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ORIGINAL INVESTIGATION

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Cerebral tryptophan hydroxylase activity, and 5-HT_{1A} receptor, 5-HT_{2A} receptor, and 5-HT transporter binding in grouped and isolated Roman RHA and RLA rats: relationships with behaviours in two models of anxiety

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Abstract Male Roman low- (RLA) and high-avoidance (RHA) rats differ when tested in the elevated plus-maze and the black/white box, but not when (isolated and) tested for their social interaction. Herein, we have analysed the impact of prior isolation on male Roman rats tested in the first two models of anxiety; moreover, because central serotonin (5-HT) systems in Roman rats have been scarcely studied, we have also analysed several anxiety-related indices of central serotonergic activity in grouped/isolated Roman rats. Group-housed RLA rats tested in the elevated plus-maze and the black/white box were less anxious than their RHA counterparts, thereby confirming our previous study. Isolation had anxiogenic (and hypolocomotor) effects, these being significant in RLA rats only. Tryptophan hydroxylase activity in midbrain (but not in cortex, hippocampus or hypothalamus) was lower in group-housed (but not in isolated) RLA rats than in RHA rats, a difference independent from changes in the regulatory properties of the enzyme. Neither midbrain and hippocampal [^{3}H]8-hydroxy-2-(di-*n*-propylamino)-tetrlin binding at 5-HT_{1A} receptors, nor midbrain [^{3}H] citalopram binding at the 5-HT transporter was different between grouped/isolated RHA/RLA rats. Alternatively, a trend toward a lower hypothalamic [^{3}H]citalopram binding in (group-housed) RLA rats than in RHA rats could be noted, whereas cortical [^{3}H]ketanserin binding at 5-HT_{2A} receptors was lower in RLA rats than in RHA rats, a difference prevented by prior isolation. This study opens the possibility that inter-line differences in 5-HT_{2A} receptors partly (or totally) underlie the respective behaviours of RHA and RLA rats in the elevated plus-maze and the black/white box.

Key words Roman (RHA/RLA) rats · Anxiety · Elevated plus-maze · Black/white box · Isolation · Tryptophan hydroxylase · 5-HT_{1A} receptors · 5-HT_{2A} receptors · 5-HT reuptake

Introduction

The Wistar-derived Roman rat lines have been selected and bred on the basis of their acquisition of a two-way active avoidance response when tested in a shuttle box, i.e. Roman high-avoidance (RHA) rats quickly acquire this response whereas Roman low-avoidance (RLA) rats fail to acquire this response (for reviews: Driscoll and Bättig 1982; Castanon and Mormède 1994). Numerous studies have shown that these line-dependent differences in two-way active avoidance behaviour are associated with differences in emotionality (Driscoll and Bättig 1982; Castanon and Mormède 1994). For instance, RHA rats placed in an open field have often been found to display increased locomotion, increased rearing, and decreased grooming and defecation, compared with RLA rats (Gentsch et al. 1981, 1982; Willig et al. 1991; Castanon et al. 1992). Compared with RHA rats, RLA rats have also been shown to be more neophobic in several tests, including hyponeophagia (Shephard and Broadhurst 1983), head-dipping (Fernandez-Teruel et al. 1992), and tunnel maze (Fernandez-Teruel et al. 1993) tests, and to display more immobility following administration of shocks in a conditioned fear test (Roozendaal et al. 1992). Beside, analyses of the reactivities of the hypothalamo-pituitary-adrenal (HPA) axis, the secretion of prolactin, and the cardiovascular system to several stressors (including exposure to an open field) have strengthened the hypothesis that RLA rats are more emotive than RHA

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rats (Gentsch et al. 1981, 1982; Driscoll and Bättig 1982; Castanon and Mormède 1994).

In a recent study aimed at analysing the behaviours of RHA and RLA rats exposed to three animal models of anxiety (namely the elevated plus-maze, the black/white box, and the social interaction test; for a review, see Lister 1990), we observed that RHA and RLA rats interacted to similar extents in the social interaction tests, whereas RLA rats, to our surprise, displayed less anxiety-related behaviours in the elevated plus-maze and the black/white box (Chaouloff et al. 1994). That Roman lines differed in the elevated plus-maze and the black/white box tests, but not in the social interaction test reinforces the finding that these tests do not recognise similar components of anxiety (File 1991); however, environmental differences which are related to the respective methodologies may also account for this discrepancy. Thus, rats are group-housed before the elevated plus-maze and the black/white box tests, whereas the rats need to be isolated for 1 week before social interaction tests (File 1980). In fact, isolating rats (but not mice: Rodgers and Cole 1993) during (post-weaning) rearing or in adulthood has proved anxiogenic in the elevated plus-maze (Jankowska et al. 1991; Wright et al. 1991b; Motta et al 1992; Maisonnette et al. 1993) and the black/white box (Ahmed et al. 1995). These findings thus open the possibility that isolation had differential anxiogenic effects in RHA and RLA rats, thereby preventing the behavioural differences initially observed between (group-housed) Roman rats.

Another key question arising from our previous study with Roman lines concerns the neurochemical bases for the aforementioned behavioural differences on exposure to the elevated plus-maze and black/white box. Indeed, numerous anxiety-related systems have been shown to differ between the Roman lines. For instance, GABA receptor-effector coupling, but not GABA and benzodiazepine binding sites, was found to be decreased in the cortex of RLA rats, compared with RHA rats (Shephard et al. 1982; Giorgi et al. 1994). Alternatively, RLA rats were found to be more sensitive than RHA rats to the positive effects of diazepam in neophagia tests (Shephard and Broadhurst 1983). In addition, RLA rats display region-selective decreases in dopamine D_1 binding sites in the nucleus accumbens (Giorgi et al. 1994), but also reduced amphetamine-induced stereotypies (Driscoll et al. 1986), compared with RHA rats. On the other hand, noradrenaline metabolism/turnover was found to be similar in numerous brain regions of (unstressed) RHA and RLA rats (Coyle et al. 1974; Driscoll et al. 1983). Regarding central serotonergic systems, the involvement of which has been repeatedly underlined in anxiety processes (for reviews: Barrett and Vanover 1993; Handley and McBlane 1993a), previous studies have reported a reduction in serotonin (5-HT) turnover in the cortex, the hypothalamus and the midbrain (but not the hippocampus) of RLA rats, compared with RHA rats (Driscoll et al. 1980; Driscoll 1988). However, whether these inter-line differences find their origins in a differential regulation of tryptophan hydroxylase, the rate limiting enzyme in 5-HT biosynthesis (see Hamon et al. 1981; Boadle-Biber 1982) is presently unknown. The present study has thus examined this hypothesis through the analysis of tryptophan hydroxylase activity in different brain regions of group-housed and isolated Roman rats.

