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Associative and nonassociative learning after chronic imipramine in rats

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Abstract

We investigated effects of 15 daily injections of imipramine (20 mg/kg; in one experiment also 10 and 30 mg/kg). The associative learning types (place learning and object recognition) as well as nonassociative learning (habituation of exploration in an open field and within the object recognition test) were studied. Tests were performed immediately after the final injection (early test) and 24 h after the final injection (late test). The 5-HT_{1A}, 5-$HT_{1B/D}$, 5-HT_{2A}, beta-adrenergic, D_2 receptors were assayed 24 h after the final injection and the 5-HT_{2A} and beta-adrenergic receptors were also measured 60 and 96 h after the final injection. While associative types of learning were impaired in early tests, they remained unaffected in late tests and, while the nonassociative learning (habituation of exploration) remained unaffected in early tests, it was changed in late tests. Measured 24 h after the final injection, imipramine (20 and 30 mg/kg per day) down-regulated the concentration of beta-adrenergic and 5-HT_{2A} receptors, while leaving all other measured receptors unaffected. However, only the down-regulation of the 5-HT_{2A} receptor outlasted the initial 24-h period after the final injection. On the basis of present and previous results, we interpret the impairment of associative types of learning in early tests as a reflection of anticholinergic effects of imipramine, while the modifications of habituation of exploration in late tests are likely primarily to be mediated by imipramine-provoked regulations of serotonergic receptors.

Keywords: Imipramine; Associative learning; Nonassociative learning; Habituation; Exploration; Open field; Place learning; Object recognition; Water maze; Serotonin; Noradrenaline; Receptor regulation; Rat

1. Introduction

The tricyclic antidepressant imipramine blocks the re-uptake of serotonin and norepinephrine (e.g., Tucker and File, 1986). Additionally, antihistaminergic (e.g., Richelson, 1979) and anticholinergic (e.g., Bohman et al., 1982; Borbe and Zierenberg, 1985; El-Fakahany and Richelson, 1983; Rana et al., 1993; Richardson et al., 1984; Shaker et al., 1981; Snyder and Yamamura, 1977; Wachtel et al., 1988) effects of imipramine have been demonstrated. Studies, in which the behavioural consequences of long-lasting or "chronic" administration of imipramine have been investigated in the rat, have produced inconsistent results. A factor, which may have contributed to such inconsistencies, is the fact that the delay between the last administration of imipramine and the behavioural testing varies greatly amongst studies. In some cases, the imipramine treatment is discontinued and a pause of at least 12 h is allowed before behavioural testing (presently, animals subjected to such a procedure will be referred to as late-test rats). Other studies are based on a procedure in which behavioural tests are administered immediately after the final imipramine injection (early test rats). The late-test design can be expected, mainly, to reflect the behavioural consequences of receptor regulations provoked by chronic imipramine treatment. The behavioural results obtained in designs of the early test type must, however, be a reflection of the combined consequences of imipramine-induced receptor regulations and more acute effects of imipramine—including anticholinergic effects. Ideally, studies of chronic imipramine effects on a particular type of behaviour should include parallel experiments, in which behavioural tests are conducted in late and early test rats, respectively.

Even if data are available from studies of both late and early test animals, the literature may be inconsistent when describing the behavioural consequences of chronic imipramine in the rat. An example of an area, in which such

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inconsistencies are prominent, is the literature on the effects of chronic imipramine on exploration and locomotion. In early test animals, exploration has been found to be increased (Kulkarni and Dandiya, 1973), reduced (Hughes and Pither, 1987), or unaffected (Harrison-Read and Steinberg, 1980), and locomotion has been described as decreased (Broitman and Donoso, 1978; Custódio Teixeira et al., 2000; Freund et al., 1979; Furgiuele et al., 1964; Hughes and Pither, 1987; Meltzer and Fox, 1971) or unchanged (Giardina and Radek, 1991; Kulkarni and Dandiya, 1973; Maj et al., 1989). In late-test rats, exploration seemed to be unaffected (Smialowski, 1987), while locomotion seemed increased (Meltzer and Fox, 1971) or unaffected (Köhler and Rauca, 1992; Smialowski, 1987). A potential source of such inconsistencies is the frequent inability to distinguish experimentally between changes in the exploratory tendencies of an animal and the more motoric symptoms reflected in an altered level of locomotion. Most exploration tests are easily affected by changed levels of locomotion and the outcome of most locomotion tests may easily be affected if the exploratory tendencies of an animal are modified (e.g., Mogensen, 2003).

In an attempt to clarify the effects of chronic imipramine on exploration and locomotion, we (Mogensen et al., 1994) subjected rats to a test-battery, including a locomotion-independent exploration test (Iversen and Mogensen, 1988) and an activity cage test, which is able to reflect locomotion in a rather exploration-independent manner (e.g., Geoffroy and Mogensen, 1988). Measured 24 h after discontinuation of imipramine, test of locomotion appeared unaffected by imipramine in the dosages of 10 and 20 mg/kg body weight per day. While exploration was only marginally affected by the lower of these dosages, the dosage of 20 mg/kg per day appeared to be associated with a significantly reduced exploration during a 15-min test period (Mogensen et al., 1994). A detailed behavioural analysis revealed that the consequences of chronic imipramine could not be described as a simple suppression of exploration, but rather, as an initial hyperexploration, followed by an overhabituation (Mogensen et al., 1994). Consequently, a likely explanation of the inconsistent results of previous studies could be that not only the method of behavioural testing, but even the duration of the individual tests might determine whether the results would indicate an increased, normal, or decreased level of exploration (Mogensen et al., 1994). Since habituation of exploration, rather than exploratory behaviour per se, seemed to be the factor affected by chronic imipramine (when tested 24 h after the final injection of the drug), our results (Mogensen et al., 1994) seem to indicate that receptor regulations provoked by such imipramine treatment are associated with modifications of at least certain types of the nonassociative learning type habituation.

