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Region-Selective Reduction of Brain Serotonin Turnover Rate and Serotonin Agonist-Induced Behavior in Mice Treated With Clonazepam

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LIMA, L. *Region-selective reduction of brain serotonin turnover rate and serotonin agonist-induced behavior in mice treated with clonazepam*. PHARMACOL BIOCHEM BEHAV **39**(3) 671–676, 1991.—Evidence supports a complex interaction between benzodiazepines and the central serotonergic system. This study attempts to correlate biochemical changes in the serotonin (5HT) system induced by clonazepam (CLON) with the behavioural response to a 5HT agonist. The acute administration of CLON to mice produced a time-dependent decrease in 5HT turnover rate in the raphe area (dorsal and medial raphe nuclei) and modified the serotonergic syndrome induced by 5-methoxy-N,N,-dimethyltryptamine (DMT). One hour after CLON administration, a dose-dependent increase in 5HT concentration was found in the raphe area, while 5-hydroxyindoleacetic acid (5HIAA) levels remained stable, leading to an increase in 5HT/5HIAA ratio, indicative of reduced 5HT turnover rate. No significant changes were detected in the frontal cortex of CLON-treated mice. After 4 days of CLON treatment, the 5HT turnover rate was still decreased in the raphe area and unchanged in the frontal cortex. Acute CLON administration produced dose-dependent alterations in locomotor activity, not observed after subchronic administration. Lateral head weaving, a motor manifestation of the serotonergic syndrome produced by DMT, was less intense in CLON-treated animals. The modifications in the 5HT system induced by CLON are region selective, suggesting differences in the receptors implicated in the interaction. Altered synaptic availability of 5HT as a result of CLON administration may be responsible for the differential response to DMT in control and CLON-treated mice.

Clonazepam Serotonin Turnover rate Lateral head weaving Benzodiazepine

CLONAZEPAM (CLON) is a potent benzodiazepine (BZD) (3) known to increase serotonin (5HT) concentration in synaptic sites (23). CLON has been reported to increase whole-brain 5HT levels without affecting 5-hydroxyindoleacetic acid (5HIAA), and to elevate noradrenaline and dopamine brain concentrations (12). However, increases in 5HIAA three hours after CLON administration have also been reported (23). In mouse brain, CLON increases tryptophan, 5HT and 5HIAA levels and counteracts the decrease of 5HT that results upon inhibition of tryptophan hydroxylase (40). After blockade of L-aromatic acid decarboxylase, there is no accumulation of 5-hydroxytryptophan by CLON treatment, suggesting that its synthesis might be unaltered (40). Moreover, in the rat, acute or chronic diazepam (DZP) administration increases levels of synaptosomal 5HT without modifying the ^{3}H-5HT uptake or 5HT rate of synthesis (41), stimulates the release of ^{3}H-5HT from midbrain slices and decreases it from cerebral cortex (6), and increases 5HIAA in the hippocampus (9). The regional differences in the effects of BZDs on 5HT pathways might be a consequence of the autoregulation of 5HT system.

In mice, CLON, DZP, oxazepam and clobazam potentiate the head twitches induced by the 5HT agonist 5-methoxy-N,N-dimethyltryptamine (DMT), an effect that is not modified by the GABA antagonist bicuculline (31), and CLON evokes head twitches that are blocked by $5HT_2$ receptor antagonists (33,34). Administration of Ro 23-0364, a BZD with anxiolytic activity, increases salt intake in rats, as also do the $5HT_{1A}$ receptor agonists 8-hydroxy-2(di-N-propylamino)tetralin (DPAT), gepirone and ipsapirone (8,11). On the other hand, the BZD-induced wet-dog shakes produced by high doses of CLON are blocked by DPAT and ipsapirone (39).

BZDs produce convulsions through BZD II receptors in newborn rats (15), and decrease firing of serotonergic neurons via potentiation of GABA transmission (7, 17, 36). In addition, GABA and baclofen inhibit the 5HT-stimulation of ^{3}H-inositol phosphate formation in mouse cortex (18). The link between 5HT system and BZDs could take place through GABA-BZD receptors or might be a direct effect on GABA-independent BZD receptors.

