



Short-term erythrosine B-induced inhibition of the brain regional serotonergic activity suppresses motor activity (exploratory behavior) of young adult mammals

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ABSTRACT

Previous studies showed that repeated ingestion of erythrosine B (artificial food color) developed behavioral hyperactivity, but nothing is known about its single administration effect as well as the neurochemical (s) involvement. The present study provides evidence that a single higher dosage (10, 100 or 200 mg/kg, p.o.) of erythrosine administration to young adult male rats reduced motor activity (MA) maximally at 2 h and brain regional (medulla-pons, hippocampus and hypothalamus) serotonergic activity (measuring steady-state levels of 5-HT and 5-HIAA, pargyline-induced 5-HT accumulation and 5-HIAA declination rate and 5-HT receptor binding) under similar experimental condition. The degree of erythrosine-induced inhibition of both MA and brain regional serotonergic activity was dosage dependent. Lower dosage (1 mg/kg, p.o.) did not affect either of the above. Erythrosine (100 or 200 mg/kg, p.o.)-induced MA suppression was also observed in the presence of specific MAO-A inhibitor, clorgyline (5 mg/kg, i.p.) or MAO-B inhibitor, deprenyl (5 mg/kg, i.p.); but their co-application (5 mg/kg, i.p., each) effectively prevented the erythrosine-induced motor suppression. Altogether these results suggest that a single higher dosage of erythrosine (10–200 mg/kg, p.o.) may reduce MA by reducing serotonergic activity with modulation of central dopaminergic activity depending on the brain regions.

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1. Introduction

Artificial food colors have been utilized over the century for esthetic that make the foods attractive and stimulate appetite. Feingold (1975) initially claimed that synthetic food dyes play a major role in etiology of behavioral disturbance. Recently, a number of studies including double-blinded, placebo-controlled trials have suggested a significant link between the long-term or repeated ingestion of synthetic food colors and behavioral hyperactivity (Bateman et al., 2004; Boris and Mandel, 1994; McCann et al., 2007; Schab and Trinh, 2004). Among the various synthetic food colors/dyes, erythrosine [chemical name/synonyms: disodium 2-(2',4',5',7'-tetraiodo-3-oxido-6-oxoxanthin-9-yl)benzoate monohydrate/CI Acid Red 51; CI Food Red 14; D&C Red No. 3; FD&C Red No. 3; erythrosine B or BS] (Budavari, 1989; Food additives in Europe, 2000) is a highly lipid soluble anionic dye and acts as an organic anion (Levitan, 1977) in biological system. Initially Levitan et al. (1984) and later Hirohashi et al. (1997) have reported that erythrosine, like other fluorescent compounds, crosses the blood-brain-barrier, though its brain uptake has been found to be restricted due to dye-plasma protein complex formation depending on the age and condition of the subject (Levitan et al., 1984). In experimental animal, long-term administration of erythrosine, like other synthetic food color (Tanaka, 2006), significantly

increases the movement activity of exploratory behavior in a dosage dependent manner (Tanaka, 2001, 2006). It has also been observed that erythrosine increases Ca^{+2} permeability in neural membrane (Colombini and Wu, 1981; Heffron et al., 1984), the release of neurotransmitters like dopamine, GABA, serotonin, acetylcholine, norepinephrine etc. (Augustine and Levitan, 1983; Logan and Swanson, 1979; Wade et al., 1984) and inhibits brain Mg^{2+} - and Na^{+} - K^{+} -ATPase (Wade et al., 1984), Ca^{+2} -ATPase (Watson and Haynes, 1982), high affinity ouabain binding (Hnatowich and Labella, 1982; Silbergeld, 1981; Swann, 1982) and dopamine uptake in rat brain tissue (Lafferman and Silbergeld, 1979). Despite all these evidences which may suggest a significant link between the ingestion of artificial food color (erythrosine) and behavioral hyperactivity, the involvement of specific neurotransmitters and neural mechanisms in relation to behavioral alteration mediated by artificial food color(s) consumption (*in vivo*) are yet to be studied.

Several pharmacological evidences have implicated the involvement of the dopaminergic system in the etiology of behavioral conditions (including attention-deficit hyperactivity disorder) (Brennan and Arnsten, 2008) and hyper locomotor activity (Giros et al., 1996; Hechtman, 1994). Recent studies indicate that central serotonergic system has a positive modulating effect on the functional activities of the brain dopaminergic system (Alex and Pehek, 2007). Several recent studies have also been performed to characterize the potential role of central serotonin (5-HT) in the activation and modulation of the locomotor system and continue to be an area of major interest (Liu and Jordan, 2005; Schmidt and Jordan, 2000). Today, it is widely

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acknowledged that serotonin plays a potential role in the development of locomotor circuits and modulation of the locomotor central pattern generator (Branchereau et al., 2002; Cazalets et al., 2000; Pflieger et al., 2002) specifically the circuit involved in exploratory behavior (Grailhe et al., 1999). This role of serotonin depends upon neuroanatomical location of various classes of 5-HT receptors in brain areas related to motor control (striatum, medulla, hippocampus, frontal cortex and spinal cord). Thus, it is conceivable that brain regional 5-HT may have a role in erythrosine-induced disturbance in motor (rearing) activity. In the present study, the authors therefore investigated the effect of a single oral consumption of erythrosine with varying dosages on brain regional (medulla-pons, hypothalamus, hippocampus and corpus striatum) serotonergic activity and its pharmacological modulation by using specific monoamine oxidase inhibitor(s) [MAOI(s)] in relation to changes in motor (rearing) activity in young adult male albino rats.

2. Experimental procedures

2.1. Reagents

Erythrosine B (dye content 90%), serotonin (5-HT)-HCl, R-(–)-deprenyl-HCl (selegiline), clorgyline, ninhydrin, semicarbazide-HCl, and bovine serum albumin (BSA) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Pargyline-HCl was obtained from Abbott laboratories (North Chicago, Illinois, USA). [³H]-5-HT (specific activity 1 Ci/mmol) was purchased from Board of Radiation and Isotope Technology (BRIT, Mumbai, India). Cocktail 'O' (liquid scintillation solution in toluene) was purchased from Spectrochem Pvt. Limited (Mumbai, India). All other reagents used in the study were of analytical grade.

2.2. Animals and animal care

Young adult male albino rats of Charles Foster strain weighing 130–140 g were taken as experimental subjects and housed in a room having constant temperature (28 ± 0.5 °C) and relative humidity (85 ± 5%) with 12 h light–dark cycle and were maintained with standard laboratory food and water *ad libitum*. In the present study, the guidelines of the animal ethical committee (Department of Biochemistry, University of Calcutta; Registration no. 797/CPCSEA) were followed and all efforts were made to minimize the number of animals used and their suffering.