As mentioned above, the neurochemical consequences of imipramine administration includes anticholinergic effects. Consequently, it may be speculated that in early tests—when relatively high concentrations of imipramine are available in the brain—such anticholinergic mechanisms may impair at least certain types of associative learning. On the basis of this assumption and our abovementioned previous results regarding modified habituation of exploration in late tests, we, as an overall hypothesis of the present study, predicted that while associative types of learning would primarily or exclusively be affected in early tests, nonassociative learning, in the form of habituation of exploration, would, primarily or exclusively, be found to be modified in late tests.

The purpose of Experiment 1 of the present paper was to test the conclusions of our previous study (Mogensen et al., 1994) in an experimental setup different from the one in which our original results were obtained. The behavioural method applied in Experiment 1 was an open-field test, in which horizontal locomotion and rearing activities were measured. In such a test, horizontal locomotion is assumed to be influenced by both the purely motoric abilities of the animal and the more "cognitive" exploratory tendencies. Rearing behaviour is supposed to reflect exploratory tendencies, in a manner that is rather locomotion-independent (obviously, major motoric impairments would reduce or eliminate the occurrence of rearing behaviour). The temporal distribution of both horizontal locomotion and rearing was studied throughout a 1-h test session. Experiment 1 included groups of rats subjected to imipramine in the dosages of 0.0, 10.0, and 20.0 mg/kg body weight per day—reproducing the three experimental groups of our previous study (Mogensen et al., 1994). Additionally, a group receiving imipramine in the dosage of 30.0 mg/kg body weight per day was included. It was decided that the dosage of imipramine found to yield the most pronounced symptoms in Experiment 1 should be utilized as the only dosage studied in Experiments 2–4.

While the focus in Experiment 1 was on aspects of the nonassociative learning process habituation in late-test animals, Experiment 2 addressed aspects of the associative learning of such rats. This was done by applying a place learning procedure in a water maze during an approximately 30-h period, starting 24 h after discontinuation of imipramine.

The third and final behavioural procedure employed in the present study was an object recognition test (Ennaceur and Delacour, 1988; Ennaceur and Meliani, 1992; Ennaceur et al., 1989). The recognition aspect of this test reflects a one-trial associative learning process. Other parameters of the test are—like the open-field test of Experiment 1—able to reflect exploration and habituation of exploration. The object recognition test was administered to late-test animals in Experiment 3.

To examine our hypothesis regarding differential effects of "chronic" imipramine administration on associative and nonassociative learning in early and late tests, the present study included not only the three abovementioned

late-test experiments, but also experiments in which the same three behavioural methods were applied to early test animals. Early and late tests of a particular behavioural procedure had to be performed in separate experiments, since the outcome of late tests performed in animals already subjected to an early test would have been influenced by the animals' previous experience with the same test procedure.

Imipramine-provoked behavioural symptoms, which outlast the initial 24 h after discontinuation of the drug, must be expected mainly—though not necessarily exclusively—to reflect receptor regulations provoked by the chronic imipramine treatment. The pattern of receptor regulations provoked by chronic administration of imipramine and the "postimipramine" duration of individual receptor up- or down-regulations have still not been fully clarified. The problem has, however, been addressed in a number of studies and it has, for instance, been demonstrated that 24 h after discontinuation of imipramine (administered in the dosage of 40 μmol/kg per day for 26 days), both the 5-HT_{1B} and 5-HT_2 receptors had been down-regulated when measured in a forebrain homogenate (Johanning et al., 1992). Twenty-four hours after discontinuation of imipramine (administered for 21 days in the dosage of 20 mg/kg per day), the 5-HT_1, 5-HT_{1A}, 5-HT_{1C}, and 5-HT_2 receptors were down-regulated in the frontal cortex, hippocampus, and choroid plexus (Mizuta and Segawa, 1989). In the present study, we conducted a quantitative assay of the serotonergic 5-HT_{1A}, 5-$HT_{1B/D}$, and 5-HT_{2A} receptors, the dopaminergic D_2 receptor, and the beta-adrenergic receptors on forebrain homogenates, after a "postimipramine" period of approximately 24 h. Furthermore, the 5-HT_{2A} and beta-adrenergic receptors were also assayed approximately 60 or 96 h after discontinuation of the drug.

2. Methods

2.1. Subjects

2.1.1. General

The subjects were experimentally naive, male Wistar albino rats with an initial body weight of approximately 275 g. They were housed in single cages with commercial rat chow and water always available. The animals' living quarters were maintained on a 12-h light–dark cycle (on 0600 h). The rats were randomly assigned to one of the treatment groups of the particular experiment: the group which would only receive vehicle (saline) control injections and the group(s) which would receive daily intraperitoneal injections of imipramine (imipramine hydrochloride, Tofranil, Ciba-Geigy, Switzerland).