The anxiety-reducing activity of BZDs has been proposed to be mediated by the reduction of brain 5HT turnover in model animals (45). Central $5HT_3$ receptors might be involved in the control of anxiety, because their antagonists possess antianxiety properties as well as buspirone (9, 11, 35) and ritanserin (35). CLON has been useful in the treatment of mania and schizoaffective and panic disorders (5, 43, 44), probably by its dual action as agonist of GABA and modulator of 5HT functional state.

If CLON is modifying the turnover rate of 5HT, the response to a 5HT agonist administered to CLON-treated animals might be altered by the influence of 5HT over its own system. The aim of this study is to evaluate the effect of CLON on brain 5HT turnover rate and on the response to the agonist DMT, in

TABLE 1

ACUTE EFFECT OF CLON ON LATERAL HEAD WEAVING AND HEAD SHAKING PRODUCED BY DMT

	Lateral Head Weaving				Head Shaking			
Treatment	0	+	++	+++	0	+	++	+++
CLON 1 h								
Control	—	100	—	—	—	80	20	—
1 mg/kg	20	80	—	—	—	40	60	—
Control	—	30	40	30	—	30	40	30
5 mg/kg	10	80	10	—	—	10	80	10
Control	—	20	70	10	—	50	20	30
10 mg/kg	—	50	30	20	—	30	50	20
CLON 4 days								
Control	—	40	50	10	—	20	40	40
1 mg/kg	20	20	50	10	—	20	70	10
Control	—	50	40	10	—	40	40	20
5 mg/kg	20	30	50	—	20	30	50	—
Control	—	10	50	40	—	—	50	50
10 mg/kg	10	40	50	—	—	10	50	40

Values are percentage of animals expressing the head movements according to scales described in the Method section. n = 9–14.

order to correlate pre- and postsynaptic events related to BZD treatment.

ABBREVIATIONS

GABA, gamma-aminobutyric acid; BZD, benzodiazepine(s); CLON, clonazepam; DZP, diazepam; HS, head shaking; 5HIAA, 5-hydroxyindoleacetic acid; 5HTP, 5-hydroxytryptophan; LHW, lateral head weaving; NA, noradrenaline; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5HT, serotonin; DPAT, 8-hydroxy-2(di-N-propylamino)tetralin; DMT, 5-methoxy-N,N-dimethyltryptamine.

METHOD

Animals

Male NMRI-IVIC mice (25 ± 3 g) were housed in individual cages and maintained on a 12-h light-dark cycle with free access to standard diet and water.

Tissue

Animals were sacrificed by decapitation; the brain was removed and placed on ice. The raphe area (corresponding to dorsal, B7, and medial, B8, raphe nuclei) and frontal cortex were dissected according to the procedure of Aghajanian et al. (1). The tissue was kept at −20°C until homogenization was carried out in 500 μl of buffer solution for chromatography without acetonitrile. Protein concentration was determined by the method of Lowry et al. (29). The homogenate was centrifuged at 40,000 × g for 10 min, and the supernatant was used for chromatographic determinations.

Drugs

Clonazepam (CLON, Roche) is one of the most potent BZDs reported to modify the 5HT system (23). It was suspended in 0.5% methylcellulose (Merck) and administered intraperitoneally (IP) in doses of 1, 5, or 10 mg/kg. DMT (Sigma) is a well-documented drug used to induce a syndrome to evaluate 5HT-mediated movements (22,26) and was chosen to observe any changes produced by the BZD in the motor response to this type of 5HT agonist. It was dissolved in 0.9% NaCl and administered IP in a dose of 2.5 mg/kg 1 min before starting observation. Control animals received the identical number and regime of injections of the vehicles. A time-course experiment was carried out by the administration of CLON 1, 2 or 3 hours prior to sacrifice; the vehicle was injected in mice that are represented at 0 time. For acute experiments, CLON was injected 1 h prior to observation, sacrifice or DMT administration. For subchronic experiments, CLON was injected once daily during four consecutive days in order to avoid tolerance (28), and animals were observed, sacrificed or administered DMT 1 h after the last dose.

Serotonergic Syndrome

The syndrome produced by DMT was scored according to the criteria of Jacobs (22) by detection of resting tremor, rigidity or hypertonicity, reciprocal forepaw treading, hind-limb abduction, Straub tail, lateral head weaving (LHW) and head shaking (HS). These signs were evaluated as absent (0), mild (+), moderate (++), and intense (+++). Locomotor activity and resting time (total time mice did not express locomotor activity for more than 30 seconds) were measured in control and CLON-injected animals, as well as DMT alone or CLON plus DMT. Animals were observed for 6 min by two persons at a time, one of them a "blind" observer. The mice were gently placed in a test box divided into 36 squares, surrounded by 30-cm walls, provided with internal illumination (1.5-volt bulbs), and covered by a Plexiglas piece with a central window. The observations were carried out in a room under dim light.

