreased during postnatal

development. However, in the adult spinal cord, activation of neurosteroidogenesis was still possible after stimulation of the PBR. This situation leads to an increased inhibitory synaptic drive within lamina II, which could be part of an endogenous modulatory/compensatory mechanism in response to a strong and/or sustained activation of the spinal nociceptive system. Finally, the PBR, which controls the local production of 5α-reduced neurosteroids, might represent an interesting therapeutic target to limit the consequences of excessive activation of spinal pain nociceptive pathways during inflammatory or neuropathic pain situations.

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