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Neonatal N-(-2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) treatment modifies the vulnerability to phenobarbital- and ethanol-evoked sedative-hypnotic effects in adult rats

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Abstract:
To study the influence of the central noradrenergic system on sensitivity to sedative-hypnotic effects mediated by the aminobutyric acid (GABA) system, intact rats were contrasted with rats in which noradrenergic nerves were largely destroyed shortly after birth with the neurotoxin DSP-4 [N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine; 50 mg/kg *sc* x2, P1 and P3]. At 10 weeks, loss of the righting reflex (LORR) was used as an index to study the acute sedative-hypnotic effects of phenobarbital (100 mg/kg *ip*) and ethanol (4 g/kg *ip*, 25% v/v). Additionally, GABA concentration in the medial prefrontal cortex (PFC), hippocampus, cerebellum and brainstem was estimated by an HPLC/ED method. Neonatal DSP-4 treatment diminished the sedative-hypnotic effects of both phenobarbital and ethanol in adult rats. While the endogenous GABA content in the PFC, hippocampus, brainstem and cerebellum of DSP-4-treated rats was not altered, phenobarbital significantly decreased GABA content of both intact and DSP-4-lesioned rats by ~40% in the hippocampus and by ~20% in other brain regions at 1 h. Ethanol reduced GABA content by ~15–30% but only in the hippocampus and brainstem of both intact and lesioned rats. These findings indicate that the noradrenergic system exerts a prominent influence on sedative-hypnotics acting *via* GABAergic systems in the brain without directly altering GABA levels in the brain.

Key words:
noradrenergic, lesion, phenobarbital, ethanol, GABA, rats

Abbreviations: DOPAC – 3,4-dihydroxyphenylacetic acid, DSP-4 – N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine, GABA – γ-aminobutyric acid, 5-HT – 5-hydroxytryptamine, 5-HIAA – 5-hydroxyindoleacetic acid, LC – locus coeruleus, MOPEG – 3-metoxy-4-hydroxyphenylglycol, NA – noradrenaline, PFC – prefrontal cortex

Introduction

The midbrain locus coeruleus (LC) contains most noradrenergic neurons that project to the forebrain [8]. LC neurons modulate numerous functions (e.g., sleep-wake cycle, attention, learning and memory, vigilance, mood, and opioid withdrawal) [2], and represent a cellular target for a large array of drugs used in the treatment of neurological and psychiatric disorders [34, 39, 41]. LC activity is tightly controlled by presynaptic α_2 autoreceptors [38] and also by afferent pathways from several brain areas [24]. Among these, serotonin- and γ-aminobutyric acid (GABA)-containing neurons seems to play a major role [1]. Anatomical relationships between the LC and the GABA neurons suggest the existence of a functional interaction between noradrenergic and GABAergic systems. It was shown that the discharge rate (of neurons) and release of noradrenaline (NA) were under the inhibitory control of GABA, acting *via* $GABA_A$ receptors [22, 37, 44]. Conversely, the noradrenergic system is also involved in the regulation of basal GABA release that is possibly mediated *via* α_{1A} and α_2-adrenoceptor activation [25, 27].

Recently, we re-established a neonatal NA lesion model [5, 31]. In brief, N-(-2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4; 50 mg/kg *sc* per day) injected two times (on the 1st and 3rd days of postnatal life) alters noradrenergic input to the hippocampus and prefrontal cortex (PFC) (endogenous NA content was reduced by 98.5% and 95.0%, respectively), without impairing dopaminergic and serotonergic input to these regions. In the present paper, we also demonstrated the elevated NA level in the brainstem and cerebellum in those DSP-4-treated rats. As we have shown, this model represents a good "tool" for studying interactions between particular neurotransmitter systems [10, 31]. Examination of the GABA-NA interaction in neonatally lesioned rats enables us to better understand compensatory processes arising consequent to ontogenetic noradrenergic neuronal denervation. This phenomenon may be of major importance, because NA deficiency is associated with a number of neurological and neuropsychiatric disorders, including depression [40], Parkinson's disease [29], and Alzheimer's disease [16]. Conversely, there is a paucity of data concerning the function of the GABAergic system in DSP-4-lesioned animals. Recently, we determined that neonatal DSP-4 treatment modified the convulsant effect of bicuculine and pentetrazole in adult rats [4]. We also established that administration of the GABA transaminase inhibitor (vigabatrin) caused an increase in GABA level in PFC in control and DSP-4 groups of animals; however, it contributed to a 2-fold higher increase in the extracellular GABA concentration in DSP-4-lesioned rats compared to control animals [3]. Taking the above into consideration, the aim of the present study was to analyze the compensatory processes which develop during postnatal ontogeny in the GABAergic system, following chemical ablation of noradrenergic neurons. It is believed that this work provides greater in-depth analysis of the consequences of NA perturbation to GABAergic systems.