5HT and 5HIAA Determination

The monamine and the metabolite were determined by high-pressure liquid chromatography with electrochemical detection. The system consisted of a 2150 pump, 2154 manual sample injector and 2134 electrochemical detector (LKB, Bromma). The potential of the glassy carbon working electrode versus the H+/H (LKB, Bromma) reference electrode was +0.70 V. The column used was an Altex Ultrasphere-ODS (25 cm × 4.6 mm i.d., 5 μm average particle), and the mobile phase was a modified solution (25,32): 90% of buffer 0.02 M sodium acetate/0.0125 M citric acid buffer, pH 3.9, with 0.01 mM EDTA and 0.03% octanylsulphonate, and 10% of acetonitrile. The amounts of the substances were calculated by the external standard method with a 3380A Hewlett-Packard integrator. The 5HT/5HIAA ratio was used as an indirect index of 5HT turnover rate (25).

Statistical Tests

Results are mean ± standard error. Analysis of variance (ANOVA) was performed, and significance of the difference between means was calculated from the mean square for the remainder (42).

RESULTS

Acute CLON Administration and Turnover Rate of 5HT

The acute administration of 5 mg/kg CLON produced a time-dependent increase in 5HT concentration and in 5HT/5HIAA ra-

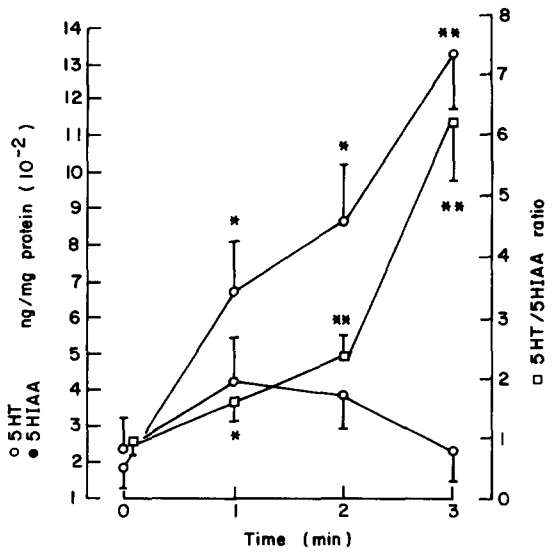

FIG. 1. Time course of the acute effect of 5 mg/kg CLON, on 5HT, 5HIAA and 5HT/5HIAA ratio in the raphe of mice. Time 0 corresponds to control (animals injected with the vehicle), n=3. The values of F by ANOVA were: 5HT, F(3,12)=8.18, $p<0.01$; 5HIAA, F(3,12)=0.79; 5HT/5HIAA, F(3,12)=24.79, $p<0.001$. *$p<0.025$ and **$p<0.01$ with respect to control.

tio (Fig. 1). Due to the effect of CLON on the response to DMT in 1-h CLON-treated mice (Fig. 4), subsequent determinations were carried out 1 h after CLON administration. The concentration of 5HT in the raphe area of mice treated with CLON 1 h prior to sacrifice (Fig. 2A) increased in a dose-dependent manner ($p<0.01$, 5 and 10 mg/kg), 5HIAA levels were not significantly modified, and there was an increase in the 5HT/5HIAA ratio with 10 mg/kg ($p<0.05$). No significant changes were observed in the frontal cortex (Fig. 2B).

Subchronic CLON Administration and Turnover Rate of 5HT

The concentration of 5HT in the raphe area of rats treated with CLON injections on 4 consecutive days was significantly increased with 10 mg/kg ($p<0.05$) while there was no change in the concentration of 5HIAA with any dose, and there was a significant increase in the ratio 5HT/5HIAA with the three doses (Fig. 3A). The 5HT turnover rate in the frontal cortex was not modified (Fig. 3B).

