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Developmental regulation of regional functionality of substantia nigra $GABA_A$ receptors involved in seizures

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Abstract

GABAergic (γ-aminobutyric acid) transmission in the substantia nigra pars reticulata is critical for seizure control. We tested the hypothesis that there is a differential regional distribution and functionality of nigral $GABA_A$ receptor sites that is developmentally regulated. In adult rats, we determined the effects on flurothyl seizures of (*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid (ZAPA, a presumed agonist of the low-affinity $GABA_A$ receptor site), bicuculline (an antagonist of the low-affinity $GABA_A$ receptor site) and γ-vinyl-GABA (a GABA-transaminase inhibitor), infused bilaterally in anterior or posterior substantia nigra pars reticulata. ZAPA infusions (8 μg) were anticonvulsant in anterior substantia nigra but proconvulsant in posterior substantia nigra. Bicuculline infusions (100 ng) were proconvulsant in anterior substantia nigra but ineffective in posterior substantia nigra. An anticonvulsant dose of γ-vinyl-GABA, when infused in anterior substantia nigra, was proconvulsant when infused in posterior substantia nigra. In 15 day old rats, the effects of ZAPA were biphasic: 2 μg was anticonvulsant while 8 μg was proconvulsant. There was no regional specificity. The data suggest that with maturation there is functional regional segregation of specific $GABA_A$ receptor subtypes involved in substantia nigra-mediated seizure control.

Keywords: Substantia nigra; Seizure; GABA (γ-aminobutyric acid); $GABA_A$ receptor; Development; (Rat)

1. Introduction

The substantia nigra pars reticulata is one of the structures controlling the spread of seizure activity via its GABAergic (γ-aminobutyric acid) neurotransmission (Gale, 1985; Iadarola and Gale, 1982) especially through $GABA_A$ receptors. Recently, two topographically distinct regions with different effects on seizures were demonstrated within the adult rat substantia nigra pars reticulata (Moshé et al., 1994; Niemi et al., 1994; Redgrave et al., 1992a,b). These regions also differ in the amount of the mRNA expression of the $GABA_A$ receptor α1-subunit and are using divergent projection networks to produce their effects on seizures (Moshé et al., 1994). In adult rats, muscimol (the prototypic $GABA_A$ receptor agonist on both high- and low-affinity receptors) has anticonvulsant effects against flurothyl-induced seizures if microinfused into the anterior substantia nigra, a site with low α1-subunit mRNA expression (Moshé et al., 1994). Similar muscimol infusions into the posterior substantia nigra pars reticulata of adult rats (a region with high expression of clustered α1-subunit mRNA) have proconvulsant effects compared with site- and volume-matched saline infusions (Moshé et al., 1994). Previous studies have shown that infusions of bicuculline, an antagonist of the low-affinity $GABA_A$ receptor site (Heyer et al., 1981; Olsen et al., 1983), in the adult substantia nigra pars reticulata have proconvulsant effect in the flurothyl seizure model (Sperber et al., 1987). However, in that study the regional specificity of the bicuculline effects was not explored because the infusions were mostly in the anterior substantia nigra pars reticulata. The effects of bicuculline infusions in the posterior substantia nigra have not yet been reported.

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In contrast, in young rats (15 days old), there is no anticonvulsant area in the substantia nigra pars reticulata with respect to muscimol. In this age group, muscimol infusions have only proconvulsant effects (Moshé and Albala, 1984; Sperber et al., 1987) and utilize the same neuronal circuit used by proconvulsant infusions of muscimol in the adult posterior substantia nigra (Moshé et al., 1994). In the 15 day old substantia nigra pars reticulata, the mRNA expression of the α1-subunit for the $GABA_A$ receptor does not display the regional topographic organization of the adult substantia nigra (Moshé et al., 1994). We have therefore suggested that the immature substantia nigra may have similar features as the adult posterior substantia nigra and that the anterior substantia nigra network becomes functional or unmasked with maturation (Moshé et al., 1994). Interestingly, bicuculline infusions into the immature substantia nigra pars reticulata have uniform proconvulsant effects (Sperber et al., 1987).