Binding assays of imipramine (a non-selective 5-HT reuptake blocker: Marcusson et al. 1986) have initially suggested that 5-HT reuptake sites are diminished in the hypothalamus, striatum, and cortex of RLA rats, compared with RHA rats (Gentsch et al. 1983). However, the hypothesis that Roman lines differ in other key serotonergic modulators of anxiety (Barrett and Vanover 1993; Handley and McBlane 1993a), such as the presynaptic 5-HT_{1A} (auto)receptors (which are located in midbrain raphe nuclei), the postsynaptic 5-HT_{1A} receptors (especially in the hippocampus), and the (postsynaptic) 5-HT_{2A}, 5-HT_{2C}, and 5-HT_3 receptors, has never been tested so far. The present study has thus analysed, in the (group-housed and isolated) RHA and RLA rats tested in the elevated plus-maze and the black/white box, several biochemical indices of central 5-HT transmission. Hence, we measured presynaptic 5-HT_{1A} receptor binding in the midbrain, postsynaptic 5-HT_{1A} receptor binding in the hippocampus, and 5-HT_{2A} receptor binding in the frontal cortex (a brain region providing a reliable index of central 5-HT_{2A} receptor regulation: Leysen 1992). Moreover, in keeping with the aforementioned imipramine-related data, the binding of the selective 5-HT reuptake inhibitor citalopram (see Hyttel 1994 for a review) was measured in the midbrain and the hypothalamus (D'Amato et al. 1987). Note, however, that whilst this manuscript was in preparation, Charnay et al. (1995) reported a reduction in the selective (see Johnston 1991) binding of [^{3}H]paroxetine to hippocampal and cortical 5-HT reuptake sites in RLA rats, compared with RHA rats.

Materials and methods

Animals and housing conditions

The animals used in the present study have been bred in our laboratory since 1991 from rats of RHA/Verh and RLA/Verh lines kindly provided by Dr. P. Driscoll (Swiss Federal Institute of Technology, Zürich, Switzerland). Animals were weaned at 3 weeks and housed by litter and sex in collective cages that were placed in a room with constant temperature (22 ± 1°C) and a 12 h:12 h cycle of illumination (lights on: 0600 hours). When 11-week old, male rats were placed by four per cage with each rat originating from a litter different from that of the other rats. At the onset of the experiments, the male rats used in this study were 15 weeks old. At that time, RHA and RLA rats were either kept in four in their collective cages (19.7 × 25.5 × 40 cm) or isolated in individual cages

(18.4 × 18 × 30.5 cm) with food and water ad libitum. All grouped and isolated (singly-housed) animals were kept in the same animal room. For each Roman line, 12 group-housed rats (three collective cages) and ten isolated rats were used. Rats were handled only twice before the onset of the first series of experiments (elevated plus-maze). All experiments (behavioural tests, death) were conducted between 1400 and 1700 hours. Note that the RHA and RLA lines from our laboratory, but not the animals used herein (to avoid the interfering influence of shocks with anxiety-related behaviours: see Zhukov and Vinogradova 1994), have been repeatedly checked in the past for their differences in shuttle-box behaviours (see Castanon and Mormède 1994). This study was conducted in conformity with the French publication on animal experimentation (N° 87–848).

Elevated plus-maze

Apparatus

The elevated plus-maze was made of Perspex, with two opposite open arms (45 × 10 cm), and two opposite closed arms of the same size with walls 50-cm high. The arms were connected by a central square (10 × 10 cm). In addition, because the floor surface of the maze was smooth, rubber ridges bordering the open arms (0.5 cm) were added to provide additional grip for the animals. The whole apparatus was elevated 66 cm above a white floor and exposed to dim illumination (70 lux).

Behavioural procedure

The procedure was similar to that previously described (Chaouloff et al. 1994); thus, group-housed and (6–7 days) isolated RHA and RLA rats were randomly placed in the central square of the elevated plus-maze, facing an enclosed arm. The number of entries onto and time spent on each arm was scored for 5 min. An arm entry was defined as all four feet in the arm; the distance of observation was 150 cm from the maze. Rats were then returned to their home (collective or individual) cages.

Black/white box

Apparatus

The black/white box was made of Perspex, and divided into two compartments, one (27 × 27 × 27 cm high) painted white and illuminated by a 60 W white incandescent bulb (900 lux), and the other (27 × 18 × 27 cm high) painted black and lit by a 60 W red incandescent bulb (70 lux). Both white and red bulbs were located 37 cm above the respective floors. The two compartments were connected by a small opening (7 × 7 cm), and the white and black floors were divided into nine and six squares (9 × 9 cm), respectively.

Behavioural procedure

The procedure was similar to the one previously described (Chaouloff et al. 1994). Thus, 3 days after being tested in the elevated plus-maze, each grouped/isolated RHA/RLA rat was placed in the center of the white compartment facing the small opening. Behaviours were video-recorded for 5 min and included latency to enter the black compartment and the number of shuttles from the black compartment to the white compartment. In addition, because behaviours in the white compartment could not be video-recorded (due to light reflection), the number of squares crossed and rears displayed in the white compartment, and the time spent in the white compartment were recorded by an observer located 1 m above the white compartment. Rats were then returned to their home (collective or individual) cages.