Locomotor Activity in CLON-Treated Rats

Spontaneous locomotor activity was measured 1 h after acute CLON administration or after the last of 4 daily injections. In the acute experiments, administration of 5 mg/kg of CLON decreased this activity, and 10 mg/kg increased it (Fig. 4A). The subchronic treatment did not modify the locomotor activity of the mice (Fig. 4B).

Effect of DMT in CLON-Treated Rats

DMT (2.5 mg/kg IP) was administered to animals that had received a single injection of CLON 1 h earlier (Fig. 4A) or daily injections during 4 days (Fig. 4B). Locomotor activity was decreased by the administration of DMT to control animals. Acutely CLON-treated mice (Fig. 4A) showed a dose-dependent resistance to the decrease in locomotor activity produced by

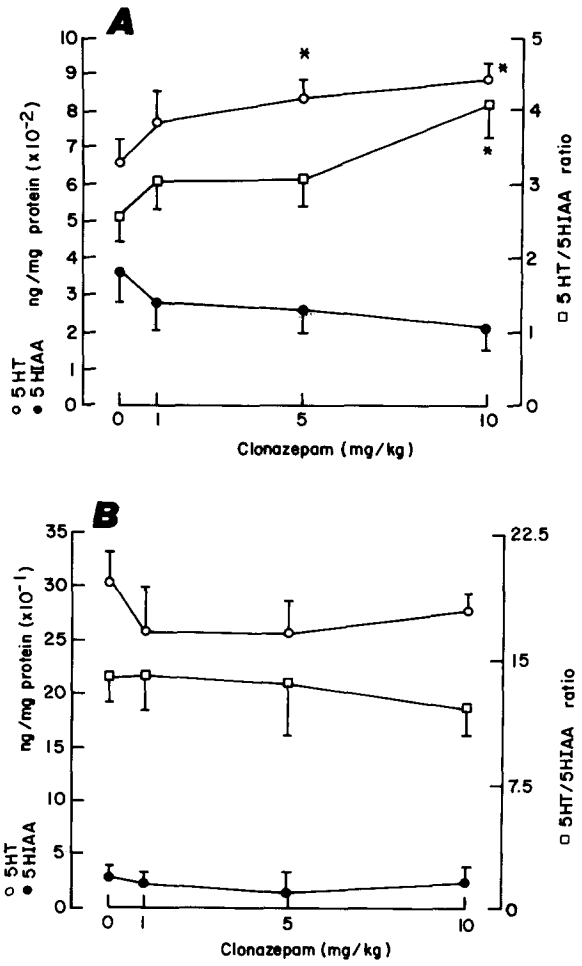

FIG. 2. Dose-dependent effect of IP administration of CLON 1 h prior to sacrifice on 5HT and 5HIAA concentration, n=7–8: (A) Raphe area: 5HT, F(3,29)=3.97, $p<0.05$; 5HIAA, F(3,31)=1.37; 5HT/5HIAA, F(3,29)=1.15. *$p<0.01$ with respect to control. (B) Frontal cortex: 5HT, F(3,30)=0.25; 5HIAA, F(3,29)=1.28; 5HT/5HIAA, F(3,29)=0.38.

DMT, which was statistically significant with 10 mg/kg of CLON. The subchronic treatment (Fig. 4B) with CLON did not change the response to DMT. The percentages of animals showing the evaluated signs of the serotonergic syndrome (LHW and HS) varied with acute or subchronic CLON administration (Table 1). There is a displacement to the zone of 0 and + in the case of LHW in animals treated with CLON in comparison to the respective control group.

DISCUSSION

The increase in 5HT concentration after acute administration of BZDs (23, 28, 41, 45) is an effect that has been reported to occur via a decrease in serotonergic activity (36,40). Tolerance, correlated to the sedative effect by chlordiazepoxide and to the effect of CLON on the 5HT system, has been also reported to occur in the hippocampus and frontal cortex (23,28). BZDs produce biphasic effects in locomotor activity in mice (13), with tolerance for the depressant effect after 5 days (14). BZDs are