In mammals, central $GABA_A$ receptors are a heterogeneous family of related proteins comprising several subunits (α1–6, β1–4, γ1–3, δ, and probably ρ1–2) (Lüddens et al., 1995). Different combinations of these subunits form functionally distinct receptors in the brain; in situ hybridization studies have revealed that the different subunits have characteristic topographic distribution in the brain and that their expression is regulated during ontogeny in a distinct fashion (Dunn et al., 1994; MacLennan et al., 1991; Wisden et al., 1992). The subunit composition of recombinant rat $GABA_A$ receptors may affect the binding characteristics. The α-subunit composition may be critical. The presence of α3-subunit is associated with low-affinity binding while the α1-subunit is usually responsible for high-affinity GABA binding (Dunn et al., 1994). Recombinant receptors containing the α3-subunit are characterized by a relatively low GABA sensitivity (EC_{50} = 270 μM) (Backus et al., 1993), while recombinant receptors containing the α1-subunit have a high sensitivity to GABA (EC_{50} = 7–12 μM) (Verdoorn et al., 1990). In the mammalian brain, similar sites may well correspond to the low- (K_d = 100 nM to 1 μM) and high- (K_d = 10–20 nM) affinity $GABA_A$ receptors (Dunn et al., 1994).

Recently, ZAPA {(*Z*)-3-[(aminoiminomethyl)thio]prop-2-enoic acid}, a relatively specific and potent agonist of the $GABA_A$ low-affinity receptor site (Allan et al., 1991) has become available. ZAPA is a conformationally restricted analog of GABA. At higher doses, ZAPA can be a substrate for the GABA neuronal uptake system, thereby increasing synaptic GABA levels (Allan et al., 1991). Similarly γ-vinyl-GABA (vigabatrin, a GABA-transaminase inhibitor) enhances levels of endogenous GABA by limiting its degradation (Jung et al., 1977).

The findings of the functional compartmentalization of the adult substantia nigra pars reticulata to anterior and posterior regions raised several new questions. First, what is the effect of the low-affinity $GABA_A$ receptor site antagonist bicuculline in the adult posterior substantia nigra on seizures? Second, are the effects of regional intranigral infusions of ZAPA on seizures opposite to the effects of bicuculline or similar to the muscimol effects? And finally, what are the effects of regional intranigral infusions of ZAPA in rat pups?

2. Materials and methods

Male adult (180–200 g) and 13 day old Sprague-Dawley rats (Taconic Farms, New York) were used. The adult rats were housed in groups of four, while 13 day old pups were housed with their respective dams on 12 h dark : 12 h light cycle in our accredited animal facility. All rats had free access to food and water. All the experiments were performed between 8 a.m. and 1 p.m. All rats were operated under anesthesia with the i.p. mixture of ketamine (70 mg/kg) and xylazine (10 mg/kg) (Xu et al., 1991). In adult rats (n = 71), two cannulae were stereotactically lowered either into the anterior substantia nigra pars reticulata (coordinates in mm with respect to bregma: anterior-posterior = 5.3; lateral = 4.0; depth = 7.0 (from the skull); incisor bar at −3.5 mm) or into the posterior substantia nigra (coordinates: anterior-posterior = 5.8; lateral = 4.0; depth = 7.1; incisor bar at −3.5 mm) (Paxinos and Watson, 1988). The cannulae were inclined at an angle of 15° from the vertical sagittal plane. Rat pups (n = 36) were operated at 13 days of age using the following stereotaxic coordinates: anterior-posterior = 5.0; lateral = 3.3; depth = 6.5; incisor bar at −3.5 mm.

Both adults and pups were allowed 2 days for recovery, i.e., young rats were tested on postnatal day 15. All drugs used in this study (bicuculline, ZAPA, γ-vinyl-GABA) were dissolved in normal saline and microinjected bilaterally using Hamilton microsyringes and a dual infusion pump. We injected 0.25 μl into each substantia nigra via the implanted cannulae over 2 min. After the infusion was completed, the infusion cannulae remained inserted in the guide cannulae for an additional minute.