Materials and Methods

Animals and treatment

Wistar rats (University Animal Department, Katowice, Poland) were housed under controlled environmental conditions, in a well-ventilated room, at 22 ± 2°C and under a 12 h light : 12 h dark cycle (lights on from 7:00 a.m. to 7:00 p.m.). Animals received food and water *ad libitum*. Offspring rats were weaned at 21 days, and segregated by sex. Experiments were carried out in the morning in only male rats, handled in accordance with the principles and guidelines described in the *NIH Guide for the Care and Use of Laboratory Animals*. All procedures were reviewed and approved by the Local Bioethical Committee for Animal Care.

The central noradrenergic system of newborn rats was lesioned with DSP-4 (Sigma, St. Louis, MO, USA). Rats were injected on the 1st and 3rd day of postnatal life with either DSP-4 (50 mg/kg *sc*) or 0.9% NaCl (1.0 ml/kg *sc*). DSP-4 was dissolved in distilled water immediately before injection. The dose and the days of injection were chosen on the basis of the works of Jonsson et al. [21] and Brus et al. [5], and were consistently proven to reduce endogenous NA content in the PFC and hippocampus by 95–99%. Rats continued to be housed as above until 8th–10th week, for further experimentation.

Assessment of GABA content in the brain

At 8th week after birth, groups of control and DSP-4-treated rats (6 per group) were decapitated 60 min after administration of phenobarbital (100 mg/kg *ip*) or ethanol (4.0 g/kg *ip*). Respective controls received 0.9% NaCl (1.0 ml/kg *ip*). The PFC, hippocampus, brainstem and cerebellum were rapidly dissected and placed on dry ice, weighed, and stored at –70°C, pending assay. Samples were homogenized for 15–20 s in ice-cold 0.1 M $HClO_4$ (Fluka, Steinheim, Switzerland) and left thereafter for 20 min at 4°C to be deproteinized. Then they were centrifuged (5,000 × g, 5 min) to remove precipitated protein. Supernatants were filtered through 0.2 μm cellulose membranes (Titan MSF Microspin filters, Scientific Resources Inc., Eatontown, GB) and were derivatized with OPA/Sulfite. Stock solutions of the OPA/Sulfite derivatizing reagent were prepared by dissolving 22.0 mg *o*-phthaldialdehyde (OPA) (Sigma, St. Louis, USA) in 0.5 ml of absolute ethanol (POCH S.A. Gliwice, Poland). A 0.5 ml volume of 1.0 M Na_2SO_4 (Z.Ch. "Organica-Sarzyna", Nowa Sarzyna, Poland) was added followed by 9.0 ml of 0.1 M $Na_2B_4O_7$ (POCH S.A., Gliwice, Poland) adjusted to pH 10.4 with 2.0 M NaOH. The working OPA/Sulfite solution was prepared by diluting 50 ml of OPA/Sulfite stock solution with 5 ml of deionized water. Precolumn GABA derivatization was performed by mixing 20 μl of the working OPA/Sulfite solution with 20 μl of GABA standard or sample for 10 min at room temperature before analysis. Samples prepared in this way were injected onto the HPLC column. GABA levels were assayed by HPLC/ED [11, 43]. The composition of the mobile phase was: 100 mM NaH_2PO_4 (Sigma, St. Louis, USA) and 25% (v/v) methanol (Sigma, St. Louis, USA), pH 5.2 adjusted with 2.0 M NaOH. The flow rate was maintained at 1.0 ml/min, temperature at 22°C, oxidation potential at +700 mV and sensitivity at 100 nA/V. Peaks were automatically integrated by universal chromatographic interface UCI-100 (Dionex Softron GmbH, Germering, Germany). The instrumentation included an electrochemical detector (Gilson Villiers-le-Bel, France) model 141 with flow cell, piston pump model 302 with head 5SC (Gilson, Villiers-le-Bel, France), manometric module model 802 (Gilson, Villiers-le-Bel, France), thermostat for STH 595 column (Dionex Softron GmbH, Germering, Germany) and chromatographic column HR-80 C18, 80 × 4.6 mm, 3 μm (ESA Inc., Chelmsford, USA).