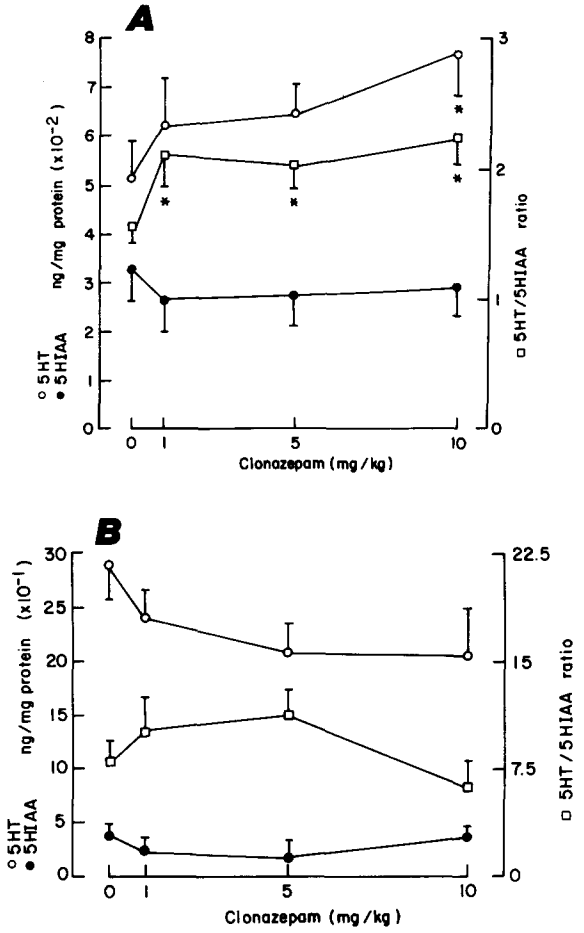

FIG. 3. Dose-dependent effect of daily IP administration of CLON for 4 days on 5HT and 5HIAA concentration, n=4–8: (A) Raphe area: 5HT, $F(3,31)=1.80$; 5HIAA, $F(3,31)=0.44$; 5HT/5HIAA, $F(3,31)=3.07$, $p<0.05$. *$p<0.05$ with respect to control. (B) Frontal cortex: 5HT, $F(3,15)=0.64$; 5HIAA, $F(3,17)=0.60$; 5HT/5HIAA, $F(3,15)=0.50$.

reported to potentiate the head twitches evoked by DMT but not those evoked by 5HTP, which are inhibited (31). The effect of CLON is not blocked by the BZD inhibitor flumazemil, the GABA antagonist bicuculline or the inhibitor of 5HT synthesis *p*-chloro-phenylalanine, suggesting a postsynaptic effect on 5HT receptors (31). While $5HT_2$ receptors are involved in head twitches (20), ipsapirone, a $5HT_{1A}$ agonist, potentiates the head twitches produced by DMT (19). BZDs have been reported to interact with $5HT_{1B}$ receptors in a behavioral paradigm (16). Moreover, $5HT_3$ receptor antagonists possess anxiolytic activity and are effective in preventing the syndrome that follows withdrawal from treatment with DZP (10).

The emerging picture is quite complex, even more so if we add that wet-dog shakes induced by CLON are blocked by the $5HT_{1A}$ receptor agonists DPAT and ipsapirone, but not by DMT, and that the $5HT_2$ receptor agonist DOI [1-(2,5-dimethoxy-4-iodophenylaminopropane)] induces the wet-dog shakes and increases the effect of CLON (39). Moreover, the $5HT_2$-mediated shaking behavior produced in mice is reduced by DZP (21).

The decrease in locomotor activity in the 5HT syndrome produced by DMT is counteracted by the acute administration of CLON, but this effect is not observed after 4 days of treatment. The LHW and HS were differentially expressed in control and CLON-treated mice. Acute subchronic administration led to a displacement to the left mainly in the LHW, that is to say that there was a diminished expression of the syndrome.

Drugs modifying synaptic availability of 5HT can indirectly affect the corresponding receptors (30). Thus the effects of CLON on DMT behavioral signs could be mediated through the modifications known to be produced by this BZD in the 5HT system, by direct effects on 5HT receptors or by parallel opposing effects on a given target. One component of the action of DMT could be the increase in 5HT supply at the postsynaptic site, while CLON treatment reduces it. The partial inhibition of the 5HT syndrome produced by DMT in CLON-treated mice suggests that the BZD is only affecting some of the mechanisms involved in the complex production of the syndrome. The interactions of BZDs and 5HT differ according to the region of the brain, so it seems appropriate to compare terminal and cell body areas.