Neurochemical assays

Two or 3 days after the completion of the black/white box tests (i.e. 12 days after the onset of isolation), each group-housed/isolated RHA/RLA rat was killed (decapitation), and the hypothalamus, the frontal cortex, the hippocampus, and the midbrain rapidly dissected out on an ice cold plate. All cerebral structures were immediately kept in dry ice, and 1–3 h later, stored at −80°C. Hippocampi from two or three animals ($n = 4$ pools/housing condition/Roman line), cortices from two animals ($n = 5$ pools/housing condition/Roman line), hypothalami from two animals ($n = 5$ pools/housing condition/Roman line), and individual midbrains ($n = 6$ tissues/housing condition/Roman line) were then homogenised in ice-cold TRIS-acetate buffer (pH 7.6) containing 2 mM dithiothreitol, and then centrifuged (12 000 g for 30 min at 4°C). The supernatants were immediately stored at − 80°C for subsequent analysis of tryptophan hydroxylase activity, as previously described (Kulikov et al. 1992; Chaouloff et al., in press. Cortical, hippocampal, and part of midbrain pellets (to be used for 5-HT_{1A} and 5-HT_{2A} receptor binding assays, which were performed as previously described: Popova et al. 1991; Chaouloff et al., in press were suspended in 40 vol cold 50 mM TRIS-HCl (pH 7.7) whereas hypothalamic (and the remaining part of) midbrain pellets (to be used for 5-HT transporter binding assays according to D'Amato et al. 1987) were suspended in 40 vol cold 50 mM TRIS-HCl (pH 7.4). Following an incubation at 35°C for 15 min (hippocampal and midbrain 5-HT_{1A} receptor binding), the suspensions were then spun at 20 000 *g* for 15 min. All resulting pellets were resuspended in 40 vol of the respective buffers, and centrifuged (15 000 *g* for 15 min). The final pellets were then stored at −80°C until binding analyses. Protein concentrations were estimated using bovine serum albumin as a standard (Bradford 1976).

Tryptophan hydroxylase activity

These assays were carried out at 35 °C in 25 µl of a 50 mM TRIS-acetate buffer (pH 7.6) containing 1 mM dithiothreitol, 50 U catalase, 1 mM m-hydroxybenzyl-hydrazine, 0.025–0.8 mM L-tryptophan, 0.3 mM of its cofactor 6-methyl-5,6,7,8-tetrahydropterin (6-MPH_4) (all compounds from Sigma, Paris, France), and 15 µl of the supernatant (0.08–0.12 mg protein). The reaction was stopped after 15 min by adding 10 µl of a 50% solution of trichloracetic acid, and the tubes centrifuged at 10 000 rpm for 20 min. The resulting supernatant was diluted in 1% trichloracetic acid (1/10), and the 6-MPH_4-protected solution kept at −20°C until analysis (2–3 days later) of 5-HTP (the reaction product of tryptophan hydroxylase activity).

Phosphorylation and dephosphorylation assays (which were performed according to Kulikov et al. 1992, modified from Boadle-Biber et al. 1989) were carried out by incubating for 3 min at 35°C the initial supernatants either with 0.5 mM ATP, 5 mM $MgCl_2$, and 0.2 mM $CaCl_2$ (phosphorylation) or with 0.2 U alkaline phosphatase (Type IIIS, *E. coli*) (dephosphorylation) prior to the addition of *L*-tryptophan (0.4 mM) and dithiothreitol, catalase, *m*-hydroxybenzyl-hydrazine, 6-methyl-5,6,7,8-tetrahydropterin (respective concentrations as above). The following steps were then performed as described above.

Tissue 5-HTP levels were analysed by means of HPLC (Kromasil C8 column; flow rate: 0.8 ml/min) coupled with electrochemical detection (700 mV) using a sodium phosphate buffer (50 mM, pH 3.25) containing sodium dodecyl sulfate (100 mg/l), EDTA (148 mg/ml) and methanol (10%). 5-HTP standard solutions

(200 pmol) were repeatedly assayed throughout the entire procedure. Values of apparent K_M and V_{max} were provided by means of Lineweaver-Burk plot analyses.

5-HT$_{1A}$ and 5-HT$_{2A}$ receptor binding

The binding assays were performed as previously described (Popova et al. 1991; Chaouloff et al., in press, excepted that methysergide was used instead of ritanserin for the estimation of non specific binding of [^{3}H]ketanserin. The pellets were suspended in 40 vol of a 50 mM TRIS-HCl buffer (pH 7.7) either containing (5-HT$_{1A}$ receptor binding) or not (5-HT$_{2A}$ receptor binding) 5 mM $CaCl_2$ and 0.1% ascorbic acid. The suspension (0.1–0.3 mg protein) was transferred to glass tubes (total volume: 0.5 ml) and the reaction carried out for 15 min at 35°C in the presence of the respective tritiated and cold ligands. For 5-HT$_{1A}$ receptor binding assays, six concentrations ranging from 0.25 to 2 nM [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, 147 Ci/mmol; Dupont/NEN, Les Ulis, France), and 1 µM bufotenin (Sigma, Paris, France) were used, whereas six concentrations ranging from 0.25 to 4 nM [^{3}H]ketanserin (63.7 Ci/mmol; Dupont/NEN, Les Ulis, France), and 10 µM methysergide (Sandoz, Paris, France) were used for 5-HT$_{2A}$ receptor binding assays. All reactions were stopped by adding 4 ml cold buffer followed by a rapid filtration through Whatman GF/B glass fiber filters. The filters were washed twice with 4 ml of the buffer, and radioactivity measured by liquid scintillation. All samples were assayed in duplicate, and the data analysed by means of Scatchard plots.

5-HT transporter binding

The assays were performed according to D'Amato et al. (1987). Thus, the pellets were suspended in 40 vol of a 50 mM TRIS-HCl buffer (pH 7.4) containing 120 mM NaCl and 5 mM KCl. The suspension (0.05–0.15 mg protein) was transferred to glass tubes (total volume: 0.25 ml) and the reaction carried out for 60 min at 25°C in the presence of six concentrations ranging from 0.5 to 16 nM of [^{3}H]citalopram (85 Ci/mmol; Dupont/NEN, Les Ulis, France), and 1 µM paroxetine (Smith Kline & Beecham, Harlow, England). All reactions were stopped by adding 4 ml of the cold buffer followed by a rapid filtration through Whatman GF/B glass fiber filters. The filters were washed twice with 4 ml of the buffer, and radioactivity measured by liquid scintillation. All samples were assayed in duplicate, and the data analysed by means of Scatchard plots.

Statistics

All values are shown as means ± SEMs. Because the data obtained in the elevated plus-maze and black/white box tests did not obey normality rules, Kruskal-Wallis analyses of variance were used for all behavioural comparisons. These analyses were followed by Mann-Whitney *U*-tests. Tryptophan hydroxylase activity and 5-HT receptor/transporter binding characteristics (V_{max}/K_M, B_{max}/K_D) were calculated by means of linear regression analyses using the least squares method, and compared by two-way analyses of variance (with the Roman line and the housing condition as main factors). Midbrain tryptophan hydroxylase regulation was analysed by means of a two-way analysis of variance (with the Roman line and the housing condition as main factors) with repeated treatment (assay condition). If significant, all these analyses of variance were followed by Tukey's multiple comparison procedure.