2.1.2. Experiment 1

Thirty-eight subjects were divided into four treatment groups: vehicle control injections ($n=10$) and imipramine in the dosages of 10 ($n=10$), 20 ($n=9$), and 30 ($n=9$) mg/kg body weight per day, respectively.

2.1.3. Experiment 2

Twenty-three subjects were divided into two experimental groups: vehicle control injections ($n=12$) and imipramine injections in the dosage of 20 mg/kg body weight per day ($n=11$).

2.1.4. Experiment 3

Twenty-four subjects were divided into two experimental groups: vehicle control injections ($n=11$) and imipramine injections in the dosage of 20 mg/kg body weight per day ($n=13$).

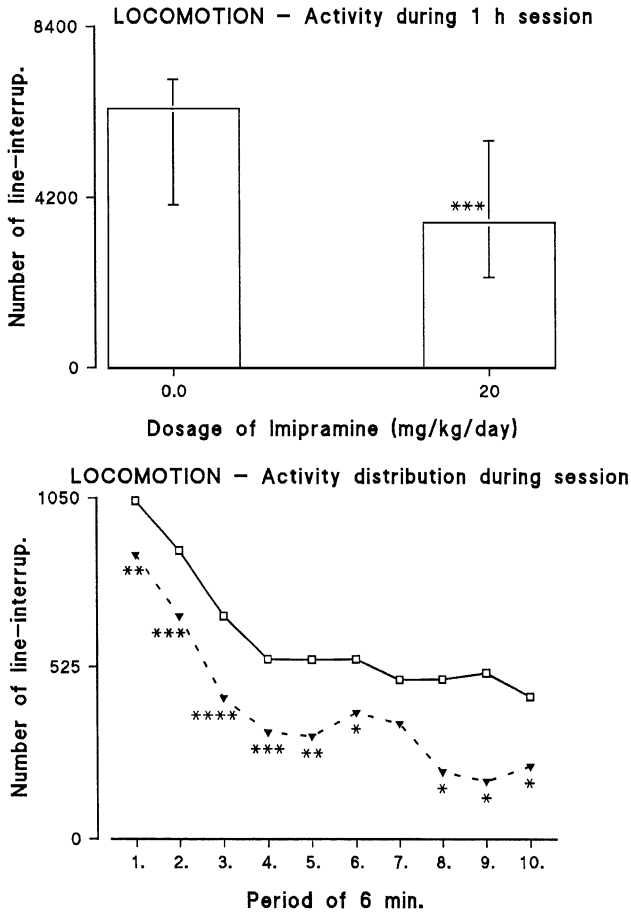

Fig. 1. Open field (early test, Experiment 4). Locomotion measured as number of line interruptions (see Methods) during the 1-h open-field test for each of the two dosage-defined experimental groups (symbols in the lower panel: open squares for vehicle-injected control group and downward pointing triangles for imipramine in the dosage of 20 mg/kg body weight per day). Values are given as medians (in the upper panel with ranges). While the upper panel illustrates the total number of line interruptions during the 1-h session, the lower panel represents the temporal distribution of such activity. * Significantly ($P<.05$) different from the vehicle-injected control group. ** Significantly ($P<.01$) different from the vehicle-injected control group. *** Significantly ($P<.001$) different from the vehicle-injected control group. **** Significantly ($P<.0001$) different from the vehicle-injected control group.

2.1.5. Experiment 4

Twenty-four subjects were divided into two experimental groups: vehicle control injections ($n = 12$) and imipramine injections in the dosage of 20 mg/kg body weight per day ($n = 12$).

The experimental protocol was approved by the Danish National Review Committee for the Use of Animal Subjects (Dyreforsøgstilsynet) and all procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Behaviour

2.2.1. Apparatus

2.2.1.1. Open field. The open field, measuring 80.0 × 80.0 cm, was surrounded by 38.5-cm-high wooden walls. A grid of 15 × 15 infrared beams divided the open field area into 256 evenly sized squares. The infrared grid was represented at two levels: the lower level is 3 cm above the floor and the higher level is 12.7 cm above the floor. The open field was connected to a computer, which registered interruptions of infrared lines and stored the data. The open field was situated in the middle of a well-lit room, in which no other activity took place during open-field testing.

2.2.1.2. Object recognition. The basic design of the object recognition equipment was similar to that described in Experiment 3 of Ennaceur and Delacour (1988). The test was conducted in an open plastic box, the floor of which measured 65.0 × 40.0 cm and was surrounded by 25.0-cm-high walls. The objects to be discriminated between were made of plastic, glass, and metal. One of the objects existed