Assessment of biogenic amine and metabolite content

At 8th week after birth control and DSP-4-treated rats (6 per group) were decapitated. The PFC, hippocampus, brainstem and cerebellum were rapidly dissected and placed on dry ice, weighed and stored at –70°C, pending assay. Samples were homogenized for 15–20 s in ice-cold trichloroacetic acid (0.1 M), containing 0.05 mM ascorbic acid. After centrifugation (5,000 × g, 5 min), supernatants were filtered through 0.2 μm cellulose membranes (Titan MSF Microspin filters, Scientific Resources Inc., Eatontown GB) and supernatants was injected onto the HPLC/ED column. Levels of noradrenaline (NA), 3-methoxy-4-hydroxyphenylglycol (MOPEG), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were assayed by HPLC/ED [31]. The composition of the mobile phase was: 75 mM NaH_2PO_4, 1.7 mM 1-octanesulfonic acid, 5 μM EDTA (Avocado, Research Chemical Ltd., Morecambe, GB), 100 μl triethylamine (Sigma, St. Louis, USA), 9.5% acetonitrile (J.T. Baker, Deventer, Holland), pH 3 adjusted with phosphoric acid (Fluka, Steinheim, Switzerland). The flow rate was maintained at 0.7 ml/min, at a temperature of 22°C, and the oxidation potential was fixed at +700 mV, 10 nA/V sensitivity. Peaks were automatically integrated by universal chromatographic interface UCI-100 (Dionex Softron GmbH, Germering, Germany). The instrumentation included an electrochemical detector (Gilson, Villiers-le-Bel, France) model 141 with flow cell, piston pump model 302 with head 5SC (Gilson, Villiers-le-Bel, France), manometric module model 802 (Gilson, Villiers-le-Bel, France), thermostat for STH 595 column (Dionex Softron GmbH, Germering, Germany), precolumn Hypersil BDS C18, 10 × 4 mm, 3 μm (ThermoQuest, Waltham, GB) and chromatographic column Hypersil BDS C18, 250 × 4.6 mm, 3 μm (ThermoQuest, Waltham, GB).

Loss of righting reflex test

Loss of righting reflex test is a simple and reliably measured behavior which has been widely used to study mechanisms of action of sedative-hypnotic drugs. Rats of both tested groups (control and DSP-4) were observed for hypnotic sensitivity to phenobarbital and ethanol by measuring both duration of the loss of righting reflex and sleep time. The testing procedure

consisted of *ip* injection of either phenobarbital (100 mg/kg) or ethanol (4 g/kg, 25% v/v) to rats, and placing them on their back in a V-shaped trough after loss of the righting reflex. Sleep time was recorded as the time period between loss and regaining of the righting response. Animals were considered to have regained the righting response after righting themselves three times in 30 s [9, 14].

Statistical analysis

Group differences in GABA were assessed by an analysis of variance (ANOVA) and the *post*-ANOVA test of Newman-Keuls. Group differences in behavioral studies were analyzed by Student's *t*-test. A p value < 0.05 was taken as the level of significant difference.

Results

Effect of DSP-4 treatment on GABA concentration in the PFC, hippocampus, brainstem and cerebellum of rats

In rats treated on the 1st and 3rd days of postnatal life with DSP-4 (50 mg/kg *sc*), and sacrificed at 8 weeks, the endogenous GABA content in the frontal cortex, hippocampus, brainstem and cerebellum was not different from control (Fig. 1, 2).

Phenobarbital (100 mg/kg *ip*) significantly reduced GABA concentration at 1 h in the PFC, hippocampus and brainstem of control and DSP-4 treated rats, and the magnitude of that effect was similar in both groups. Similar-effect was observed in the cerebellum, but the GABA level was significantly decreased only in control rats (Fig. 1).

Ethanol (4.0 g/kg *ip*) also reduced GABA concentration at 1 h in the hippocampus and brainstem, to a similar extent in control and DSP-4 groups, but was without effect in the PFC and cerebellum (Fig. 2).