Results

Elevated plus-maze tests

Analyses of variance revealed overall differences between the four rat groups (group-housed/isolated RHA/RLA rats) on the percent number of open arm entries ($H = 10.56$, $P = 0.014$), on the duration of open arm visits ($H = 11.04$, $P = 0.012$), and on the number of total (closed + open) arm entries ($H = 12.16$, $P = 0.007$) (Fig. 1). Post hoc tests showed that all these behavioural measures were reduced in group-housed RHA rats, compared with group-housed RLA rats; beside, isolation reduced all behavioural scores in Roman lines (Fig. 1). However, due to low baseline levels in group-housed RHA rats and high heterogeneity of the data in group-housed RLA rats, the inhibitory

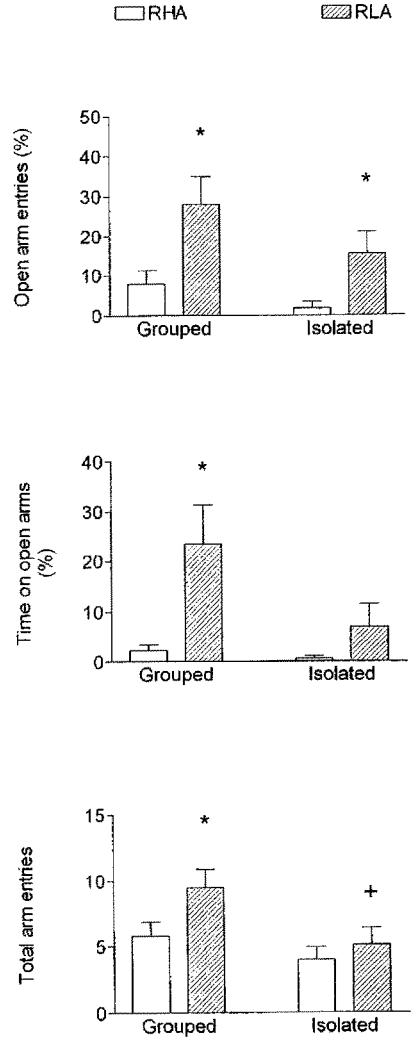

Fig. 1 Behaviours of group-housed and (6- or 7-day) isolated RHA and RLA rats exposed for 5 min to an elevated plus-maze. Values are the mean ± SEM of 10 (isolated) or 12 (group-housed) rats. $^*P < 0.05$ for the difference between RHA and RLA rats; $^+P < 0.05$ for the effect of isolation

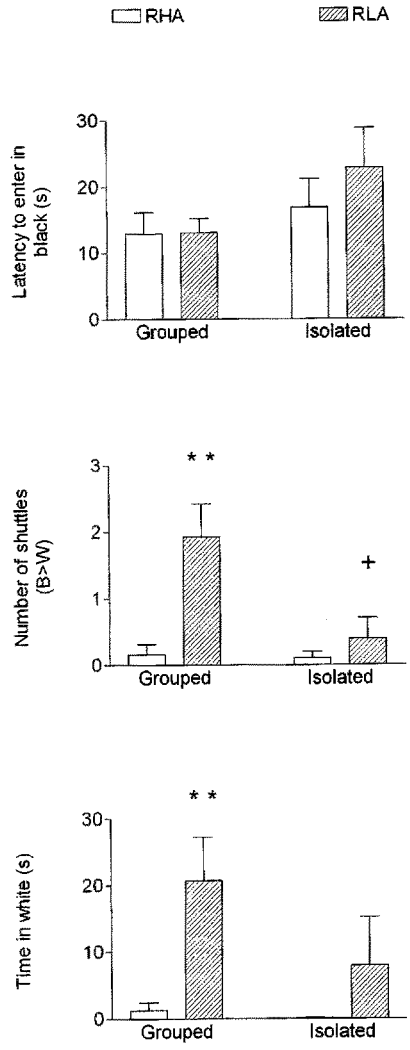

Fig. 2 Behaviours of group-housed and (9- or 10-day) isolated RHA and RLA rats exposed for 5 min to a black/white box (note that the time in the white compartment does not take into account the initial latency to enter in the black compartment). Values are the mean ± SEM of 10 (isolated) or 12 (group-housed) rats. $^{**}P < 0.01$ for the difference between RHA and RLA rats; $^{+}P < 0.05$ for the effect of isolation

effect of isolation proved significant only for the number of total arms visited by the RLA rats (Fig. 1).

In keeping with the observation that both open and total arm entries varied between (group-housed) RHA and RLA rats, the respective numbers of closed arm visits by the Roman lines were also analysed: in fact, the difference between the four rat groups was at the limit of significance ($H = 7.8$, $P = 0.05$) with group-housed RHA and RLA rats visiting, respectively, 5.1 ± 0.86 and 6.25 ± 0.60 closed arms, and isolated RHA and RLA rats visiting, respectively, 3.9 ± 0.9 and 3.7 ± 0.67 closed arms ($P = 0.021$ for the effect of isolation in RLA rats). In addition, a correlation analysis of the open arm visits against the closed arm visits in the ten isolated RLA rats yielded non-significant results ($r = 0.57$; $P = 0.084$).

Black/white box tests

The latency to enter for the first time in the black compartment was not significantly affected either by the rat line or the housing condition; alternatively, the four groups of rats differed in the number of (black to white compartment) shuttles ($H = 17.67$, $P < 0.001$) and the duration of time spent in the white compartment ($H = 17.33$, $P < 0.001$) (Fig. 2). In fact, the number of visits to the white compartment, and the time spent therein, were lower in (group-housed) RHA rats than in RLA rats. In addition, due to low baseline levels in group-housed RHA rats, isolation had a significant inhibitory effect on the number of shuttles displayed by RLA rats only (Fig. 2). In confirmation, the number of squares crossed in the white compartment differed between rat groups ($H = 16.53$, $P < 0.001$), with group-housed RLA and RHA rats crossing, respectively, 11.33 ± 3.6 and 1.17 ± 1.17 squares ($P = 0.006$) and isolated RLA and RHA rats crossing, respectively, 3.7 ± 3.1 and 0.1 ± 0.1 squares ($P = 0.036$ for the influence of the housing condition in RLA rats).