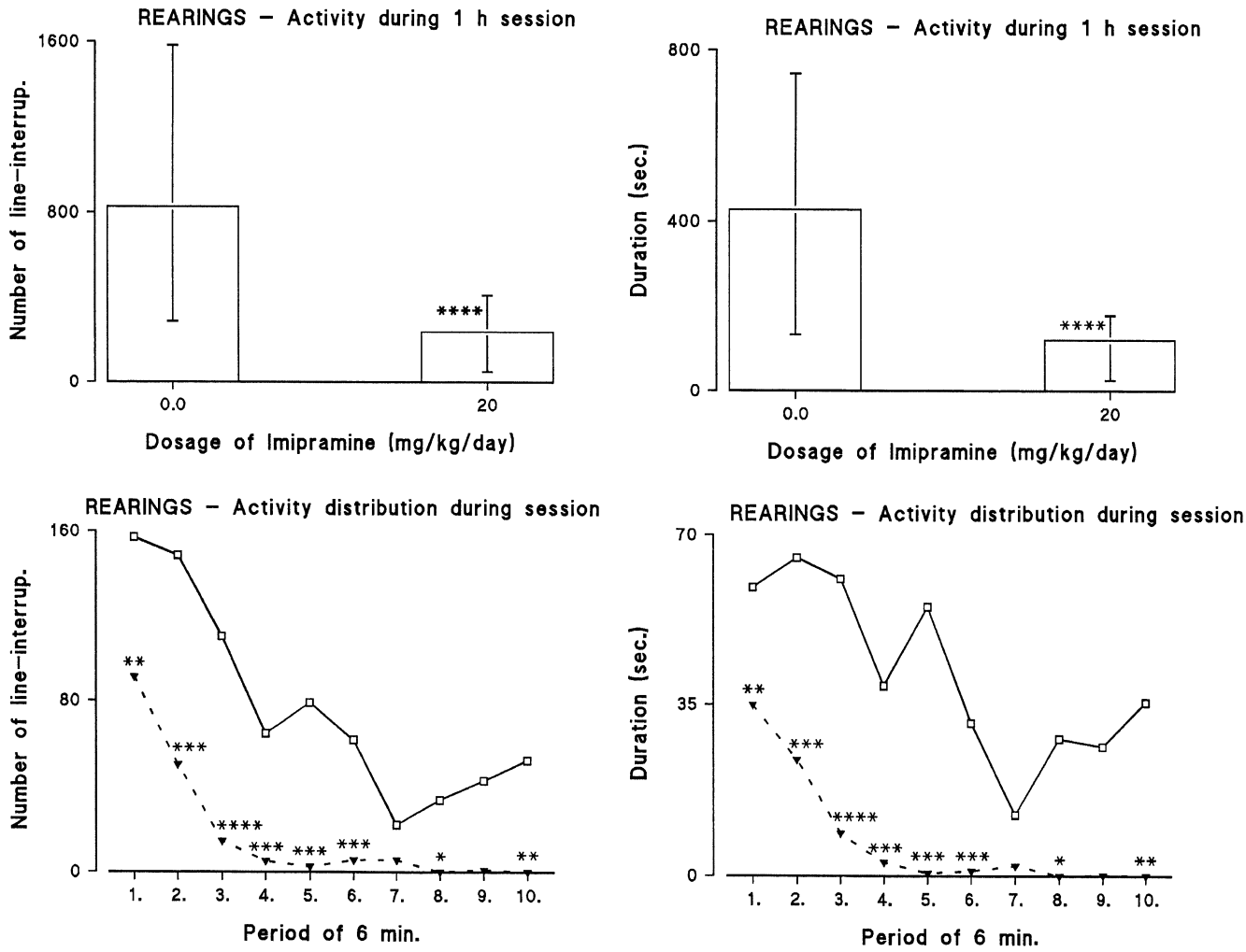

Fig. 2. Open field (early test, Experiment 4). Rearings (exploration) measured as number and duration of "upper level" line interruptions (see Methods) during the 1-h open-field test for each of the two dosage-defined experimental groups (symbols in the lower panels as indicated for Fig. 1). Values are given as medians (in the upper panels with ranges). While the upper panels illustrate the total number and duration of line interruptions during the 1-h session, the lower panels represent the temporal distribution of such activities. * Significantly ($P < .05$) different from the vehicle-injected control group. ** Significantly ($P < .01$) different from the vehicle-injected control group. *** Significantly ($P < .001$) different from the vehicle-injected control group. **** Significantly ($P < .0001$) different from the vehicle-injected control group.

in triplicate. The weights of the discrimination objects were such that they could not easily be moved by the rats. The object recognition equipment was placed in a well-lit room, in which no other activities took place during object recognition testing.

2.2.1.3. Water maze. The water maze—a circular water tank measuring 1.85 m in diameter—was constructed according to a basic design similar to that of Morris (1984) and has been described in details elsewhere (e.g., Mogensen et al., 1995a,b). Four points along the circumference of the water tank were arbitrarily designated North (N), South (S), East (E), and West (W), thus dividing the maze into four quadrants. Throughout all parts of the experiments, one circular, submerged platform (diameter, 12.5 cm) remained in a fixed position in the middle of the SE quadrant. While all parameters involving time were measured in seconds, all distances were measured in arbitrary units (pixels).

2.2.2. Behavioural procedures

2.2.2.1. General procedures

All experiments. On each of the 15 days of the injection period, all animals were weighed and intraperitoneal injections of either vehicle or imipramine were administrated.

Experiment 1. On the 16th day of the experiment, no injections were administered and the open-field test was performed (24 h after the final injection). Immediately after termination of the open-field test, all animals were sacrificed in high concentration CO_2 and the brains were promptly removed for biochemical analysis.

Experiment 2. On the 16th and 17th days of the experiment, no injections were administered and the six sessions of the place learning procedure were performed (the initial session performed 24 h after the final injection—Sessions 1, 2, and 3 administered on Day 16 and Sessions 4, 5, and 6 administered on Day 17 of the experiment). After termination of the final place learning session, all animals were sacrificed in high concentration of CO_2 [60 or 96 h after the final injection of imipramine, depending on which of the (equally big) receptor assay groups the individual animal belonged to—affiliation of the individual rat being randomly selected] and the brains were promptly removed for biochemical analysis.