In the bicuculline experiments, adult rats received microinfusions of either saline or 100 ng (in 0.25 μl of saline) bicuculline methiodide in saline (n = 7) into the posterior substantia nigra pars reticulata. To reconfirm our previous results showing proconvulsant effects of bicuculline in the adult anterior substantia nigra, two additional rats were infused with 100 ng of bicuculline into the anterior substantia nigra. The controls (n = 8) were infused with saline, 0.25 μl per site. There was a 5 min latency between the infusion and flurothyl testing (Sperber et al., 1989).

In the ZAPA experiments, in the adult rats (n = 29), we used the doses 2, 4 or 8 μg of ZAPA per site (in 0.25 μl of saline). In the rat pups (n = 27), we infused 1, 2 or 8 μg of ZAPA per site. Controls received saline (0.25 μl) microinfusions (adults n = 16, pups n = 9). Seizure testing was performed 5 min post-infusion. The time delay be-

tween the infusion and seizure testing was determined by pilot studies.

In the γ-vinyl-GABA experiment, adult rats received microinfusions of either saline (0.25 μl; $n = 8$) or 20 μg of γ-vinyl-GABA in saline (0.25 μl) per site into the posterior substantia nigra ($n = 9$) 24 h prior to the flurothyl challenge (Xu et al., 1991). Due to differences in the flurothyl chamber design which occurred after the γ-vinyl-GABA but before the ZAPA and bicuculline experiments were performed, these experiments cannot be directly compared using absolute values. However, we can compare differences from respective controls and direction of the main effect.

For seizure testing, the rats were challenged with flurothyl (20 μl/min constant flow rate) in an air-tight chamber (9.38 l) until clonic seizures consisting of facial and forelimb clonus with preservation of the righting reflex occurred (Velíšek et al., 1995). We determined the latency to the onset of clonic seizures. Because the flurothyl was infused at a constant rate, the latency to the onset of seizures allowed us to recalculate the amount of infused flurothyl necessary to elicit the seizure; i.e., flurothyl seizure threshold for our chamber size.

After completion of the experiments, the animals were killed and their brains were removed and frozen. Serial sections were cut coronally at 30 μm and stained with thionin for histological examination of the placements.

The data were compared by one-way Analysis of Variance with post-hoc Fisher Protected Least Square Degree test within each age group. All values are means ± S.E.M. The level of significance has been preset to $P < 0.05$.

3. Results

Only the data from rats with verified placements of both cannulae in the substantia nigra pars reticulata were further analyzed. In adult rats, there was an additional requirement for bilaterally symmetric placement of the cannulae in either the anterior or posterior area of the substantia nigra (Fig. 1).

All drugs influenced the rats behavior. Rats treated with ZAPA or bicuculline had excessive sniffing, hyperreactivity, head nodding and rearing. Rats treated with γ-vinyl-GABA were lightly sedated and hypoactive. The elicited behavior was not age specific.

In adult rats, there were no differences in the flurothyl seizure thresholds for clonic seizures between anterior and posterior saline infusions into the substantia nigra pars reticulata (anterior substantia nigra threshold = 130.6 ± 2.8 μl of flurothyl, $n = 8$; and posterior substantia nigra threshold = 133.2 ± 5.8 μl of flurothyl, $n = 6$). Therefore, the anterior and posterior substantia nigra pars reticulata saline data were combined in subsequent analyses (131 ± 3.0 μl of flurothyl).

In the adults, bicuculline (100 ng) microinfused into the posterior substantia nigra had no effects on clonic flurothyl-induced seizures (threshold = 139.0 ± 2.5 μl of flurothyl, $n = 7$) (Fig. 2A). When microinfused into the anterior substantia nigra, bicuculline had proconvulsant effects (threshold = 85.0 + 2.0 μl of flurothyl, $n = 2$).