Table 1 Tryptophan hydroxylase activity in the midbrain, the frontal cortex, the hippocampus, and the hypothalamus of group-housed or isolated RHA and RLA rats. V_{max} (pmol 5-HTP/mg protein/min) and K_M (μM) are given as means ± SEM of four (hippocampus), five (frontal cortex, hypothalamus), or six (midbrain) independent experiments, and calculated from analyses of Lineweaver-Burk plots (with six concentrations of L-tryptophan ranging from 0.025 to 0.8 mM). Midbrains were assayed individually whereas two or three rats were pooled for the analysis of cortical, hippocampal, and hypothalamic tryptophan hydroxylase activity

		RHA		RLA	
		Grouped	Isolated	Grouped	Isolated
Midbrain	V_{max}	244 ± 14	233 ± 16	169 ± 8**	233 ± 18^{++}
	K_M	95 ± 14	81 ± 11	61 ± 6	94 ± 16
Fr. cortex	V_{max}	124 ± 10	138 ± 30	120 ± 10	165 ± 13
	K_M	158 ± 21	148 ± 21	135 ± 14	150 ± 15
Hippocampus	V_{max}	96 ± 12	105 ± 8	97 ± 3	98 ± 5
	K_M	198 ± 25	177 ± 21	172 ± 28	204 ± 57
Hypothalamus	V_{max}	163 ± 11	186 ± 23	135 ± 9	174 ± 13
	K_M	245 ± 20	216 ± 16	244 ± 18	220 ± 8

$^{**}P < 0.01$ for the difference between grouped RHA and RLA rats; $^{++}P < 0.01$ for the effect of isolation in RLA rats

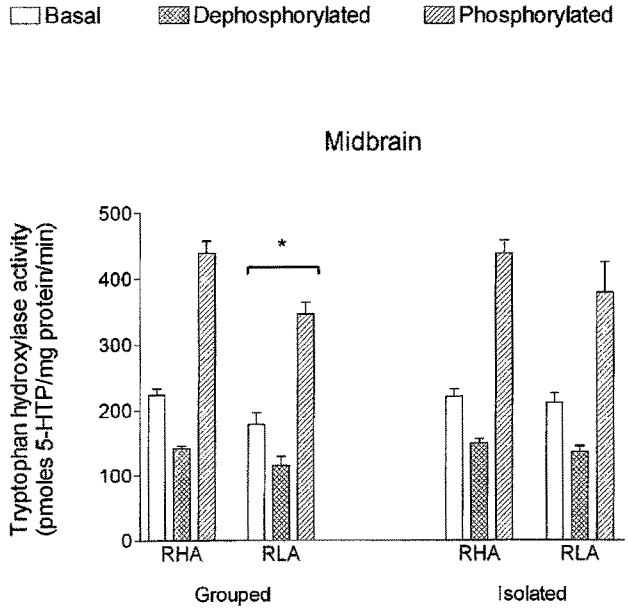

Fig. 3 Midbrain tryptophan hydroxylase activity under basal, dephosphorylating, and phosphorylating conditions in group-housed and 12-day isolated RHA and RLA rats. Values are the mean ± SEM of five rats. $^{*}P < 0.05$ for the differences between group-housed RHA and RLA rats (under the three assay conditions)

Tryptophan hydroxylase activity

Table 1 depicts the respective influences of the housing conditions upon midbrain, cortical, hippocampal, and hypothalamic tryptophan hydroxylase activity in RHA and RLA rats. Analyses of variance revealed significant line ($F_{1,20} = 6.679$, $P = 0.018$) and line × housing condition ($F_{1,20} = 6.477$, $P = 0.019$) effects upon midbrain tryptophan hydroxylase activity (as assessed by means of V_{max} analyses). Hence, group-housed RLA rats displayed a lower enzymatic activity than group-housed RHA rats, a difference that vanished in isolated animals (Table 1). Alternatively, a tendency toward a stimulatory effect of isolation upon hypothalamic tryptophan hydroxylase activity in RHA and RLA rats ($F_{1,16} = 4.01$, $P = 0.063$) could be noted, whereas neither the line nor the housing condition affected tryptophan hydroxylase activity in the cortex and the hippocampus (Table 1).

In keeping with the aforementioned line and line × housing condition effects on midbrain tryptophan hydroxylase, the regulatory properties of the enzyme were then studied under dephosphorylating and phosphorylating conditions. As shown in Fig. 3, the line ($F_{1,16} = 6.56$, $P = 0.021$), the assay condition ($F_{2,32} = 474.37$, $P < 0.001$), and the line × assay condition interaction ($F_{2,32} = 6.07$, $P = 0.006$) had significant effects upon midbrain tryptophan hydroxylase activity. Moreover, post-hoc tests revealed that whatever the assay condition, tryptophan hydroxylase activity was lower in RLA grouped rats, compared with RHA grouped rats (Fig. 3). In isolated rats, this phenomenon did not reach significance due to the high heterogeneity of the data obtained in RLA rats (especially under phosphorylating conditions) (Fig. 3).

[^{3}H]8-OH-DPAT binding to 5-HT$_{1A}$receptor sites

As shown in Table 2, neither midbrain presynaptic 5-HT$_{1A}$ receptor binding nor hippocampal postsynaptic 5-HT$_{1A}$ receptor binding (as assessed through [^{3}H]8-OH-DPAT specific binding) were different between RHA and RLA rats, whether group-housed or isolated.