Effect of DSP-4 treatment on biogenic amine and metabolite levels in the PFC, hippocampus, brainstem and cerebellum

DSP-4 treatment on the 1st and 3rd days of postnatal life with a dose of 50 mg/kg *sc*, reduced NA contents of the PFC and hippocampus by 95.0% and 98.5%, respectively ($p < 0.01$). Conversely, the concentration of NA in the brainstem was elevated by approx. 42.8% and in the cerebellum by 34.6% in comparison to control ($p < 0.05$). MOPEG and DA were increased only in the brainstem of DSP-4-treated rats. In the

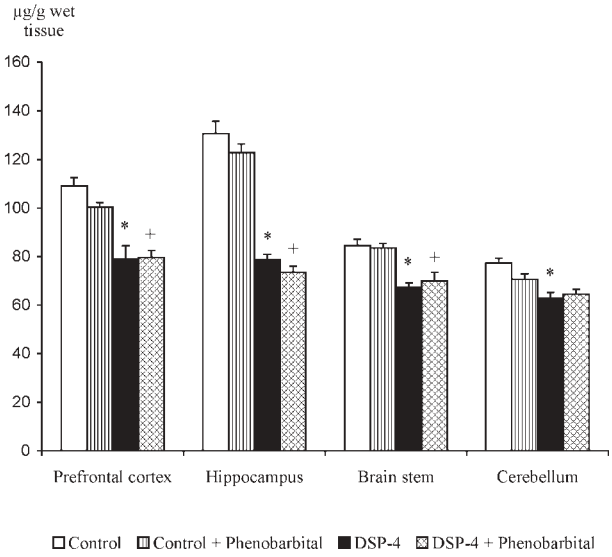

Fig. 1. Effect of neonatal DSP-4 (50 mg/kg *sc* on the 1st and 3rd day of postnatal life) lesioning on GABA content in the prefrontal cortex, hippocampus, brainstem and cerebellum after acute phenobarbital (100 mg/kg *ip*) treatment of adult rats (n = 6). * $p < 0.05$ Control *vs.* Control + Phenobarbital; $^{+}$ $p < 0.05$ DSP-4 *vs.* DSP-4 + Phenobarbital

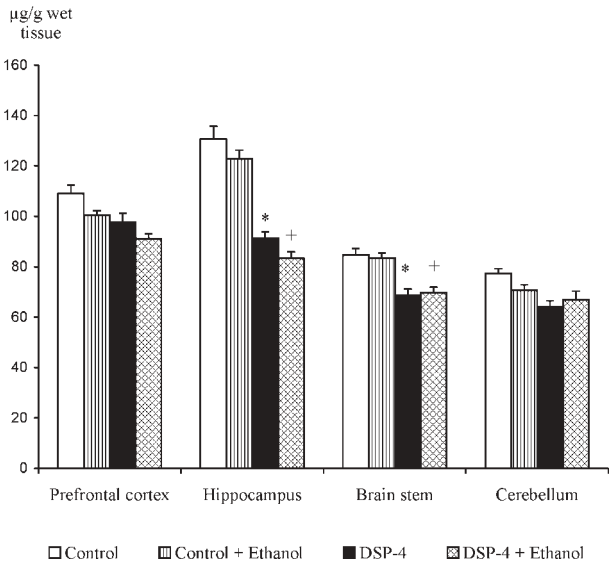

Fig. 2. Effect of neonatal DSP-4 (50 mg/kg *sc* on the 1st and 3rd day of postnatal life) lesioning on GABA content in the prefrontal cortex, hippocampus, brainstem and cerebellum after acute ethanol (4 g mg/kg *ip*) treatment of adult rats (n = 6). * $p < 0.05$ Control *vs.* Control + Ethanol; $^{+}$ $p < 0.05$ DSP-4 *vs.* DSP-4 + Ethanol

Tab. 1. Effect of DSP-4 (50 mg/kg *sc* on the 1st and 3rd day of postnatal life) administration on biogenic amine level in the PFC, hippocampus, brainstem and cerebellum (x ± SEM; n = 6)

Examined brain structures	Groups	NA (ng/g)	MOPEG (ng/g)	DA (ng/g)	DOPAC (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)
Frontal cortex	Control	483 ± 29	208 ± 13	264 ± 29	54 ± 4	281 ± 21	119 ± 10
	DSP-4	24 ± 3**	163 ± 11	271 ± 42	61 ± 8	216 ± 32	115 ± 17
Hipocampus	Control	392 ± 13	140 ± 6	8 ± 1	Not detected	275 ± 9	146 ± 6
	DSP-4	6 ± 1**	117 ± 7	2 ± 0	Not detected	208 ± 15	150 ± 7
Brainstem	Control	513 ± 41	275 ± 23	28 ± 2	13 ± 2	376 ± 55	270 ± 23
	DSP-4	733 ±45*	351 ± 25*	33 ± 1*	12 ± 1	382 ± 11	236 ± 15
Cerebellum	Control	234 ± 22	503 ± 18	5 ± 1	6 ± 1	47 ± 8	60 ± 5
	DSP-4	315 ± 19*	537 ± 22	3 ± 0	6 ± 0.5	45 ± 2	37 ± 1*