2.3. Experimental design

Young adult male albino rats were randomly divided into four groups (Groups I, II, III and IV). Each group was further divided into five (05) sub-groups (sub-group 1 was considered as control and sub-groups 2–5 were considered as experimental). Experimental sub-groups (2, 3, 4 and 5) were treated orally (p.o.) with a single dosage of erythrosine (1, 10, 100 and 200 mg/kg, p.o. respectively in 0.5 ml distilled water) and their corresponding control rats of sub-group 1 were treated with the equivalent volume of vehicle of erythrosine (distilled water) through the same route under similar condition.

Group I: In order to observe the optimum time required for the maximum erythrosine-induced behavioral response, rats of Group I (containing 8–12 rats in each sub-group) were treated with a single dosage of erythrosine (1, 10, 100 or 200 mg/kg, p.o. in 0.5 ml) and motor activity (MA) was measured at different time points (0 to 9 h). In the present investigation dosages of erythrosine (1 to 200 mg/kg, p.o.) for single oral exposure were selected considering the previous studies reported by Abdel Aziz et al. (1997), Sasaki et al. (2002), Tanaka (2001) and Tsuda et al. (2001).

Group II: Rats of Group II were used for neurobiochemical parameters. Each neurobiochemical parameter (using 4–6 rats in each sub-group of Group II) was measured following 2 h or 7 h of erythrosine consumption.

Group III: Animals of Group III (containing 12 rats in each sub-group and pretreated with a single dosage of erythrosine or its vehicle as mentioned above) were used for determination of pargyline (75 mg/kg, i.p. in 0.2 ml saline)-induced 5-HT accumulation and 5-HIAA declination rates. Control rats of Group III were treated with an equal volume (0.5 ml) of vehicle of erythrosine (distilled water) and saline (when required) through the same route under similar experimental conditions.

Group IV: Animals of Group IV (containing 8–12 rats in each sub-group and pretreated with a single dosage of erythrosine or its vehicle as mentioned above) were treated with specific MAOIs [clorgyline (5 mg/kg, i.p.) and/or deprenyl (5 mg/kg, i.p.)] and used for behavioral (motor activity) study. Clorgyline and/or deprenyl (dissolved in normal saline) were administered to Group IV rats after 10 min of erythrosine treatment. MA was measured (0 to 9 h) at every 30 min interval. Control rats of Group IV were treated with an equal volume (0.5 ml) of vehicle of erythrosine (distilled water) and saline (when required) through the same route under similar experimental conditions.

2.4. Behavioral rating of rat motor activity (MA)

The number of vertical (rearing) motor activity of each animal was measured during 5 min observation period to monitor the MA as mentioned in Jamaluddin and Poddar (2003). The animals treated with either erythrosine or its vehicle (distilled water) considered as corresponding control or erythrosine in the presence of specific MAOI (s) or their corresponding vehicle(s) considered as corresponding control, were gently transferred to a transparent plastic chamber (24 × 24 × 20 cm³) illuminated with an electrical lamp at the top. The vertical rearing frequency was measured by an electrical device based on the capacitance change proportional to the distance between the animal's head and probe following the method of Keenan and Johnson (1972) and then they were gently placed back in their home cages.

2.5. Collection of brain tissue

Rats of both control and experimental groups were sacrificed by cervical dislocation between 2:00 and 2:30 pm to avoid circadian effect, if any. After decapitation brains were immediately taken out and immersed in liquid nitrogen for estimation of steady-state levels of 5-HT, 5-HIAA or kept in ice-cold condition (0–4 °C) for the enzyme (MAO-A) activity and 5-HT receptor binding studies. Immediately after collection of the brains, different brain regions (e.g. medulla-pons, hypothalamus, hippocampus and corpus striatum) were dissected out following the method described by Poddar and Dewey (1980).

2.6. Estimation of neurobiochemical parameters

2.6.1. Steady-state levels of 5-HT and 5-HIAA

Brain regional (medulla-pons, hypothalamus, hippocampus and corpus striatum) steady-state levels of 5-HT and its metabolite 5-HIAA were estimated spectrophotofluorometrically according to the method of Scapagnini et al. (1969). Rat brain tissues were homogenate in 20 volume of 0.4 N perchloric acid (PCA). 1 ml of tissue homogenate was mixed with 2.5 mg of EDTA and 2.5 mg of ascorbic acid and then centrifuged at 4500 rpm for 5 min. The clear supernatant was used for determination of 5-HT and 5-HIAA. The pH of the supernatant was adjusted to 6.8–7.0 with solid K₂CO₃, 10% ZnSO₄, and 1 M NaOH. Then mixed well with solid NaCl, 6 N HCl and 10 ml of butyl acetate and centrifuged for 5 min at 4500 rpm.

Aqueous phase was used for the determination of 5-HT and organic phase was used for 5-HIAA determination. In the organic phase 0.1 M phosphate buffer was added, shaken and centrifuged for 5 min at 4500 rpm. Concentrated HCl was then added to the buffer phase and the fluorescence was measured by spectrophotofluorometer (Hitachi F-3010) with an excitation and emission 305 and 540 nm respectively. The pH of the aqueous phase was increased by means of solid K₂CO₃. Then 0.5 M

borate buffer (pH 10), n-butanol and NaCl were added, mixed well and centrifuged for 5-min at 4500 rpm. In 5-HT determination, the organic layer was transferred to another centrifuged tube containing n-heptane and phosphate buffer. After shaking thoroughly the mixture was then centrifuged at 4500 rpm for 5 min. A known amount buffer layer was taken, mixed with ninhydrin solution (0.1 M) and incubated at 75 °C for 30 min. The formed fluorescence was measured at room temperature in a spectrophotofluorometer (Hitachi F-3010) with an excitation and emission 385 and 490 nm respectively. Steady-state levels of 5-HT and 5-HIAA were expressed as $\mu\text{g}/\text{mg}$ protein.

2.6.2. Assay of monoamine oxidase-A (MAO-A) activity

Brain regions were homogenized in 10 volumes of 0.32 M sucrose solution (pH 7.4). The homogenate was centrifuged at 600 \times g for 10 min, and the supernatant was retained and the residue was discarded. The supernatant was centrifuged again at 8500 \times g for 20 min and the residue was retained and the supernatant was discarded. The crude mitochondrial pellet from brain tissue was then resuspended in the 0.32 M sucrose. The pure mitochondria were then obtained by sucrose density gradient centrifugation as described by Gray and Whittaker (1962). The pellet at the bottom of the density gradient, containing mitochondria, was resuspended in 0.25 M sucrose and used as mitochondrial enzyme source.