[^{3}H]Citalopram binding to 5-HT reuptake sites

Table 2 shows that neither midbrain nor hypothalamic [^{3}H]citalopram specific binding were affected by the housing condition or the rat line; beside, the tendency for a housing condition × line effect on hypothalamic

Table 2 [^{3}H]8-OH-DPAT and [^{3}H]citalopram binding in the midbrain, the hippocampus and/or the hypothalamus of group-housed or isolated RHA and RLA rats. B_{max} (fmol/mg protein) and K_D (nM) are given as means ± SEM of three or four independent experiments, and calculated from analyses of Scatchard plots (with six concentrations of [^{3}H]8-OH-DPAT ranging from 0.25 to 2 nM or six concentrations of [^{3}H]citalopram ranging from 0.5 to 16 nM). Midbrains were assayed individually whereas two or three rats were pooled for the analysis of hippocampal [^{3}H]8-OH-DPAT binding and hypothalamic [^{3}H]citalopram binding

		RHA		RLA	
		Grouped	Isolated	Grouped	Isolated
[^{3}H]8-OH-DPAT binding					
Midbrain	B_{max}	57 ± 7	56 ± 5	65 ± 6	59 ± 1
	K_D	1.02 ± 0.21	0.98 ± 0.14	1.16 ± 0.20	1.01 ± 0.17
Hippocampus	B_{max}	646 ± 46	633 ± 62	580 ± 30	626 ± 34
	K_D	0.84 ± 0.02	1.01 ± 0.19	0.87 ± 0.05	0.94 ± 0.17
[^{3}H]Citalopram binding					
Midbrain	B_{max}	389 ± 52	426 ± 38	428 ± 16	402 ± 16
	K_D	1.64 ± 0.28	1.26 ± 0.17	1.59 ± 0.22	1.53 ± 0.38
Hypothalamus	B_{max}	405 ± 25	355 ± 26	317 ± 22	388 ± 41
	K_D	0.72 ± 0.13	0.62 ± 0.10	0.60 ± 0.06	0.81 ± 0.15

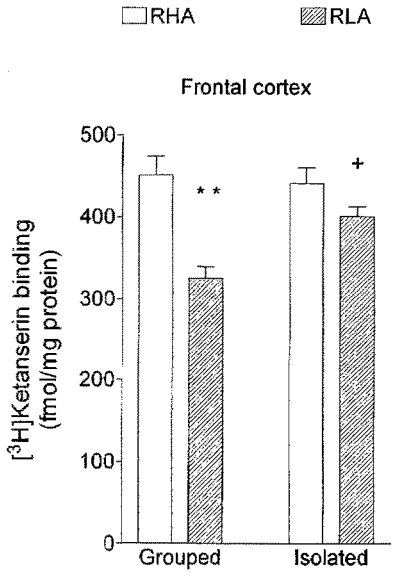

Fig. 4 [^{3}H]Ketanserin specific binding (B_{max}) in the frontal cortices of group-housed and 12-day isolated RHA and RLA rats. Values are the mean ± SEM of four determinations (two frontal cortices/determination). Saturation binding curves with six concentrations of radioligand (0.25–4 nM) were performed in duplicate; note that the respective K_{Ds} did not differ between groups (see text for details). $^{**}P < 0.01$ for the difference between RHA and RLA rats; $^{+}P < 0.05$ for the effect of isolation

[^{3}H]citalopram binding did not reach significance ($F_{1,12} = 4.23$, $P = 0.062$).

[^{3}H]Ketanserin binding to cortical 5-HT_{2A}receptor sites

As shown in Fig. 4, there was a line effect ($F_{1,12} = 22.17$, $P < 0.001$) and a housing condition × line interaction ($F_{1,12} = 5.75$, $P = 0.034$) upon the B_{max} values of cortical [^{3}H]ketanserin specific binding; indeed, grouped RHA rats displayed a higher number of cortical 5-HT_{2A} receptors than grouped RLA rats, a difference which was prevented by prior isolation (Fig. 4). Alternatively, the K_D values (in nmol/l) were not different between grouped RHA (1.58 ± 0.29), grouped RLA (1.12 ± 0.17), isolated RHA (1.31 ± 0.07), and isolated RLA (1.58 ± 0.24) rats.

Discussion

When successively placed for the first time in the elevated plus-maze and the black/white box, group-housed RHA and RLA rats were found to display marked behavioural differences, an observation in line with previous data (Chaouloff et al. 1994). Thus, in the elevated plus-maze, both the number of open arm visits and the duration of these visits were more prominent in RLA rats than in RHA rats. Interestingly, Koltushi low-avoidance (KLA) rats have been recently shown to spend more time on the open arms of an elevated plus-maze than their high-avoidance (KHA) counterparts (Zhukov and Vinogradova 1994), hence confirming that high- and low-avoidance rat lines may display paradoxical differences in models of anxiety. Alternatively, the rats used in the present study, but not those used in our previous study (Chaouloff et al. 1994), displayed also a line-dependent difference in the number of total (open and closed) arms visited, with RLA rats exploring a higher number of total arms than RHA rats. In keeping with this result, it is however noteworthy (see below) that the number of closed arm visits did not differ between Roman rats (whether grouped or isolated). A huge number of studies using pharmacological tools and/or stressful stimuli (see Pellow et al. 1985; Handley and McBlane 1993b), but also principal component analyses (Cruz et al. 1994), have indicated that the percent number of open arm visits and the time spent thereon are inversely linked to (state: Lister 1990) anxiety. On the other hand, principal component analyses have revealed that the number of closed arm entries, rather than the total number of entries, reflects locomotion (Cruz et al. 1994), thus suggesting that grouped RHA and RLA rats do not differ in their locomotor activities in this particular paradigm. This last observation is noteworthy in view of the differences in open field locomotion (i.e. RHA rats are more active than RLA rats) that have been repeatedly reported (see Driscoll and Bättig 1982).In effect, this suggests either that our Roman rat lines have diverged from those initially used in previous open field studies or, more likely, that locomotion in the (dimly-lit) elevated plus-maze and the (brightly-lit) open field does not represent similar behavioural entities. Lastly, in the black/white box tests (Crawley 1981) (group-housed) RLA rats displayed less aversion to the wide and brightly lit compartment (as assessed by the number and the duration of the visits in the white compartment) than (group-housed) RHA rats. Because anxiogenic and anxiolytic drugs (which could not be tested herein due to the limited number of animals available) respectively decrease and increase the number of visits to the white compartment (Crawley 1981; Costall et al. 1989), our results, which confirm those obtained in our previous study (Chaouloff et al. 1994), reinforce the hypothesis of an anxiety-related difference between RHA and RLA rats (at least under our experimental conditions: but see the Introduction). However, in keeping with the observation (in mice) that a single exposure to an elevated plus-maze may decrease (9 days later) the aforementioned indices of anxiety in the black/white box (Rodgers and Shepherd 1993), the possibility that repeated testing had differential effects in RHA and RLA rats cannot be excluded.