Experiment 3. Two additional injections (substances and dosages as indicated for the injection period) were administered on the 16th and 17th days of the experiment. On these 2 days, the six sessions of the place learning procedure were administered (Sessions 1, 2, and 3 administered on Day 16 and Sessions 4, 5, and 6 on Day 17). On the 18th day of the experiment (the first day after termination of the slightly expanded injection period), the object recognition test was performed (24 h after the final injection). After termination of the object recognition test, all animals were sacrificed in high concentration of CO_2 [60 or 96 h after the final injection of imipramine, depending on which of the (equally big) receptor assay groups the individual animal belonged to—affiliation of the individual rat being randomly selected] and the brains were promptly removed for biochemical analysis.

Experiment 4. Two additional injections (substances and dosages as indicated for the injection period) were administered on the 16th and 17th days of the experiment. On the 16th day of the experiment, the open field test was performed. On the 17th day of the experiment, the object recognition test was performed. After termination of the

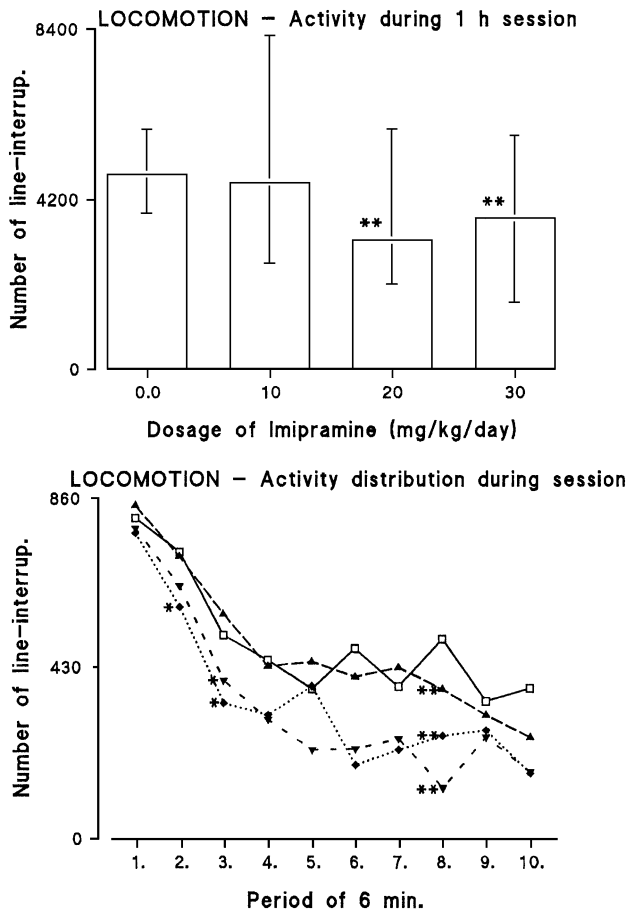

Fig. 3. Open field (late test, Experiment 1). Locomotion measured as number of line interruptions (see Methods) during the 1-h open-field test for each of the four dosage-defined experimental groups (symbols in the lower panel: open squares for vehicle-injected control group, upward pointing triangles for imipramine in the dosage of 10 mg/kg body weight per day, downward pointing triangles for imipramine in the dosage of 20 mg/kg body weight per day, and diamonds for imipramine in the dosage of 30 mg/kg body weight per day). Values are given as medians (in the upper panel with ranges). While the upper panel illustrates the total number of line interruptions during the 1-h session, the lower panel represents the temporal distribution of such activity. * Significantly ($P<.05$) different from the vehicle-injected control group. ** Significantly ($P<.01$) different from the vehicle-injected control group.

object recognition test, all animals were sacrificed in high concentration of CO_2 [60 or 96 h after the final injection of imipramine, depending on which of the (equally big) receptor assay groups the individual animal belonged to—affiliation of the individual rat being randomly selected] and the brains were promptly removed for biochemical analysis.

2.2.2.2. Test procedures

Open field. At the beginning of the session, the animal was placed in the middle of the open field area and the experimenter immediately left the room, allowing the animal 60 min of undisturbed activity in the open-field area. The timing started 10 s after the moment the experimenter let the animal go. During the session, all interruptions of infrared lines at both the lower and higher levels were counted by the computer and the duration of periods, during which at least one infrared line at the upper level was interrupted, was measured. The parameters considered were "locomotion", as evaluated by the number of line interruptions at the lower level and "rearings", as evaluated by two parameters: the number of line interruptions at the upper level and the duration (in seconds) of line interruptions at the upper level. The program registered these three parameters for the entire duration of the 1-h session, as well as for the duration of the 10 individual 6-min periods, into which the sessions was arbitrarily divided. It should be emphasized that the division into 6-min periods was conducted for analytical purposes only, and that the animal remained undisturbed throughout the 60-min session.