Brain regional mitochondrial MAO-A activity was measured spectrophotometrically using serotonin as substrate, following the method of Green and Haughton (1961) and Guha (1966). The standard incubation mixture consisted of 0.1 M phosphate buffer (pH 7.0), appropriate enzyme source containing 0.2 mg protein, 0.125 M semicarbazide and 0.1 M serotonin in a final volume of 2 ml and incubated at 37 °C for 30 min. The reaction was then stopped by adding 0.1 M 2,4 DNPH in 2 N HCl. Enzyme blank was prepared by adding 2,4 DNPH before substrate serotonin (0.1 M). The reaction products were then extracted with benzene. The organic (benzene) layer was then added into NaOH, mixed well and centrifuged for 5 min at 5000 rpm. The aqueous layer was warmed at 80 °C for 10 min, cooled at room temperature and the color was read at 450 nm against a reagent blank. All values were corrected against enzyme blanks and the MAO-A activity was expressed as Δ O.D./mg protein/h.

2.6.3. Measurement of accumulation rate of 5-HT and declination rate of 5-HIAA in brain regions

Following the method of Trozer et al. (1966) monoamine oxidase (MAO) inhibitor pargyline (75 mg/kg) was injected intraperitoneally (i.p.) into rats at various time intervals (30, 60 and 90 min) following erythrosine (1–200 mg/kg, p.o.) or its vehicle administration. Rats (both experimental and corresponding control) were then sharply decapitated after 2 h of erythrosine or its vehicle (control) treatment and steady-state levels of 5-HT and 5-HIAA in brain regions were determined according to the method of Scapagnini et al. (1969) as mentioned above. Accumulation rate of 5-HT and declination rate of 5-HIAA in brain regions were calculated accordingly (Trozer et al., 1966).

2.6.4. [³H]-5-HT receptor binding assay

In-vitro [³H]-5-HT binding to its receptor in different brain regional membranes was measured according to the method of Bennett and Snyder (1976) and Peroutka and Snyder (1979). Brain regions were separately homogenized in 20 vol of ice-cold 0.32 M sucrose solution and membrane was prepared according to the method of Bennett and Snyder (1976). The membrane prepared from different brain regions was suspended individually in 50 mM Tris-HCl buffer (pH 7.4) containing 10 μM pargyline and 0.1% ascorbic acid and these membrane suspensions were immediately used for [³H]-5-HT (serotonin) binding assay. The specific binding of [³H]-serotonin to the brain regional membrane was assayed according to the method of Peroutka and Snyder (1979). Briefly assay mixture containing 0.1 ml [³H]-ligand [final concentration 2.0 nM [³H]-5-HT, (Sp. activity 1 Ci/mmol)], 0.1 ml

50 mM Tris-HCl buffer (pH 7.4) or unlabelled 5-HT (10 μM) in the same buffer and 0.8 ml membrane suspension (containing 0.1 mg protein/ml) was incubated (total incubation volume 1.0 ml) in triplicate at 37 °C for 10 min and it was then rapidly filtered under vacuum using Whatman GF/B filters with two 5 ml washing using 50 mM Tris-HCl buffer (pH 7.4). Radioactivity was measured by liquid scintillation β -counter (WALLAC 1409) in 5 ml of scintillation fluid (Cocktail 'O'). Specific binding was calculated by subtracting the non-specific (in the presence of unlabelled 5-HT) binding from total (in absence of unlabelled 5-HT) binding and expressed as pmol/mg protein.

2.7. Protein estimation

Protein content of tissues was estimated spectrophotometrically following the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.8. Statistical analysis

Statistical significance between the mean values was assessed by analysis of variance (ANOVA) using Scheffe's multiple comparison F test unless otherwise mentioned. Statistical significance between mean values was considered as $p < 0.05$.

3. Results

3.1. Erythrosine-induced changes in MA

Fig. 1 depicts that erythrosine at single low dosage (1 mg/kg, p.o.) did not significantly influence the motor activity (MA) in young adult male rat. But a single higher dosage (10–200 mg erythrosine/kg, p.o.) reduced MA and showed a maximum effect following 2 h of erythrosine administration [38.53%–100%, $F(3, 34) = 355.00$, $p < 0.01$] which was then gradually restored to basal level with time. The degree of erythrosine-induced motor suppression was increased with the increase of erythrosine dosages. No significant difference was observed in motor activity following 7 h of erythrosine administration in any of the dosage group with respect to their corresponding control under above mentioned condition (Fig. 1).

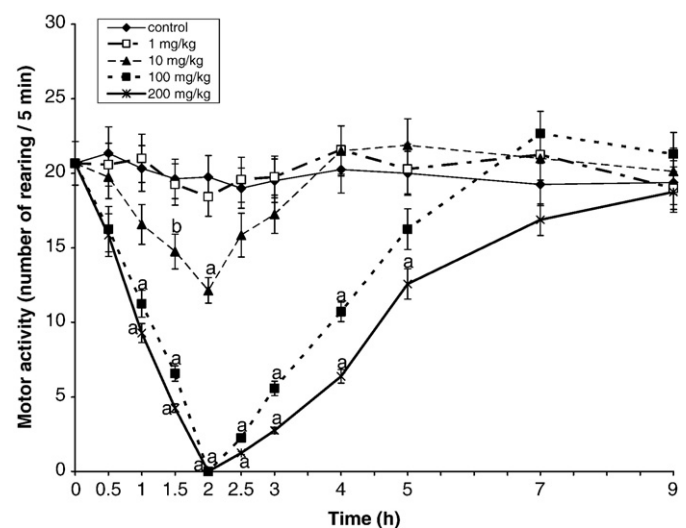


Fig. 1. Effect of single administration of erythrosine (1, 10, 100 or 200 mg/kg, p.o.) on motor activity of rats at different time intervals. Each point represents mean \pm SEM of 8–12 separate observations (each observation was made from a single rat). Vertical line represents \pm SEM. X axis indicates time (h) after administration of erythrosine. No significant change was observed between the control values corresponding to time of erythrosine exposure. Significantly different from corresponding control ^a $p < 0.01$ and ^b $p < 0.05$.