* p < 0.01, ** p < 0.05

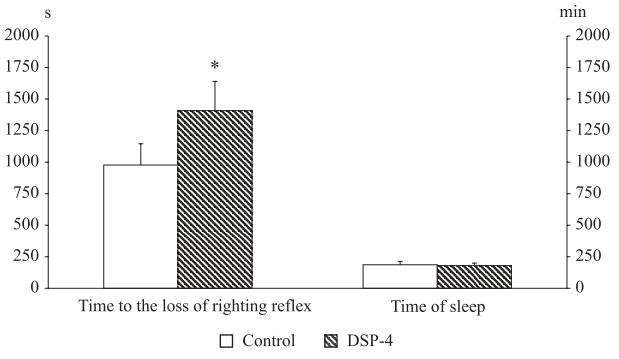

Fig. 3. Effect of neonatal DSP-4 (50 mg/kg *sc* on the 1st and 3rd day of postnatal life) lesioning on time to phenobarbital (100 mg/kg *ip*)-induced loss of the righting reflex and duration of sleep in adult rats (n = 7). * p < 0.05

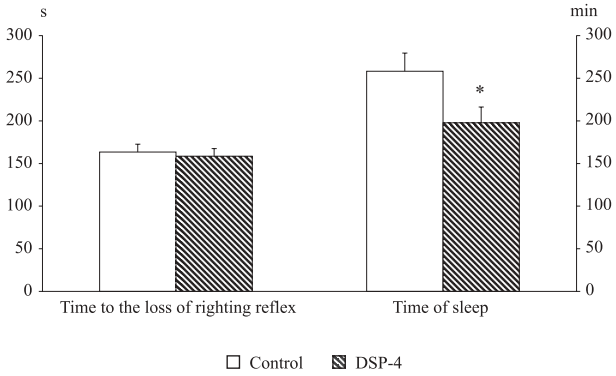

Fig. 4. Effect of neonatal DSP-4 (50 mg/kg *sc* on the 1st and 3rd day of postnatal life) lesioning on time to ethanol (4 g/kg *ip*)-induced loss of the righting reflex and duration of sleep in adult rats (n = 7). * p < 0.05

PFC, hippocampus brainstem and cerebellum, there was no consistent change in the DA and its metabolite DOPAC nor in 5-HT and its metabolite 5-HIAA (Tab. 1).

Effect of DSP-4 treatment on the hypnotic effect of ethanol and phenobarbital

Phenobarbital (100 mg/kg *ip*) produced a greater loss of the righting reflex in DSP-4 rats versus controls (1450.1 ± 231.7 *vs.* 976.8 ± 169.2 s, respectively). Conversely, phenobarbital-induced sleep time was virtually identical in both groups (179.1 ± 21.2 *vs.* 186.4 ± 25.9 s, respectively) (Fig. 3).

After ethanol injection (4.0 g/kg *ip*) time to loss of righting reflex was comparable in control and DSP-4-treated rats (163.4 ± 18.3 *vs.* 158.5 ± 17.8), while sleep time was significantly shortened in the DSP-4 group (258.1 ± 42.7 *vs.* 197.7 ± 37.4) (Fig. 4).

Discussion

The major findings of the present study are that 1) neonatally DSP-4-lesioned rats are less vulnerable to sedative-hypnotic effects of phenobarbital and ethanol. Additionally, 2) there was no significant change

in GABA concentration of the PFC, hippocampus, cerebellum and midbrain of DSP-4-lesioned rats.