The biochemical studies were carried out 1 h after CLON treatment in order to correlate them to the behavioral effects. After acute CLON administration, the concentration of 5HT in the raphe increased but not that in frontal cortex. This suggests that the effect of BZDs on neuronal 5HT is functionally related, not only to the nature of the BZD receptors implicated, but also to the effects that changes in availability of the monoamine produce on its own system. After subchronic treatment, the concentration of 5HT in the raphe was significantly affected, and there was a reduction of its turnover rate not observed in the frontal cortex. Thus the modification of 5HT function in the raphe is not necessarily expressed in the projections to the forebrain, although a decrease in 5HT turnover rate in the hippocampus of rats can be seen after similar treatment (unpublished results).

While $5HT_{1A}$ receptors in the midbrain and the hippocampus are abundant (2,37), the cerebral cortex possesses a mixed population of 5HT receptors (38). This might explain the differences observed between the raphe and frontal cortex. Also, the nature of BZD receptors differ among these regions. Whether the modifications of 5HT turnover rate are due to interaction with BZD-specific receptors needs to be clarified. However, the fact that acute CLON treatment produced a reduction in 5HT turnover rate in the raphe and modifications of the behavioral effects produced by DMT at the same time may be an indication of correlated phenomena, but does not exclude the participation of other brain regions or systems. DMT is a $5HT_1$ and $5HT_2$ agonist (38), and both may be involved in the effects of CLON. It seems that the CLON-mediated decrease in 5HT turnover rate in the raphe area is affecting some target in the 5HT system, resulting in a reduction of 5HT release by DMT, so that the response to the 5HT agonist is smaller in CLON-treated mice.

The involvement of specific BZD and 5HT receptors is yet to be clarified. The efficacy of CLON and some other BZDs, which are useful in the treatment of long-term and intractable seizures, myoclonus, mania and panic disorder (4, 24, 27, 43, 44), may be enhanced by its effects on the 5HT system.

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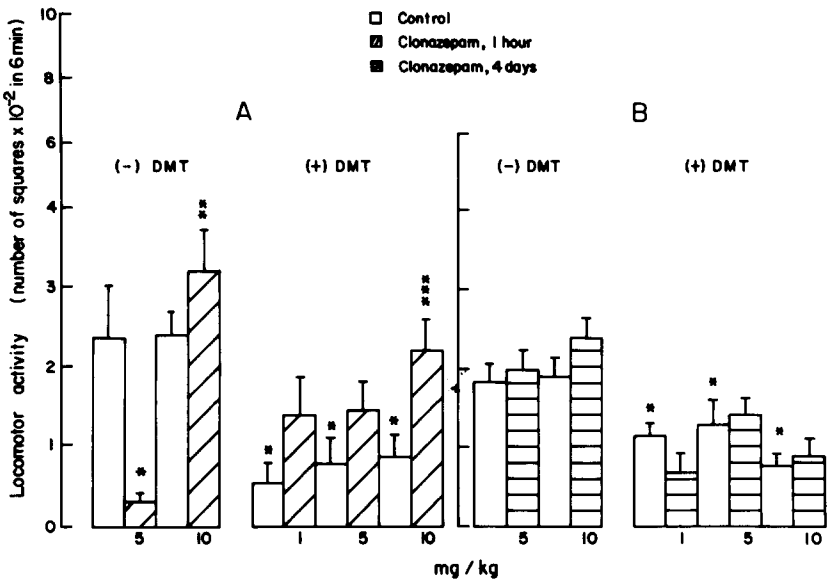

FIG. 4. Acute and chronic effects of CLON and DMT on locomotor activity, n=9–14. ANOVA, CLON 1 h (−)DMT, $F(3,36)=18.12$, $p<0.001$. CLON 1 h (+)DMT, $F(5,54)=3.07$, $p<0.05$. CLON 4 days (−)DMT, $F(3,36)=0.36$. Control 4 days (+)DMT, $F(5,54)=0.87$. CLON 1 h (−) (+)DMT, $F(4,45)=8.55$, $p<0.001$. CLON 4 days (−) (+) DMT, $F(4,45)=4.35$, $p<0.01$. Control 1 h (−) (+)DMT, $F(4,45)=11.04$, $p<0.001$. Control 4 days (−) (+)DMT, $F(4,45)=2.60$, $p<0.05$. *$p<0.001$ with respect to control (−) DMT; **$p<0.001$ with respect to CLON 5 mg/kg 1 h (−)DMT; ***$p<0.025$ with respect to control (+) DMT.

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