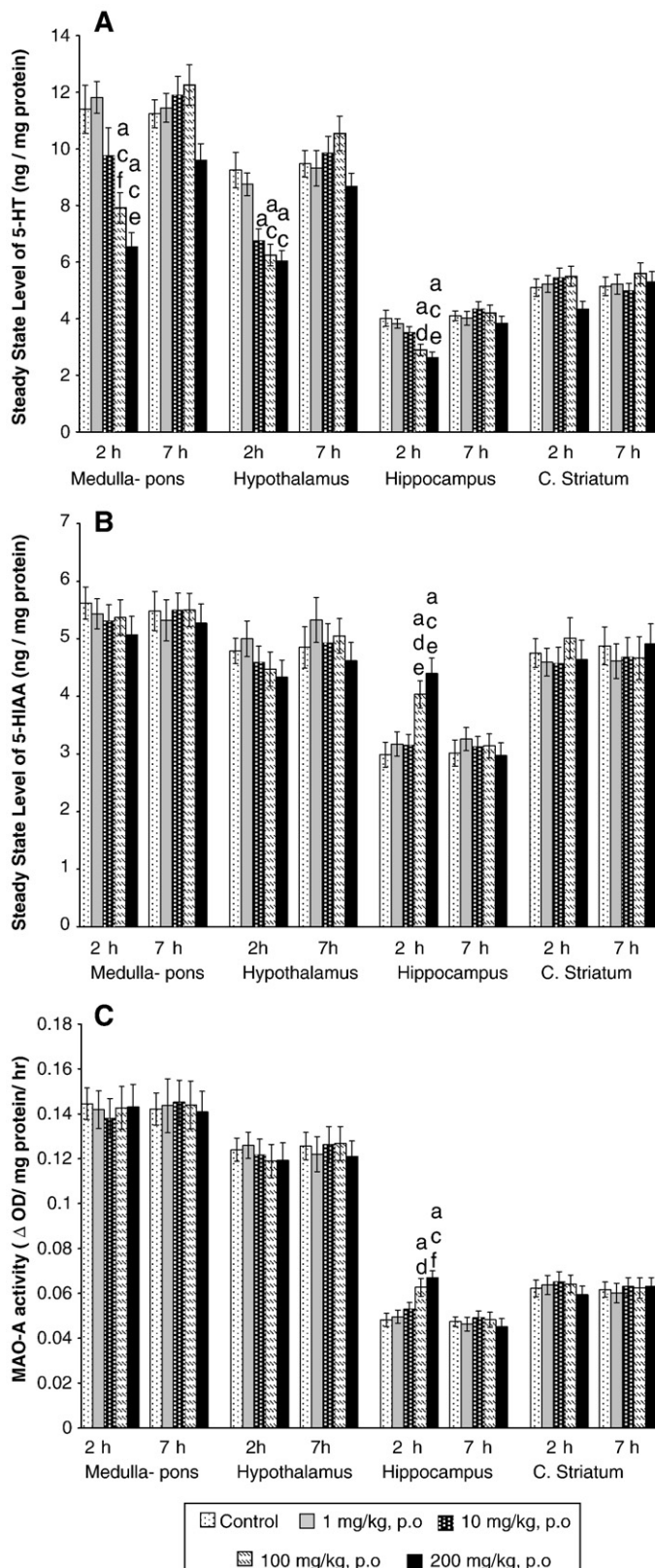


Fig. 2. Effect of single administration of erythrosine (1–200 mg/kg, p.o.) on rat brain regional steady-state level of 5-HT (A) and 5-HIAA (B) and the activity of mitochondrial MAO-A (C) following 2 h and 7 h of exposures. Results are expressed as mean ± SEM of 4–6 separate observations (each observation was made from a single rat). Significantly different from corresponding (i) control ^a $p < 0.01$; (ii) 1 mg/kg (p.o.) dosage group ^c $p < 0.01$, ^d $p < 0.05$ and (iii) 10 mg/kg (p.o.) dosage group ^e $p < 0.01$, ^f $p < 0.05$.

3.2. Erythrosine-induced changes in steady-state levels of 5-HT, 5-HIAA and MAO-A activity

Fig. 2 represents that a single low dosage of erythrosine (1 mg/kg, p.o.) did not significantly alter steady-state levels of 5-HT, 5-HIAA and mitochondrial MAO-A activity in any of the brain regions studied at any time. Single higher dosages of erythrosine (10–200 mg erythrosine/kg, p.o.), on the other hand decreased steady-state level of 5-HT in medulla-pons [14.44%–42.61%, $F(3, 18) = 7.5$, $p < 0.01$], hypothalamus [27.11%–34.69%, $F(3, 18) = 9.37$, $p < 0.01$], and hippocampus [12.67%–34.4%, $F(3, 18) = 7.32$, $p < 0.01$] without affecting corpus striatum after 2 h of erythrosine exposure with respect to their corresponding controls (Fig. 2A). No such effect was observed in steady-state level of 5-HIAA in any of the brain regions studied except in hippocampus where 5-HIAA level was increased [5.55%–47.17%, $F(3, 18) = 8.12$, $p < 0.01$] with higher dosage of erythrosine (10–200 mg/kg, p.o.) after 2 h of exposure with respect to their corresponding controls (Fig. 2B). It is evident from Fig. 2C that MAO-A activity in only hippocampus was increased [9.98%–39.29%, $F(3, 18) = 5.64$, $p < 0.01$] at 2 h of its (erythrosine) single higher dosage with respect to the corresponding control group. Fig. 2 further represents that no significant effect was observed in brain regional steady-state level of 5-HT, 5-HIAA and MAO-A activity with respect to their corresponding controls following 7 h of erythrosine treatment (1–200 mg/kg, p.o.).

3.3. Erythrosine-induced changes in 5-HT accumulation rate

Table 1 represents that the lowest experimental dosage of erythrosine (1 mg/kg, p.o.) did not significantly affect pargyline-induced 5-HT accumulation rate in any of the brain region studied; whereas, increase of erythrosine dosages (1–200 mg/kg, p.o.) decreased pargyline-induced 5-HT accumulation rate in medulla-pons [11.08%–50.05%, $F(3, 8) = 18.06$, $p < 0.01$] and hypothalamus [29.15%–48.14%, $F(3, 8) = 15.31$, $p < 0.01$] without showing any significant change in hippocampus and corpus striatum after 2 h of erythrosine administration with respect to their corresponding control groups.

3.4. Erythrosine-induced changes in 5-HIAA declination rate

Table 2 represents that a single erythrosine exposure (1–200 mg/kg, p.o.) did not significantly affect the pargyline-induced 5-HIAA

Table 1

Pargyline-induced accumulation rate of rat brain regional 5-HT following single administration of erythrosine (1–200 mg/kg, p.o.).

Dosage of erythrosine (mg/kg, p.o.)	Pargyline-induced accumulation rate of brain regional 5-HT (ng/mg protein/h)			
	Medulla-pons	Hypothalamus	Hippocampus	Corpus striatum
Control (treated with vehicle of erythrosine)	10.29 ± 0.62	9.16 ± 0.70	3.06 ± 0.16	4.04 ± 0.18
1 mg/kg	10.57 ± 0.62	8.77 ± 0.47	3.00 ± 0.19	4.27 ± 0.27
10 mg/kg	9.15 ± 0.71	6.49 ± 0.52 ^{a, d}	3.28 ± 0.22	4.51 ± 0.28
100 mg/kg	6.22 ± 0.47 ^{a, c}	5.17 ± 0.39 ^{a, c}	2.83 ± 0.17	4.48 ± 0.29
200 mg/kg	5.14 ± 0.43 ^{a, c, e}	4.75 ± 0.35 ^{a, c, f}	2.67 ± 0.18	3.35 ± 0.18

Results are expressed as mean ± SEM of 3 separate accumulation rates (each accumulation rate was calculated from 4 rats). Pargyline (75 mg/kg) dissolved in 0.2 ml of normal saline (0.9% NaCl) was administered (i.p.) at various time intervals (30, 60 and 90 min) following erythrosine (1, 10, 100 or 200 mg/kg, p.o.) or its vehicle (distilled water) administration. Rats, treated with distilled water (vehicle of erythrosine) were considered as the control of the corresponding experimental groups. All rats (experimental or corresponding control) were sacrificed following 2 h of erythrosine or its vehicle (distilled water) administration and brain regional 5-HT content was measured. Accumulation rates were calculated from graphical presentation (not shown) of both control and experimental data of 5-HT contents at various time intervals. Significantly different from corresponding (i) control ^a $p < 0.01$; (ii) 1 mg/kg (p.o.) dosage group ^c $p < 0.01$, ^d $p < 0.05$ and (iii) 10 mg/kg (p.o.) dosage group ^e $p < 0.01$, ^f $p < 0.05$.