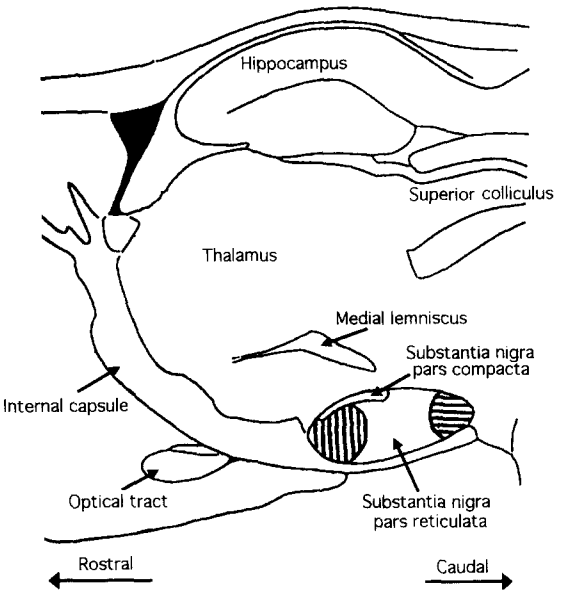

Fig. 1. A diagram of a representative section showing the anterior-posterior differences in the cannula placements in adult rats. Parasagital brain section at the level 2.40 mm lateral from midline (Paxinos and Watson, 1988). The cannula tip placements accepted to data analyses were located in either the anterior third (anterior group, vertically hatched) or the posterior third (posterior group, horizontally hatched) of the substantia nigra pars reticulata.

In the adult rats, 2 and 4 μg ZAPA did not alter the flurothyl-induced clonic seizure threshold irrespective of the exact intranigral localization (anterior substantia nigra: 2 μg of ZAPA – threshold = 141.2 ± 4.1 μl of flurothyl, $n = 5$, 4 μg of ZAPA – threshold = 128.4 ± 6.3 μl of flurothyl, $n = 3$; posterior substantia nigra: 2 μg of ZAPA – threshold = 120.0 ± 6.9 μl of flurothyl, $n = 5$, 4 μg of ZAPA – threshold = 138.7 ± 9.9 μl of flurothyl). However, the largest dose of ZAPA (8 μg per site) was significantly anticonvulsant after microinfusion into the anterior substantia nigra pars reticulata (threshold = 212.1 ± 18.4 μl of flurothyl) while it was proconvulsant in the posterior substantia nigra pars reticulata (threshold = 97.8 ± 4.9 μl of flurothyl) (Fig. 2B).

Microinfusions of 20 μg of γ-vinyl-GABA into the posterior substantia nigra pars reticulata were significantly proconvulsant in clonic seizures compared with saline-infused controls (saline threshold = 141.0 ± 10.8 μl of flurothyl, $n = 5$; γ-vinyl-GABA – threshold = 110.1 ± 6.9 μl of flurothyl, $n = 7$) (Fig. 2C).

In 15 day old rats, a single anterior-posterior coordinate was used. The cannulae were found to be in both substantia nigra pars reticulata regions. Analysis of the cannulae placements did not reveal any evidence that the effects of ZAPA were specific for a particular substantia nigra pars