Object recognition. The basic behavioural procedures were similar to those described in Experiment 3 of Ennaceur and Delacour (1988). However, for the present experiment, the separation between acquisition and retention sessions

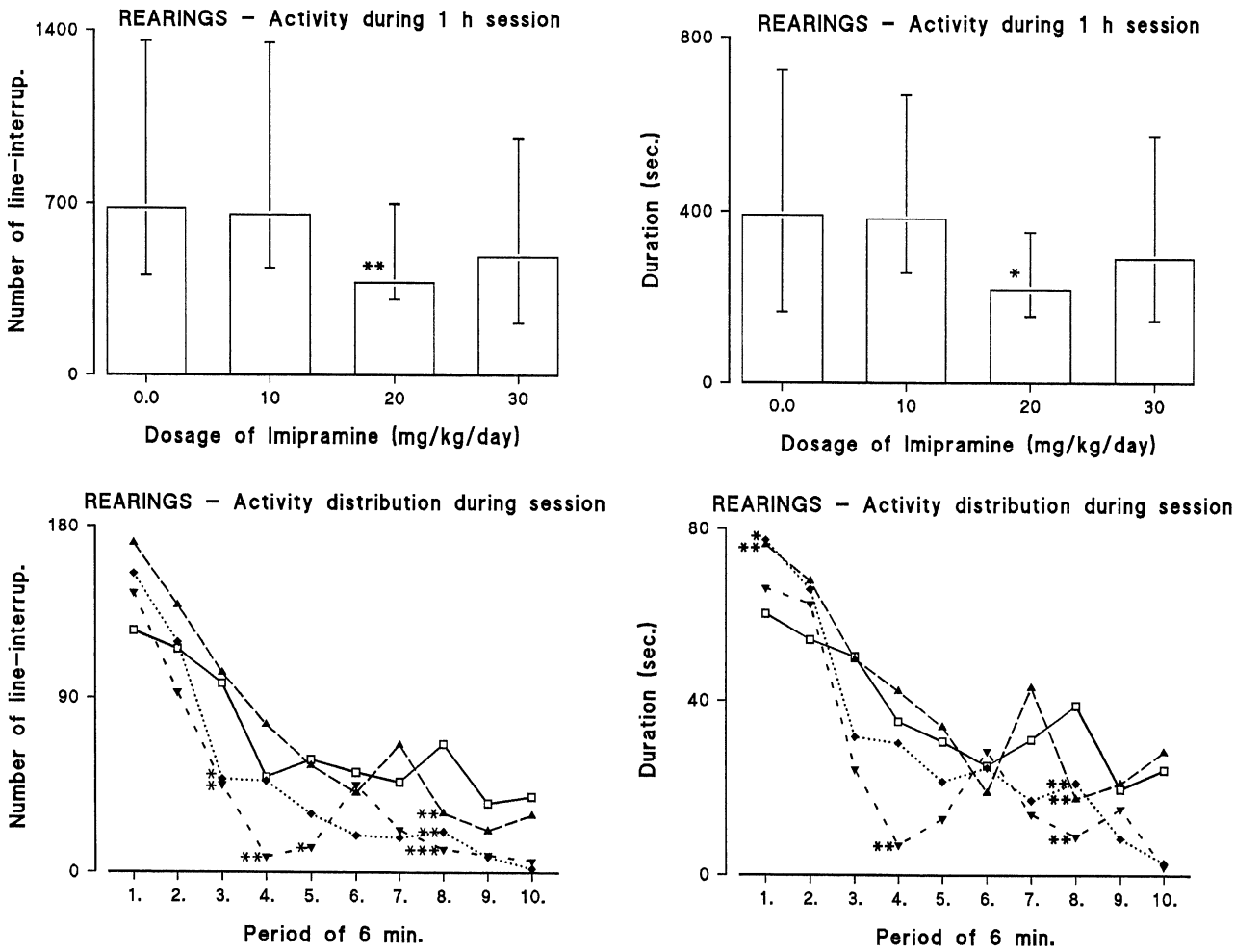

Fig. 4. Open field (late test, Experiment 1). Rearings (exploration) measured as number and duration of "upper level" line interruptions (see Methods) during the 1-h open-field test for each of the four dosage-defined experimental groups (symbols in the lower panels similar to those indicated for Fig. 3). Values are given as medians (in the upper panels with ranges). While the upper panels illustrate the total number and duration of line interruptions during the 1-h session, the lower panels represent the temporal distribution of such activities. * Significantly ($P<.05$) different from the vehicle-injected control group. ** Significantly ($P<.01$) different from the vehicle-injected control group. *** Significantly ($P<.001$) different from the vehicle-injected control group.

was 15 min. Animals were initially habituated for 20 min to the apparatus. During this habituation session, no discrimination objects were present. Twenty-four hours after the habituation session, animals were subjected to a 3-min acquisition session, followed by a 3-min retention session. During the 15-min pause between the acquisition and the retention sessions, animals were left undisturbed in their home cages. During the acquisition session, two identical objects (objects A1 and A2) were present. During the retention session, two dissimilar objects were presented. One of these objects (A3) was, in all respects, similar to objects A1 and A2, while the other object (B) was clearly dissimilar to objects previously encountered by the animals. During the two test sessions, the experimenter observed the animal throughout the 3-min period and registered the duration of periods during which the animal explored each of the four objects. Exploration was defined as all physical contacts between the animal and the object, if such a contact seemed to be the result of activities directed towards the object (if, for instance, the tail of the rat would touch an object while the animal was engaged in activities directed elsewhere, such a contact would not be registered as exploration). The parameters considered from the object recognition test were Exploration 1 (E1; the sum of the periods during which the animal explored the objects A1 and A2); Exploration 2 (E2; the sum of the periods during which the animal explored the objects A3 and B); Habituation 1 (H1; E2 subtracted from E1); Habituation 2 (H2; the time spent exploring object A3 subtracted from half the value of E1); Discrimination 1 (D1; the duration of the