Table 2
Pargyline-induced declination rate of rat brain regional 5-HIAA following single administration of erythrosine (1–200 mg/kg, p.o.).

Dosage of erythrosine (mg/kg, p.o.)	Pargyline-induced declination rate of brain regional 5-HIAA (ng/mg protein/h)			
	Medulla-pons	Hypothalamus	Hippocampus	Corpus striatum
Control (treated with vehicle of erythrosine)	9.02 ± 0.42	8.67 ± 0.45	3.19 ± 0.16	3.53 ± 0.18
1 mg/kg	8.79 ± 0.46	8.38 ± 0.54	3.06 ± 0.20	3.68 ± 0.21
10 mg/kg	9.25 ± 0.57	8.88 ± 0.59	3.40 ± 0.19	3.77 ± 0.24
100 mg/kg	9.28 ± 0.62	8.98 ± 0.63	2.81 ± 0.15	3.44 ± 0.17
200 mg/kg	9.81 ± 0.76	8.58 ± 0.60	2.60 ± 0.14	3.43 ± 0.23

Results are expressed as mean ± SEM of 3 separate declination rates (each declination rate was calculated from 4 rats). Pargyline (75 mg/kg) dissolved in 0.2 ml of normal saline (0.9% NaCl) was administered (i.p.) at various time intervals (30, 60 and 90 min) following erythrosine (1, 10, 100 or 200 mg/kg, p.o.) or its vehicle (distilled water) administration. Rats, treated with distilled water (vehicle of erythrosine) were considered as the control of the corresponding experimental groups. All rats (experimental or corresponding control) were sacrificed following 2 h of erythrosine or its vehicle (distilled water) administration and brain regional 5-HIAA content was measured. Declination rates were calculated from graphical presentation (not shown) of both control and experimental data of 5-HIAA contents at various time intervals. No significant difference was observed with respect to their corresponding control.

declination rate in any of the brain regions studied under the present experimental condition with respect to their corresponding controls.

3.5. Erythrosine-induced changes in [³H]-5-HT receptor binding

Table 3 represents that there was no significant change in specific binding of [³H]-5-HT to its receptors in any of the brain regions studied following 2 h of erythrosine exposure at low dosage (1 mg/kg, p.o.) with respect to their corresponding controls. Higher dosages of erythrosine decreased specific binding of [³H]-5-HT to its receptors in medulla-pons [11.43%–39.06%, $F(3, 18) = 16.78, p < 0.01$], hypothalamus [27.12%–37.15%, $F(3, 18) = 13.30, p < 0.01$] and in hippocampus [10.24%–31.95%, $F(3, 18) = 10.18, p < 0.01$] without affecting corpus striatum with respect to their corresponding controls under similar condition.

3.6. Erythrosine-induced changes in MA in the presence of MAOIs

Fig. 3 represents that separate application of clorgyline (5 mg/kg, i.p.) or deprenyl (5 mg/kg, i.p.) to control rats (treated with distilled water) produces no significant change in MA following 9 h of post treatment period. Co-application of clorgyline (5 mg/kg, i.p.) and deprenyl (5 mg/kg, i.p.) to control rats (treated with distilled water) significantly

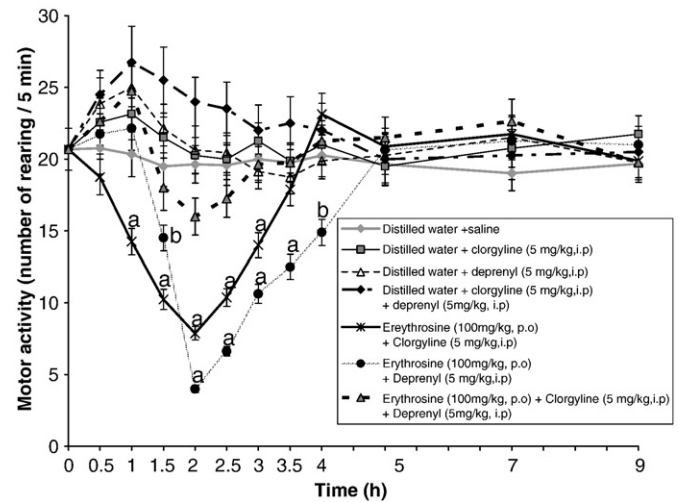


Fig. 3. Effect of clorgyline and/or deprenyl on erythrosine (100 mg/kg, p.o.)-induced inhibition of motor activity of rats (shown in Fig. 1). Results are expressed as mean ± SEM of 8–12 separate observations (each observation was made from a single rat). Clorgyline or deprenyl or clorgyline along with deprenyl in 0.2 ml saline was injected 10 min after erythrosine (100 mg/kg, p.o., in 0.5 ml distilled water) or its vehicle administration. Control rats were treated with 0.5 ml distilled water (vehicle of erythrosine) and 0.2 ml saline (vehicle of clorgyline/deprenyl). X axis indicates time (h) after administration of erythrosine. Dosages and route of administration of clorgyline and deprenyl are mentioned in the corresponding parenthesis. No significant difference between the control values of the corresponding experimental groups. Significantly different from corresponding control [vehicle of erythrosine (distilled water) + vehicle of clorgyline/deprenyl (saline)] ^a $p < 0.01$ and ^b $p < 0.05$.

increased MA following only 1 h and 1.5 h of post drug administration period (Fig. 3). Application of clorgyline (5 mg/kg, i.p.) or deprenyl (5 mg/kg, i.p.) to erythrosine (100 mg/kg, p.o.)-pretreated rats, reduced erythrosine-induced MA suppression (Fig. 2) and restored MA to the control values following 3.5 h or 5 h respectively of erythrosine exposure; whereas, co-application of clorgyline (5 mg/kg, i.p.) and deprenyl (5 mg/kg, i.p.) in erythrosine (100 mg/kg, p.o.)-pretreated rats produced no significant change in MA with respect to controls under similar experimental condition as presented in Fig. 3.