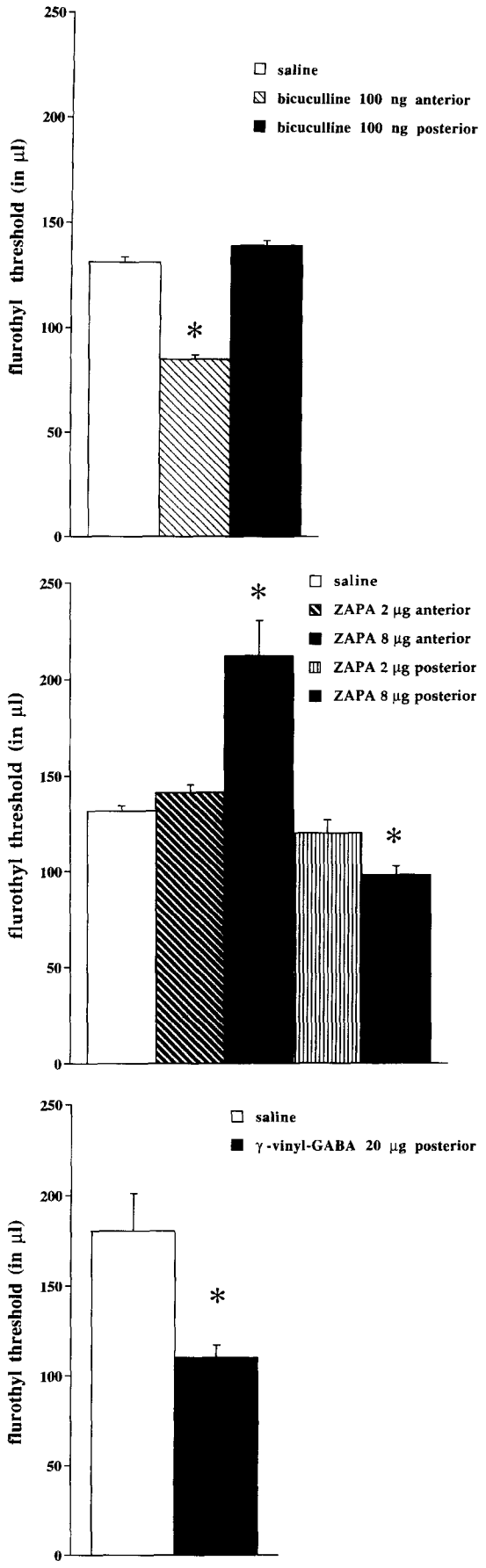

Fig. 2. Adult rats: effects of localized microinfusions of GABAergic agents in the substantia nigra pars reticulata on flurothyl-induced seizures. Mean flurothyl seizure threshold values ± S.E.M.; asterisk indicates a significant difference ($P < 0.05$) compared with the control group (Analysis of Variance with post-hoc Fisher Protected Least Square Degree test). (A) Effects of bicuculline infusions in the anterior and posterior substantia nigra pars reticulata. The dose 100 ng/0.25 µl per site of bicuculline, which is proconvulsant when infused in anterior substantia nigra had no effect in posterior substantia nigra pars reticulata. (B) Effects of localized microinfusions of ZAPA into the substantia nigra pars reticulata Only the highest dose 8 µg/0.25 µl per site produced significant effect. In the anterior substantia nigra it was anticonvulsant, while in the posterior substantia nigra was proconvulsant. The data obtained with infusions of 4 µg/0.25 µl per site did not differ from controls and the 2 µg/0.25 µl dose, and are not shown. (C) Effects of γ-vinyl-GABA infusions into the posterior substantia nigra pars reticulata on flurothyl-induced seizures. Infusions of γ-vinyl-GABA (20 µg/0.25 µl per site) in the posterior substantia nigra pars reticulata had proconvulsant effects. Due to differences in the flurothyl chamber design which occurred after the γ-vinyl-GABA but before the ZAPA and bicuculline experiments were performed, these experiments cannot be directly compared using absolute values. However, we can compare differences from respective controls and a direction of the main effect.

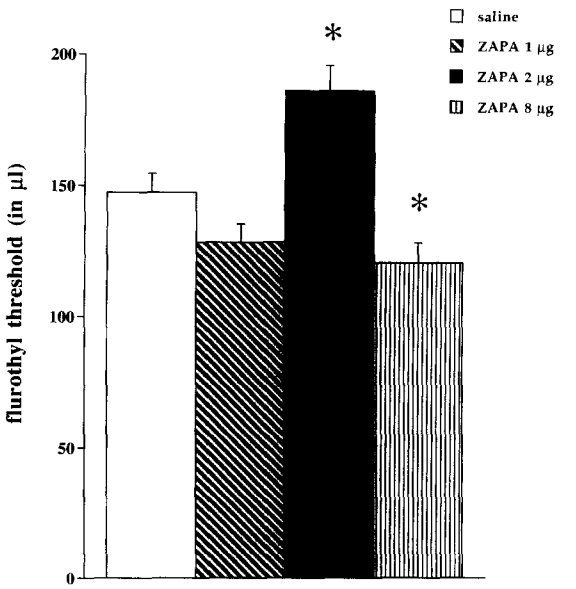

Fig. 3. 15 day old rats: effects of nigral ZAPA infusions on flurothyl-induced seizures. Mean seizure threshold values ± S.E.M.; asterisk indicates a significant difference compared with controls ($P < 0.05$; Analysis of Variance with post-hoc Fisher Protected Least Square Degree test). ZAPA infusions had biphasic effect. The lower dose (2 µg/0.25 µl per site) produce anticonvulsant effects while the highest dose (8 µg/0.25 µl per site) had proconvulsant effects.