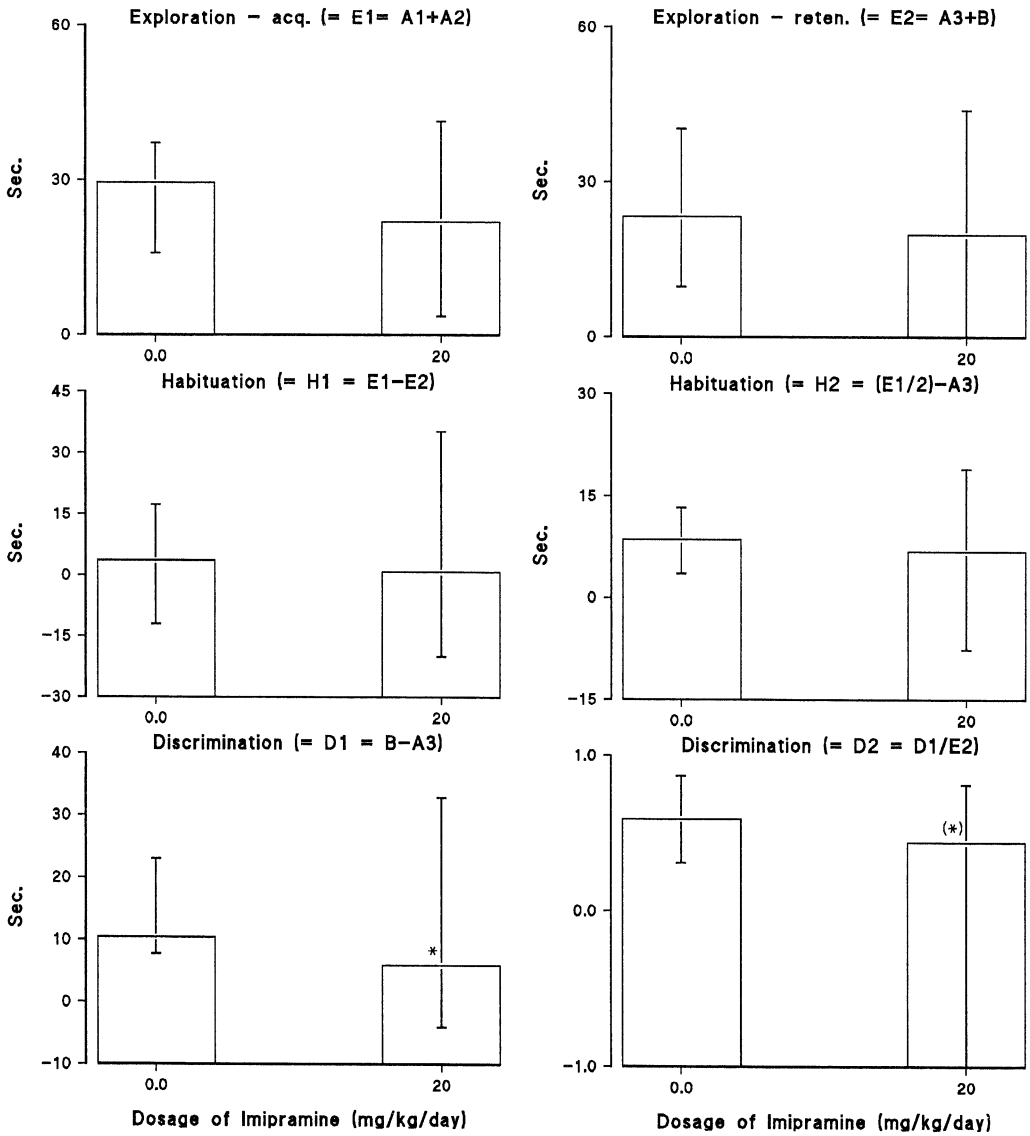

Fig. 5. Object recognition (early test, Experiment 4). Results obtained by the two dosage-defined experimental groups (0.0: the vehicle-injected control group; 20: imipramine in the dosage of 20 mg/kg body weight per day) on the two sessions of the exploration-based object recognition test. Results are given as medians with ranges. For procedural details and descriptions of calculations of individual parameters, see the Methods section. * Significantly ($P < .05$) different from the vehicle-injected control group. (*)Significantly ($P < .05$, one-tailed) different from the vehicle-injected control group.

period spent exploring A3 subtracted from the duration of the period spent exploring object B); and Discrimination 2 (D2; D1 divided by E2).

Place learning. The behavioural procedures were similar to those described by Mogensen et al., (1995a,b). In short, each animal was given five trials (swims) per session. Each trial had as its start position one of the locations N, S, E, or W. Within a session, a given start position was not allowed to be selected on more than two trials and the start positions were, otherwise, randomly selected. The following parameters were considered: the total swim distance and duration of a swim, the average speed of a swim, the mean distance to platform, the heading angle error, and the percentage of the swim duration during which the animal was found in the outer maze centered annulus. The place learning training was administered by three sessions per day.

During all behavioural procedures, the experimenter was kept ignorant about the group to which an individual rat belonged.