Table 4 represents that a single administration of erythrosine (10–200 mg/kg, p.o.) decreased MA [38.53%–100%, $F(3, 34) = 355.00, p < 0.01$] following 2 h of erythrosine exposure with respect to their corresponding controls. Separate administration of specific MAOI(s) (clorgyline or deprenyl, 5 mg/kg, i.p.) in erythrosine-treated rats (10–200 mg/kg, p.o.) reduced MA following 2 h of erythrosine administration in the presence of either clorgyline [6.3%–71.1%, $F(3, 34) = 129.97, p < 0.01$] or deprenyl [8.86%–85.3%, $F(3, 34) = 118.79, p < 0.01$] with respect to their corresponding controls. In contrast, co-

Table 3
Effect of single administration of erythrosine on specific binding of [³H]-5-HT (serotonin) to its receptors of rat brain regions.

Dosage of erythrosine (mg/kg, p.o.)	Hour (after erythrosine administration)	[³ H]-5-HT (serotonin) binding (pmol/mg protein)			
		Medulla-pons	Hypothalamus	Hippocampus	Corpus striatum
Control (treated with vehicle of erythrosine)	2	1.216 ± 0.050	0.966 ± 0.050	0.820 ± 0.036	0.840 ± 0.037
	7	1.207 ± 0.051	0.948 ± 0.041	0.858 ± 0.051	0.857 ± 0.039
1 mg/kg	2	1.247 ± 0.062	0.997 ± 0.041	0.836 ± 0.031	0.811 ± 0.042
	7	1.221 ± 0.049	0.968 ± 0.038	0.877 ± 0.046	0.875 ± 0.043
10 mg/kg	2	1.077 ± 0.052	0.704 ± 0.045 ^{a, c}	0.736 ± 0.051	0.844 ± 0.036
	7	1.214 ± 0.062	0.936 ± 0.040	0.871 ± 0.045	0.877 ± 0.039
100 mg/kg	2	0.801 ± 0.033 ^{a, c, e}	0.637 ± 0.035 ^{a, c}	0.584 ± 0.029 ^{a, c, e}	0.886 ± 0.038
	7	1.195 ± 0.062	0.935 ± 0.039	0.839 ± 0.038	0.845 ± 0.039
200 mg/kg	2	0.741 ± 0.032 ^{a, c, e}	0.607 ± 0.035 ^{a, c}	0.558 ± 0.026 ^{a, c, e}	0.775 ± 0.030
	7	1.181 ± 0.062	0.930 ± 0.036	0.832 ± 0.042	0.832 ± 0.039

Results are expressed as mean ± SEM of 4–6 separate observations (each observation was made from a single rat).

All rats (experimental or control) were sacrificed following 2 h and 7 h of erythrosine or its vehicle (distilled water) administration. Significantly different from corresponding (i) control ^a $p < 0.01$, (ii) 1 mg/kg (p.o.) dosage group ^c $p < 0.01$ and (iii) 10 mg/kg (p.o.) dosage group ^e $p < 0.01$.

Table 4

Effect of different monoamine oxidase (MAO-A and/or -B) inhibitors on motor activity (MA) of single dosage of erythrosine-treated rats.

Condition of treatment	Motor activity (%)				
	Control		Erythrosine (mg/kg, p.o.)		
	Vehicle of erythrosine (distilled water, p.o.)	Vehicle of clorgyline and/or deprenyl (saline, i.p.)	10	100	200
Control	100.0 ± 4.88	–	61.5 ± 4.36 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a
Control (vehicle of clorgyline/deprenyl)	101.3 ± 6.14	100.6 ± 6.18	61.3 ± 4.25 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a
Clorgyline (5 mg/kg, i.p.)	107.6 ± 6.98	107.0 ± 6.85	93.7 ± 3.84 ^x	40.0 ± 2.05 ^{a, x}	28.9 ± 1.54 ^{a, x}
Deprenyl (5 mg/kg, i.p.)	104.5 ± 5.51	105.1 ± 8.64	91.1 ± 6.58 ^x	20.3 ± 1.32 ^{a, x}	14.7 ± 1.04 ^{a, x}
Clorgyline + deprenyl (5 + 5 mg/kg, i.p.)	122.8 ± 8.92	121.6 ± 8.81	108.9 ± 6.68 ^x	81.0 ± 6.94 ^x	74.7 ± 6.73 ^x

Results are expressed as mean ± SEM of 8–12 separate observations (each observation was made from a single rat). MA (19.75 ± 1.23) of control rats (treated with vehicle of erythrosine, distilled water) was considered as 100.0 ± 6.23 and the results were calculated accordingly. Clorgyline or deprenyl or clorgyline along with deprenyl (dissolved in 0.2 ml saline) or their corresponding vehicle (0.2 ml saline) was injected 10 min after single oral administration (0.5 ml) of erythrosine or its vehicle. MA was measured 2 h following erythrosine or its vehicle administration. Dosages and rout of administration of clorgyline and deprenyl are mentioned in the corresponding parenthesis.

Significantly different from corresponding (i) control [vehicle of erythrosine (distilled water)] ^a*p* < 0.01; (ii) erythrosine-treated group ^x*p* < 0.01.

application of clorgyline (5 mg/kg, i.p.) and deprenyl (5 mg/kg, i.p.) in erythrosine pretreated rats (10–200 mg/kg, p.o.) under similar condition did not significantly change MA with respect to their corresponding controls (Table 4).

4. Discussion

Considerable number of previous studies suggests a link between the repeated or long-term ingestion of artificial food colors and onset of behavioral alteration including behavioral hyperactivity (McCann et al., 2007; Schab and Trinh, 2004; Stein et al., 2007; Tanaka, 2001; Tanaka, 2006; Weiss, 1986). Erythrosine-induced behavioral hyperactivity was observed previously in juvenile as well as in adult mice following long-term administration of erythrosine (Tanaka, 2001). The present study (Fig. 1) provides evidence that a single oral exposure of artificial food color erythrosine though at its very low experimental dosage (1 mg/kg, p.o.) fails to produce any significant effect on MA in young adult male rats, at higher dosages (10–200 mg/kg, p.o.) inhibits vertical motor activity in a time-dependent manner (Fig. 1). In the present study MA has been measured by counting vertical rearing number (Keenan and Johnson, 1972) which is more related to vertical exploration in addition to locomotion (Kelley, 1993). Further, it is believed that exploratory behavior may be linked to an internal drive state. Certain behavioral phenomenon such as spontaneous alteration suggests that exploratory behavior is not necessarily motivated by obvious biological needs (Kelley, 1993). Exploration has been viewed in the context of acquisition of information. This cognitive framework considers the interaction of the organism with the environment and the process associated with information gathering (Kelley, 1993). Neurobehavioral and neurochemical studies (Branchereau et al., 2002; Cazalets et al., 2000; Pflieger et al., 2002) have also suggested that brain 5-HT modulates the activity of neural circuits and thereby plays a central role in several behavioral/motor activity (Alex and Pehek, 2007; Bankson and Cunningham, 2001) specifically in exploratory behavior (Grailhe et al., 1999). Single higher dosage of erythrosine (≥ 10 mg/kg) shows maximum reduction in rearing activity at 2 h and restores to normal value after 5 or 7 h depending on the dosage and remains normal thereafter (Fig. 1). Hence the present study deals with the effect of single intake of erythrosine (1–200 mg/kg, p.o.) on brain regional serotonergic activity following 2 h (maximum reduction of MA) and 7 h (maximum time require to reach normal value of MA) of its administration.