reticulata region. In this age group, ZAPA produced dose-dependent effects. The threshold for saline was 147.4 ± 7.2 µl of flurothyl, $n = 9$. The dose of 1 µg of ZAPA had no effects on clonic seizures (threshold = 128.3 ± 7.0 µl of flurothyl). In contrast, 2 µg of ZAPA significantly increased the flurothyl seizure threshold, i.e., this dose was anticonvulsant, for clonic seizures (threshold = 186.3 ± 9.5 µl of flurothyl, $n = 11$). The largest dose of ZAPA (8 µg)

had proconvulsant effects on flurothyl seizures compared with saline infused controls (threshold = 120.5 ± 7.7 μl of flurothyl, $n = 8$) (Fig. 3).

4. Discussion

ZAPA is considered to be a relatively specific and potent agonist of low-affinity $GABA_A$ receptor sites, because 'ZAPA displaces the low-affinity binding of GABA to rat brain membranes by a factor of two better than GABA' (quoted from Allan et al., 1991). ZAPA is also a weak agonist of high-affinity $GABA_A$ sites with the IC_{50} about two-fold higher compared with GABA (Allan et al., 1986). Moreover, ZAPA is a potent substrate for neuronal GABA uptake with approximately 3 times greater affinity than GABA (Allan et al., 1991; Johnston, 1992). In contrast, bicuculline is considered to be an antagonist of the low-affinity $GABA_A$ receptor sites (Heyer et al., 1981; Olsen et al., 1983) while γ-vinyl-GABA is a GABA-transaminase inhibitor which enhances the levels of endogenous GABA by limiting its degradation, thus affects both low and high-affinity $GABA_A$ receptors (Jung et al., 1977).

In adult rats, the differential effects of ZAPA, γ-vinyl-GABA and bicuculline infusions in anterior and posterior substantia nigra provide additional strong support for the topographic and functional segregation of the substantia nigra pars reticulata-based circuits involved in the control of seizures (Moshé et al., 1994; Niemi et al., 1994; Redgrave et al., 1992a,b).

In the adult anterior substantia nigra, the effects of ZAPA and bicuculline were in the opposite direction, as ZAPA had anticonvulsant effects while bicuculline had proconvulsant effects. Since ZAPA is an agonist of the $GABA_A$ low-affinity site, and bicuculline an antagonist of the $GABA_A$ low-affinity site, the data suggest that the opposing effects of ZAPA and bicuculline are mediated by the $GABA_A$ low-affinity receptor. The role of anterior nigral low-affinity $GABA_A$ receptors on seizures is supported by previous data showing that the anticonvulsant effect of γ-vinyl-GABA infusions in this part of the substantia nigra can be blocked by nigral bicuculline infusions (Xu et al., 1991). However, it is possible that the anticonvulsant effects of ZAPA in the anterior substantia nigra pars reticulata may be due, in part, to its action on high-affinity receptor sites that mediate the anticonvulsant effects of the mixed agonist muscimol (Bartholini et al., 1985; Meldrum, 1981; Moshé and Sperber, 1990) and the specific agonist of the high-affinity $GABA_A$ receptor sites THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol) (Krogsgaard-Larsen, 1984; Xu et al., 1992). The effect of ZAPA may be direct via its albeit weak action on high-affinity $GABA_A$ receptors or by competing with endogenous GABA for transport, thus enhancing GABA concentration. We thus propose that, in adults, GABAmimetic stimulation of the anterior substantia nigra induces anticonvulsant effects in the flurothyl seizure model by activating both high- and low-affinity $GABA_A$ receptors.