As stated in the Introduction, one of the goals of this study was to assess whether isolation had a differential effect upon RHA and RLA rats tested in the two aforementioned models of anxiety. In RLA rats,

isolation decreased the number and the duration of open arm visits thereby confirming that isolation affects rat behaviours in the elevated plus-maze (Jankowska et al. 1991; Wright et al. 1991b; Motta et al. 1992; Maisonnette et al. 1993). Beside, we observed that isolation decreased also the number of total arms (and that of the closed arms), a result similar to that reported in several papers (Jankowska et al. 1991; Motta et al 1992; Maisonnette et al. 1993), but opposed to that reported in other papers (Parker and Morinan 1986; Wright et al. 1991b). It is noteworthy that isolation had a significant anxiogenic and hypolocomotor effect in RLA rats but not in RHA rats, a difference most probably linked to the already low baseline levels in grouped RHA rats. This also holds true for the effects of isolation on the behaviours of RHA rats placed in the black/white box. In this test, however, the number of shuttles to the white aversive compartment was significantly lower in isolated RLA rats than in group-housed RLA rats. This observation confirms the previous observation by Ahmed et al. (1995) showing that isolation markedly increases the latency to enter the white compartment from the dark one. Finally, our results suggest that isolation had anxiogenic effects both in RHA and RLA rats, thereby allowing us to reject the hypothesis that isolation had differential effects in Roman lines (see Introduction); rather, our results confirm that the social interaction test (in which isolated RHA and RLA rats did not differ: Chaouloff et al. 1994) does not measure the same components of the anxiety profile as those measured in the elevated plus-maze and the black/white box (File 1991). In keeping with this comparison, it is noteworthy that isolation decreased exploration toward an unfamiliar environment whereas at the opposite it increases exploration toward an unfamiliar partner in the social interaction test.

The Roman lines have been shown to display differences in several transmitter systems (e.g. the dopaminergic, the noradrenergic, the GABAergic ones: see Introduction). As far as central serotonergic systems are concerned, available data so far have substantiated a reduction in serotonin (5-HT) turnover in the cortex, the hypothalamus and the midbrain of RLA rats, compared with RHA rats (Driscoll et al. 1980; Driscoll 1988). In the present study, we measured a specific index of 5-HT synthesis, namely the activity of tryptophan hydroxylase, the rate limiting step in 5-HT biosynthesis (Hamon et al. 1981; Boadle-Biber 1982). Our results did reveal inter-line differences in midbrain (which contains the raphe nuclei where serotonergic cell bodies are located), thereby suggesting that RLA rats display a decreased 5-HT biosynthesis in serotonergic cell bodies, compared to that measured in RHA rats. Interestingly, the respective extents to which midbrain tryptophan hydroxylase activity reached its maximal and minimal levels, as assessed through phosphorylating and alkaline phosphatase-elicited dephosphorylating conditions (Boadle-Biber et al. 1989), were both lower in RLA rats, compared with RHA rats. Thus, this series of experiments strongly suggest that the aforementioned inter-line differences in enzyme activity do not result from changes in its (phosphorylation-dependent) regulatory properties; whether line-dependent differences in tryptophan hydroxylase mRNA expression actually underlie our observations remains one possibility. Alternatively, tryptophan hydroxylase activity tended to decrease in the hypothalamus (which contains both serotonergic cell bodies and nerve terminals) of RLA rats, compared with RHA rats, whereas neither cortical nor hippocampal enzyme activities differed between Roman lines. Taken together, these results suggest that tryptophan hydroxylase activity displays line-dependent differences in serotonergic cell bodies, but, for an unknown reason, not in nerve terminals.

Acute exposure to inescapable shocks increases hypothalamic (and pons/medulla) 5-HT metabolism in RLA rats, but decreases it in RHA rats (Driscoll et al. 1983). This result is noteworthy because inescapable shocks have differential effects on the elevated plus-maze behaviours of Koltushi rats: thus, KHA rats display a high number of visits in the open arms whereas KLA rats do not go into the open arms (i.e. the opposite to the behaviours observed in naive Koltushi rats: see above) (Zhukov and Vinogradova 1994). In the present study, isolation stress had a line-dependent effect upon midbrain tryptophan hydroxylase activity; thus, isolation prevented the above-mentioned decrease in enzyme activity that could be observed in the midbrains of (group-housed) RLA rats. Beside, isolation did not affect hippocampal or cortical tryptophan hydroxylase activity, a result which contrasts with the observation of decreased hippocampal 5-HT metabolism in isolation-reared animals (Parker and Morinan 1986). Due to a high heterogeneity of the data, phosphorylation-dephosphorylation experiments did not allow us to understand whether this preventive effect of isolation in RLA rats was mediated by changes in the regulatory properties of the enzyme or in the enzyme protein levels. In this context, it is noteworthy that repeated sound stress, but not immobilisation stress, increases midbrain (but also cortical) tryptophan hydroxylase activity through a glucocorticoid-mediated mechanism (for a review: Chaouloff 1993). Future experiments should then examine the possibility that our observations arise from a line-selective effect of glucocorticoids.

One previous study has evidenced increases in hypothalamic, but decreases in striatal and cortical [^{3}H]imipramine binding sites in RLA rats, compared with RHA rats (Gentsch et al. 1983). In keeping with the suggestion that [^{3}H]imipramine binding provides an index of 5-HT transporter binding (a suggestion which we now know to be incorrect: Marcusson et al. 1986; D'Amato et al. 1987), this series of results suggested that Roman lines may differ in their respective

numbers of 5-HT reuptake sites, at least in several brain regions. In confirmation, a recent study (published whilst the present one was already finished) has indicated that binding to [^{3}H]paroxetine, a selective 5-HT reuptake inhibitor (SSRI) (see Johnson 1991), was significantly decreased in the frontal cortex and the hippocampus of RLA rats, compared to RHA rats (Charney et al. 1995). In addition, this study reported that brain stem [^{3}H]paroxetine binding was not different between Roman lines whereas hypothalamic [^{3}H]paroxetine binding tended to be decreased in RLA rats (Charney et al. 1995). On the basis of the [^{3}H]imipramine-related data, but also previous results indicating that repeated administration of SSRIs may lead to anxiolysis in different animal models (Cadogan et al. 1992; Griebel et al. 1994; Lightowler et al. 1994), we have measured whether differences in anxiety-related behaviours between Roman rats were associated with differences in the (5-HT transporter) binding to another selective SSRI, namely [^{3}H]citalopram (D'Amato et al. 1987; see Hyttel 1994 for a review). Confirming the data obtained with [^{3}H]paroxetine, we observed that [^{3}H]citalopram binding was not different in the midbrain of RHA and RLA rats whereas it tended to decrease in the hypothalamus of RLA rats. Taken together, all these data suggest that at the opposite of what was observed with tryptophan hydroxylase assays (see above), 5-HT reuptake sites may be decreased in serotonergic nerve terminals, but not cell bodies, of RLA rats, compared to RHA rats. Whether this difference extends to changes in 5-HT release from terminals and whether these changes promote the inter-line differences in anxiety-related behaviours are questions which remain to be explored.