Substantial evidence have shown that functional output of 5-HT neurons in different brain regions varies depending on their origin from different groups of raphe nuclei (Jacobs et al., 2002). It is also known that the rostral portion of the midbrain raphe related to the basal-ganglia motor system and the caudal area more related to the limbic system make the variation in functional output of 5-HT neurons in brain regions (Waterhouse et al., 1986). Therefore, in the present investigation the experiments were designed to evaluate the

effect of erythrosine (1–200 mg/kg, p.o.) on serotonergic system in relation to different brain regions (medulla-pons, hypothalamus, hippocampus and corpus striatum). The ineffectiveness of lower dosage (1 mg/kg, p.o.) of erythrosine at 2 h or 7 h of its exposure in steady-state level of 5-HT, 5-HIAA, MAO-A activity (Fig. 2), synthesis rate of 5-HT (Table 1) and also specific binding [³H]-5-HT to its receptors (Table 3) in any of the brain regions studied suggests that at this experimental dosage the brain regional 5-HT system is not affected. Unlike lower dosage, higher dosage of erythrosine (10 mg/kg, p.o.) significantly reduces only hypothalamic steady-state level of 5-HT following 2 h of erythrosine exposure (Fig. 2) either by decreasing the rate of 5-HT synthesis and/or by increasing its catabolism over the synthesis rate. This thought may further be strengthened by no change in hypothalamic steady-state level of 5-HIAA (Fig. 2B), its MAO-A activity (Fig. 2C), a significant increase in the ratio of steady-state level of 5-HIAA over 5-HT (Table 5) and a significant decrease in pargyline-induced 5-HT accumulation rate (Table 1) without affecting pargyline-induced 5-HIAA declination rate (Table 2) under the present experimental condition. Higher dosage of erythrosine (100 or 200 mg/kg, p.o.) not only affects the hypothalamic serotonergic system but also the medulla-pons region in a similar manner as observed earlier in the hypothalamus. In hippocampus, on the other hand, erythrosine at this dosage (100 or 200 mg/kg, p.o.) enhances the breakdown of 5-HT to 5-HIAA (Fig. 2) instead of lowering the 5-HT synthesis (Table 1). This may be considered as a cause of low hippocampal 5-HT content with an increase of 5-HIAA (Fig. 2). The enhanced degradation of 5-HT in hippocampus may be supported by the increase of hippocampal MAO-A activity (Fig. 2C) and no significant change in pargyline-induced 5-HT accumulation rate (Table 1) in this brain region under the same experimental condition. The inhibition of hypothalamic

Table 5

Rat brain regional 5-HIAA/5-HT ratio following 2 h of single oral administration of erythrosine.

Dose of erythrosine (mg/kg, p.o.)	5-HIAA/5-HT ratio			
	Medulla-pons	Hypothalamus	Hippocampus	Corpus striatum
Control (treated with vehicle of erythrosine)	0.493 ± 0.032	0.518 ± 0.028	0.744 ± 0.042	0.931 ± 0.064
1 mg/kg	0.460 ± 0.030	0.572 ± 0.031	0.829 ± 0.045	0.879 ± 0.051
10 mg/kg	0.544 ± 0.037	0.681 ± 0.036 ^a	0.899 ± 0.046	0.840 ± 0.045
100 mg/kg	0.679 ± 0.045 ^{a, c}	0.716 ± 0.034 ^{a, d}	1.392 ± 0.057 ^{a, c, e}	0.912 ± 0.050
200 mg/kg	0.775 ± 0.061 ^{a, c}	0.718 ± 0.032 ^{a, d}	1.670 ± 0.068 ^{a, c, e}	1.068 ± 0.055

Results are expressed as mean ± SEM of 4–6 separate observations (each observation was made from a single rat). The ratios 5-HIAA/5-HT in brain regions were calculated from the respective steady-state values as mentioned in Fig. 2.

Significantly different from corresponding control ^a*p* < 0.01; (ii) 1 mg/kg (p.o.) dosage group ^c*p* < 0.01, ^d*p* < 0.05 and (iii) 10 mg/kg (p.o.) dosage group ^e*p* < 0.01.

serotonin receptor binding at 10 mg/kg, (p.o.) and inhibition of hypothalamic, medulla-pons and hippocampal serotonin receptor binding with more higher dosage (100 or 200 mg/kg, p.o.) of erythrosine (Table 3) suggest that higher dosage of erythrosine indiscriminately inhibits the serotonin receptor binding in the brain regions studied. The inhibition of 5-HT receptor binding is not unlikely, because erythrosine at the above mentioned dosages reduces 5-HT content in those brain regions (Fig. 2A) and thereby may reduce the availability of 5-HT at the synaptic cleft. The erythrosine-induced lowering of 5-HT content in the brain regions may be further explained by the fact that erythrosine enhances the catabolic rate of 5-HT in comparison to that of its anabolism irrespective of the brain regions studied except corpus striatum (Fig. 2, Tables 1–3). The higher dosages (10–200 mg/kg, p.o.) of erythrosine-induced significant reduction in 5-HT content without affecting their MAO-A activity, excepting a significant increase in hippocampus (Fig. 2A and C) further force to assume that erythrosine at higher dosages unlike its very low dosage, may produce less release of 5-HT at terminals and/or enhance the glial/non-serotonergic neuronal uptake of 5-HT (Fitzgerald et al., 1990; Levitt et al., 1982) and thereby may reduce the availability of 5-HT in the synaptic cleft to interact with its (5-HT) post synaptic receptors. The possibility of increase in glial and/or non-serotonergic compartmental uptake of 5-HT from its (5-HT) synaptic cleft, may be supported by enhancement of the 5-HIAA content particularly in hippocampus where MAO-A (mobilizes 5-HT to 5-HIAA) is activated in erythrosine-treated rats under the present experimental condition (Fig. 2).