However, in the adult posterior substantia nigra, the effects of ZAPA and bicuculline on seizures are not in the opposite direction. Bicuculline does not alter the seizure threshold while ZAPA is proconvulsant. These data suggest that the GABA effects on seizures produced by the anterior or posterior substantia nigra are not solely due to different output networks because although both networks are GABA sensitive, bicuculline can only block the anterior network. Thus, the incongruent effects of ZAPA and bicuculline suggest that there is a differential distribution of low-affinity $GABA_A$ receptors in the substantia nigra pars reticulata. Low-affinity $GABA_A$ receptors may be absent in the posterior substantia nigra pars reticulata. If this is the case, the proconvulsant effects of ZAPA may be due to its other GABAergic actions and its effects on flurothyl seizures may be mediated by a different receptor than the receptors mediating the ZAPA effects in anterior substantia nigra.

In the posterior substantia nigra, muscimol (Moshé et al., 1994), ZAPA and γ-vinyl-GABA (this study) have proconvulsant effects. We propose that the $GABA_A$ receptors located in the posterior substantia nigra have a distinct molecular subunit composition and binding characteristics (a high-affinity proconvulsant receptor subtype) (Moshé et al., 1994). The proconvulsant effects are obtained from a region with high mRNA expression of the $GABA_A$ α1-subunit suggesting that this population of receptors is comprised by different subunits than $GABA_A$ receptors in the anterior substantia nigra. The inability of bicuculline to alter the seizure threshold suggests that the high concentration of the α1 subunit may denote a high-affinity receptor subtype that mediates proconvulsant effects. However, the exact subunit composition of the receptor remains unknown.

The effects of the substantia nigra pars reticulata are age specific (Moshé and Albala, 1984; Sperber et al., 1987). Accordingly in 15 day old rats, we were not able to demonstrate that there is a topographic organization for the effects of ZAPA; this lack of topographic organization has also been reported for muscimol (Moshé et al., 1994). Furthermore, we found that in 15 day old rats, an intermediate dose of ZAPA (2 μg per site) had anticonvulsant effects against seizures, while a larger dose (8 μg per site) was proconvulsant. The anticonvulsant action of ZAPA may be mediated by $GABA_A$ low-affinity receptors either by ZAPA's direct action or indirectly due to blockade of $GABA_A$ uptake. The latter may result in a γ-vinyl-GABA-like effect and an increased amount of endogenous GABA in the synaptic cleft which enhances GABA action at the abundant low-affinity $GABA_A$ receptor. Indeed, previous studies indicate that these receptors are present in the 15 day old substantia nigra at slightly higher densities (130%) than in the adult substantia nigra (Wurpel et al., 1988). Furthermore, infusions of bicuculline are procon-

vulsant (Sperber et al., 1987) or block the anticonvulsant action of γ-vinyl-GABA (Xu et al., 1992). The effect of the higher dose may be due to the action of ZAPA on high-affinity receptors as described earlier for the adult posterior substantia nigra. We have previously described proconvulsant effects of muscimol (an agonist on both high and low-affinity $GABA_A$ receptor sites; (Bartholini et al., 1985; Meldrum, 1981)) and THIP (an agonist on the high-affinity $GABA_A$ receptor sites; (Krogsgaard-Larsen, 1984)) in 15 day old rat pups (Moshé and Albala, 1984; Sperber et al., 1987; Xu et al., 1992). Moreover, nigral muscimol infusions can also block the anticonvulsant action of γ-vinyl-GABA (Xu et al., 1992).

These results suggest that the immature, undifferentiated substantia nigra has the features of the posterior substantia nigra in terms of the presence of the proconvulsant high-affinity $GABA_A$ receptor subtype. However, in the immature substantia nigra, the $GABA_A$ low-affinity receptors are also present (Wurpel et al., 1988). We hypothesize that, in the flurothyl model, the full anticonvulsant properties of the anterior substantia nigra emerge with development as the density of high-affinity receptors of different subunit composition rises to its mature levels (Wurpel et al., 1988). In the posterior substantia nigra pars reticulata a small proportion of the existing high-affinity $GABA_A$ receptors maintain their immature features leading to their proconvulsant effects. Additional studies using recombinant GABA receptors are needed to prove our hypothesis that specific combinations of GABA subunits change the binding affinity and the pharmacological effect of GABAergic agents.

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