There is a large body of literature data associating the noradrenergic system with regulation of the sensitivity to GABA-mimetics (i.e., anesthetic response to barbiturates, hypnotic effect of ethanol, anxiolytic effect of phenobarbital, alprazolam or susceptibility to convulsant action of bicuculine and other GABAergics) [6, 13, 15, 18]. Yet, there are no data regarding such an association in neonatally DSP-4-treated animals. Neonatal treatment with neurotoxins, such as 6-hydroxydopamine or DSP-4 produces marked impairment in development of the central noradrenergic system (i.e., permanent and robust NA-denervation of the PFC and hippocampus, accompanied by NA hyperinnervation of brainstem and cerebellum [20, 28]. It is noteworthy that NA hyperinnervation is not observed in rodents lesioned with DSP-4 as adults [20, 21]. The latter treatment may serve as a model for examination of NA deficiency which is thought to be associated with a number of neurological and neuropsychiatric diseases, including depression, Parkinson's disease, and Alzheimer's disease [47]. Conversely, neonatal DSP-4 treatment model may be applied for modeling of childhood disorders. Previously, we developed an animal (rat) model of attention-deficit-hyperactivity disorder (ADHD) based on severe neonatal DA depletion with simultaneous partial serotonergic fiber lesion in adulthood. Our preliminary findings [3–5, 10] have confirmed the hypothesis that rats lesioned as neonates with DSP-4 represent another good model for such studies.

As mentioned above, NA has a prominent role in the regulation of attention, arousal, cognitive processes, anxiety, and nociception – all of which are potential targets for anesthetic actions. It is well-known that drugs, such as etomidate, ketamine, pentobarbital, diazepam, halothane, etc., strongly influence noradrenergic system activity (e.g., NA release, NA turnover and NA content throughout brain) [17, 19, 33]. In contrast, noradrenergic activity affects barbiturate anesthesia [26, 32]. Sedative-hypnotic effects relating to that, also apply to ethanol [23, 46]. Spuhler et al. [43] determined that in selectively-bred ethanol-sensitive and -insensitive mice, adult treatment with DSP-4 did not alter the sensitivity to ethanol, assessed by loss of the righting response. Therefore, although synaptically-released monoamines may influence ethanol responses, it was considered that NA probably did not directly mediate differences in behavioral sensitivity to ethanol in these mouse lines. The above findings contrast with the present results. In the neonatal model of DSP-4 treatment, we show that onset of anesthesia (loss of righting reflex) after ethanol (4.0 g/kg *ip*) was comparable in control and DSP-4 treated rats, but the duration of anesthesia (sleep time) was significantly shortened in the DSP-4 group (Fig. 3). Conversely, after phenobarbital (100 mg/kg *ip*) there was an increased duration of loss of the righting reflex in DSP-4 rats versus control, but sleep time was not different in the two groups (Fig. 4). This seemingly is in conflict with findings by Członkowski et al. [7], but it must be noted that DSP-4 lesioning was performed in adult rats in their study, but in neonatal rats in our study.

Medina and Novas [28] demonstrated that neonatal treatment of rats with DSP-4 was associated with reduced benzodiazepine (BZ) receptor number in the PFC, and increased BZ number in the cerebellum and brainstem. It is likely that changes in BZ receptor density account, at least in part, for the diminished sensitivity to phenobarbital and ethanol observed in neonatally DSP-4 lesioned rats in our study. There is also an alternative explanation of the current results. Matsumoto et al. [26] found that the intracerebroventricular administration of methoxamine (α_1-adrenoceptor agonist) or yohimbine (α_2-adrenoceptor antagonist), produced a dose-dependent decrease in pentobarbital-induced (50 mg/kg) sleeping time. Also, DSP-4 treatment resulted in profound changes in adrenoceptor density (i.e., up-regulation of α_1 and α_2 and β receptors) [12, 45]. Perhaps compensatory enhancement in NA turnover as a result of DSP-4 treatment [36], similarly to adrenergic facilitatory acting agents, contributes to the reduction of phenobarbital- and ethanol-evoked sedative-hypnotic effects.

In the present study, we also estimated the level of GABA in the PFC and hippocampus after phenobarbital and ethanol treatments. Noguchi and Kawai [30] as well as Paul and Ekambaram [35] found that phenobarbital and ethanol altered the content of GABA and other neurotransmitters (e.g., glutamic acid) in the brain. In fact, we also demonstrated that these drugs significantly reduced GABA concentration in the PFC, hippocampus and brainstem of control and DSP-4-lesioned rats, with a similar magnitude of effect in both groups (Fig. 1). An analogous effect was observed in the cerebellum, but the GABA level was significantly decreased only in control rats. Ethanol also reduced GABA content in the hippocampus and

brainstem in intact and DSP-4-lesioned rats, but was without any effect in the PFC and cerebellum (Fig. 2).

In conclusion, the present study demonstrates a prominent effect of noradrenergic neurons in regulating the sensitivity to sedative-hypnotic GABA-acting agents, being at the same time without effect on GABA concentration in the brain. It may be suggested that hypoactivity of the noradrenergic pathway may account for lower vulnerability to anesthetics.

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