Because MAO is involved in the degradation of physiologically active monoamines, it can be hypothesized that reduction in MAO activity using MAO inhibitors (MAOIs) in erythrosine pretreated rats may oppose the suppression of motor (rearing) activity by increasing monoamine(s) content in brain regions. Therefore, in the next phase of the study the consequences of specific MAOIs (clorgyline and/or deprenyl) on the motor (rearing) activity in erythrosine pretreated rats were analyzed. In brain, specific MAO and its preferred substrates are not found in the same neuron. MAO-A is predominantly found in catecholaminergic neuron, MAO-B is mostly abundant in serotonergic and histaminergic neurons (Jahng et al., 1997) but, both MAO-A and MAO-B are found abundant in glial cells (Fitzgerald et al., 1990; Levitt et al., 1982). On the basis of substrate selectivity and inhibitor sensitivity, MAO-A has higher affinity for substrate serotonin and inhibited by clorgyline; whereas, MAO-B has higher affinity for phenylethylamine (PEA), benzylamine and inhibited by deprenyl (Fowler and Tipton, 1982; Yang and Neff, 1973). It is also well known that concentration of 5-HT in extra-cellular compartment (synaptic cleft) may depend on the type of MAO present and their activities (Celada and Artigas, 1993). According to previous studies, application of clorgyline to experimental animals inhibits MAO-A activity towards its substrates serotonin and dopamine (Celada and Artigas, 1993; Green and Youdim, 1975), increases brain tissue content of 5-HT without the change of 5-HT output (release) to synaptic cleft (Celada and Artigas, 1993), inhibits the reuptake (Tekes and Magyar, 2000) and increases efflux (Butcher et al., 1990) of dopamine. In the present study it has been observed that application of clorgyline (5 mg/kg, i.p.) to erythrosine (10–200 mg/kg, p.o.)-treated rats significantly prevents the degree of erythrosine-induced (100 or 200 mg/kg, p.o., 2 h exposure) MA suppression (Table 4) and reduces the recovery/restoration time (from 7 h to 3.5 h) of erythrosine-induced (100 mg/kg, p.o.) MA suppression (Fig. 3). Therefore, the present study suggests that clorgyline application may reduce the intensity and duration of erythrosine (at higher dosages)-induced suppression of MA but not effective enough to restore MA to their basal (control) level by modulating serotonergic and/or dopaminergic systems. Application of deprenyl (specific MAO-B inhibitor) inhibits the degradation of substrate phenylethylamine (PEA) and benzylamine but not brain serotonin (Fowler and Tipton, 1982; Yang and Neff, 1973) and produces no substantial change in 5-HT content in synaptic cleft (Celada and

Artigas, 1993). Further the increase in PEA concentration in brain has been found to stimulate quick release of dopamine at post synaptic nerve terminal (Barroso and Rodriguez, 1996; Nakamura et al., 1998). These suggestions may be consistent with the fact that application of MAO-B inhibitor, deprenyl (5 mg/kg, i.p.) to the present erythrosine-treated rats does not effectively prevent suppression of MA following 2 h of erythrosine administration nor affect the time required for restoration of erythrosine-induced MA suppression to basal (control) level (Table 4, Fig. 3); rather deprenyl under this condition helps to maintain MA at basal level at the initial phase (less than 1.5 h) of post erythrosine treatment period (Fig. 3). Previous studies (Timar et al., 1993; Youdim and Finberg, 1994) have shown that deprenyl enhances the function of the dopaminergic system by a complex mechanism that may include inhibition of dopamine (DA) reuptake and increase of DA turnover (Timar et al., 1993; Youdim and Finberg, 1994); but deprenyl-induced elevation in dopamine concentration occurs more rapidly and returns to baseline within 2 h of deprenyl administration (Okuda et al., 1992). All these information may explain why application of deprenyl (5 mg/kg, i. p.) in erythrosine (100 mg/kg, p.o.) treated rats maintains the initial phase of MA at baseline/control value (Fig. 3) and it may further suggest that erythrosine-induced inhibition of serotonergic activity may be a cause of its (erythrosine) MA suppression. Co-application of both forms of MAO inhibitors, clorgyline and deprenyl increases 5-HT level in synaptic cleft by stimulating its release from serotonergic neurons and suggests that the blocking of both forms of MAO is necessary to achieve an increase in 5-HT output (Celada and Artigas, 1993). In the present experiment, suppression of MA in erythrosine (10, 100 and 200 mg/kg, p.o.) treated rats may be an effect of erythrosine-induced brain regional serotonergic inhibition (at higher dosage) that could further be strengthened more positively by co-application of clorgyline (5 mg/kg, i.p.) and deprenyl (5 mg/kg, i.p.) following erythrosine treatment (10, 100 or 200 mg/kg, p.o.). As co-application of clorgyline and deprenyl may cause a large increase in 5-HT availability in synaptic cleft (Celada and Artigas, 1993; Sleight et al., 1988) of serotonergic neurons, it may compensate the erythrosine-induced reduction of serotonergic activity and hence may maintain the MA at the baseline under the present experimental condition (Table 4, Fig. 3).

In the present study the effective lowest single oral dosage of erythrosine (10 mg/kg) that decreases exploratory behavior (vertical rearing activity) as well as brain regional serotonergic activity in young adult male rats is apparently greater than the present acceptable daily intake (ADI) of erythrosine (≤ 0.1 mg/kg b. wt) in humans (JECFA, 1991; Larsen, 2006). It would therefore, appear that the level of actual dietary intake (within ADI) of erythrosine is unlikely to produce any adverse effect on human health. Although, considering a number of previous reports it may be stated that humans are more sensitive to erythrosine-induced toxicity rather than experimental animals (Larsen, 2006; Gardner et al., 1987). Therefore human trial data are required to clarify the physiological effects of the present minimal effective dosage (10 mg/kg) of erythrosine at the level of human health and nutrition. In addition, the present observation also indicates that exposure to excess artificial food additive (over ADI) may cause a risk to human health especially in a country like India, where all kinds of artificial food colors are very popularly used throughout the year (Rao et al., 2005).

Finally, it may be concluded that single erythrosine exposure-induced MA suppression unlike repeated erythrosine exposure-induced behavioral hyperactivity (Tanaka, 2001), may be the reflection of decrease in brain regional serotonergic activity under the present experimental condition. It is also known that reduction in serotonergic activity may not be the only factor responsible for suppressing MA because complete inhibition of both MAO-A and MAO-B activities markedly inhibits the erythrosine-induced MA suppression and thereby may indicate the involvement of brain's other monoamines along with serotonin. Further, a growing body of evidences including the report from our laboratory (Jamaluddin and Poddar, 2003) suggests a complex interaction among other neurotransmitter systems in locomotor

regulation (Howell and Kimmel, 2008). Brain glutamatergic system provides an important regulation of dopamine function and inhibitory neurotransmitter, gamma-amino butyric acid (GABA) system can modulate basal dopamine and glutamate release (Howell and Kimmel, 2008; Jamaluddin and Poddar, 2003). As repeated application of a drug may lead to a robust and enduring changes in neurobiological substrates (including monoamines) along with the corresponding changes in sensitivity to acute drug effects on neurochemistry and behavior, further studies on the involvement of other neurotransmitters (including serotonin and dopamine) as well as their receptor activities in relation to a single (the present experimental condition) or long-term erythrosine consumption on motor behavior are now in progress.

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