Among indices of 5-HT function(s) in Roman lines, one study has reported that the hyponeophagic effect of 5-methoxy-dimethyltryptamine, a 5-HT$_{1A}$/5-HT$_{2A}$ receptor agonist, was more important in RLA rats than in RHA rats (Shephard and Broadhurst 1983). This could suggest hypersensitivity of central 5-HT$_{1A}$ and/or 5-HT$_{2A}$ receptors in RLA rats, compared with RHA rats. In fact, the receptor binding data presented herein do not support such an hypothesis as on the one hand pre- and postsynaptic [^{3}H]8-OH-DPAT binding did not differ between rats, whereas on the other hand cortical [^{3}H]ketanserin binding (a tool repeatedly used for the study of central 5-HT$_{2A}$ receptors: see Leysen 1992) was lower in (group-housed) RLA rats than in RHA rats. Data regarding [^{3}H]8-OH-DPAT binding are noteworthy because stimulation of midbrain 5-HT$_{1A}$ (auto)receptors and/or hippocampal 5-HT$_{1A}$ (postsynaptic) receptors have repeatedly been shown to affect behaviours in animal models of anxiety (see Schreiber and De Vry 1993; Andrews et al. 1994). Although 5-HT$_{1A}$ receptor-mediated functions have not been analysed herein, our results suggest that among the different mechanisms that may underlie anxiety-related behavioural differences in Roman rats, that related to a change in 5-HT$_{1A}$ receptor sensitivity may be discarded. Lastly, isolation did not affect 5-HT$_{1A}$ pre- and postsynaptic receptor binding in Roman rats, an observation which contrasts with the suggestion that isolation-rearing (in Lister hooded rats) up-regulates the spinal 5-HT$_{1A}$ receptors that mediate components of the "serotonergic syndrome" (Wright et al. 1991a).

Among the results obtained in this study, those related to line-dependent differences in cortical 5-HT$_{2A}$ receptors (i.e. receptors that rapidly desensitise on repeated stimulation; Leysen 1992) are noteworthy. Firstly, the observation that RLA rats displayed a decreased number of cortical 5-HT$_{2A}$ receptors, but not increased cortical 5-HT synthesis/metabolism, compared with RHA rats suggests that the former is independent from presynaptic changes. This suggestion is reinforced by the finding that isolation up-regulates these receptors and tends to hyperactivate cortical tryptophan hydroxylase in RLA rats. In this context, it is noteworthy that isolation rearing has been shown to decrease (evoked) 5-HT release (Wright et al. 1989) and to increase 5-HT$_{2A}$ receptor-mediated back muscle contractions (but not 5-HT$_{2A}$ receptor-mediated wet-dog shakes) in Lister hooded rats (Wright et al. 1991a). Taken together, all these results point to the need for future experiments aimed at investigating the mechanisms (transcriptional and/or post-transcriptional) that underlie the differences in cortical 5-HT$_{2A}$ receptors in the Roman lines, and whether these differences extend (i) to other 5-HT$_{2A}$ receptors (e.g. in striatum), and (ii) to 5-HT$_{2A}$ receptor-mediated functions. Moreover, because we used [^{3}H]ketanserin, an antagonist which labels both the high-affinity (G protein-coupled) and the low-affinity states of 5-HT$_{2A}$ receptors (Teiteler et al. 1990), rather than an agonist (which labels only the high-affinity state of the receptor: Teiteler et al. 1990), the possibility that inter-line differences only concern receptors that are under the low-affinity conformation cannot be excluded. Lastly, it is our belief that future experiments should also examine the hypothesis of differences between Roman lines in the number and/or the morphology of 5-HT neurones and their cell targets.

Secondly, the observation of a relationship between the number of cortical [^{3}H]ketanserin binding sites (in group-housed and isolated) RLA rats and the occurrence of anxiety-like behaviours in the two tests used herein is of interest. As stated above, the high baseline levels of anxiety that were noted in group-housed RHA rats did not allow us to observe a significant anxiogenic effect of isolation in these rats. If this statement also holds true for cortical 5-HT$_{2A}$ receptors in group-housed and isolated RHA rats (i.e. high densities of 5-HT$_{2A}$ receptors in RHA rats cannot be increased by isolation), then the above-mentioned relationship between cortical 5-HT$_{2A}$ receptors and anxiety-like behaviours could extend to the four rat groups. Because (i) acute/chronic 5-HT$_{2A}$ receptor blockade in rats and

humans often (but not always) leads to anxiolysis (Barrett and Vanover 1993; Handley and McBlane 1993), (ii) 5-HT_{2A} receptor down-regulation has been recently found to be associated with anxiolytic effects in the elevated plus-maze (Benjamin et al. 1992; Cadogan et al. 1992), our results suggest that differences in 5-HT_{2A} receptors between Roman lines may partly (or totally) underlie the behavioural differences observed in the elevated plus-maze and the black/white box. However, the confirmation of this hypothesis needs future experiments aimed at modifying either 5-HT_{2A} receptor mRNA expression (by means of antisense oligonucleotide technology) or 5-HT_{2A} receptor binding (by means of repeated 5-HT_{2A} receptor blockade/stimulation) and measure (through pharmacologically validated tests, including with the Roman lines) their behavioural consequences in RHA and RLA rats. Moreover, in keeping with the observation that exposure to novel environments (such as the elevated plus-maze) may alter 5-HT uptake and 5-HT release (at least in the hippocampus: File et al. 1993), the possibility that prior behavioural testing had differential neurochemical consequences in RHA and RLA rats must be taken into account. All these key issues are to be addressed soon in our laboratory.

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