2.2.3. Statistical analysis

Nonparametric statistics were chosen for the statistical analysis of the behavioural data, since normal distribution could not be expected. Whenever comparisons were to include more than two groups, the data were originally subjected to the Kruskal–Wallis nonparametric analysis of variance (ANOVA) (Siegel, 1956). If the ANOVA revealed significant group differences—or if only two groups were to be compared—individual groups were

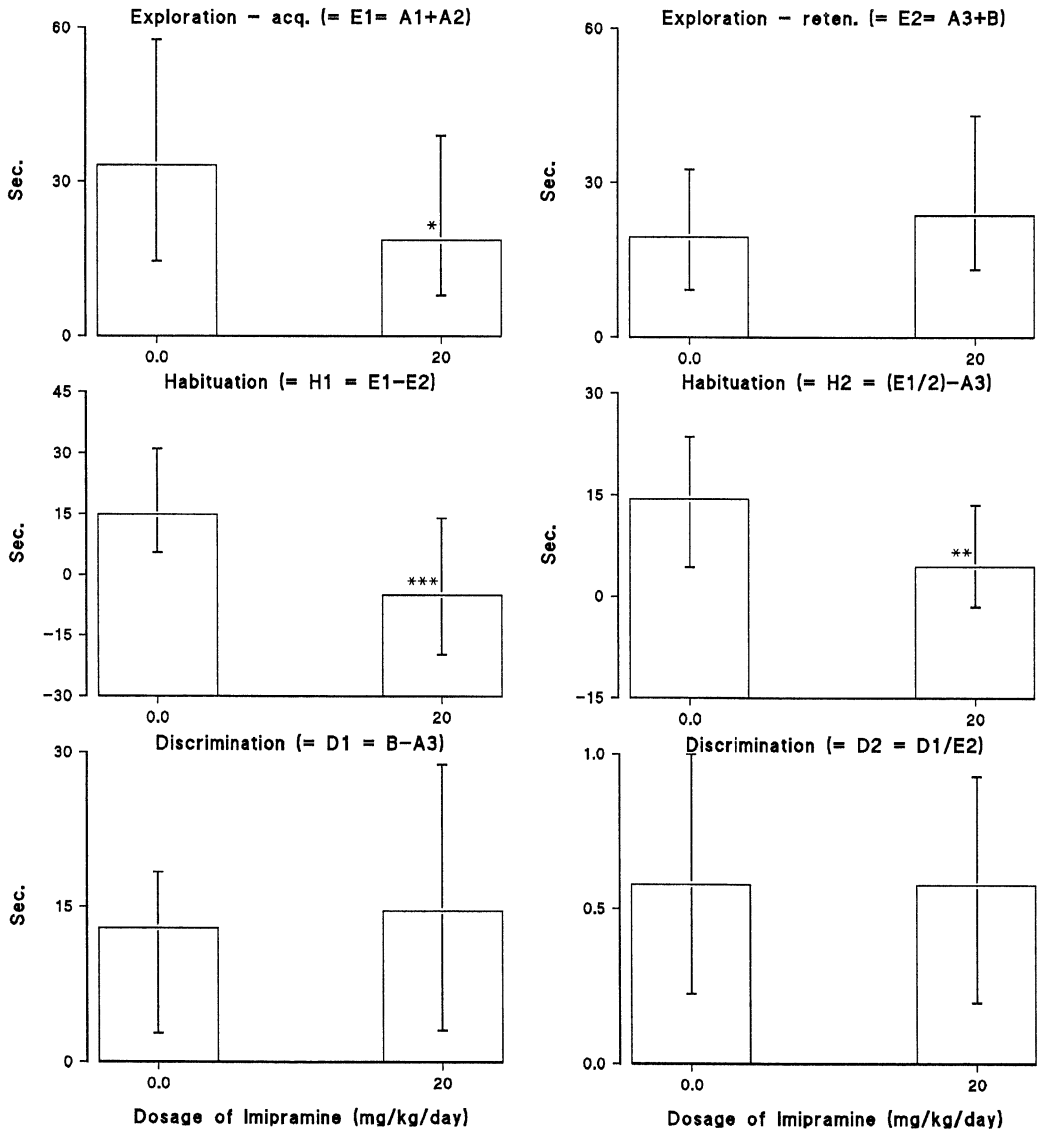

Fig. 6. Object recognition (late test, Experiment 3). Results obtained by the two dosage-defined experimental groups (indicated as in Fig. 5) on the two sessions of the exploration-based object recognition test. Results are given as medians with ranges. For procedural details and descriptions of calculations of individual parameters, see the Methods section. * Significantly ($P<.05$) different from the vehicle-injected control group. ** Significantly ($P<.01$) different from the vehicle-injected control group. *** Significantly ($P<.001$) different from the vehicle-injected control group.

compared using the Mann–Whitney *U* test (two-tailed) (Siegel, 1956).

2.3. Biochemistry

2.3.1. Receptor assay methods

Brain membranes were prepared as follows: the brains were homogenized with an Ultraturrax homogenizer for 10 s at 3/4 of maximum speed in buffer containing 150-mM NaCl, 20-mM ethylenediaminetetraacidic acid (EDTA), and 50-mM Tris (pH 7.5 at 0 °C). The homogenate was centrifuged and the membranes were washed with the same buffer. The drained membranes were lysed and then rehomogenized in buffer containing 5-mM EDTA and 5-mM Tris (pH 7.5 at 0 °C). The membranes were centrifuged and washed twice with Buffer 1 containing 120-mM NaCl, 5-mM KCl, 50-mM Tris (pH 7.5). Finally, the membranes were suspended in Buffer 1, at a protein concentration of about 6 mg/ml and kept at −80 °C, until required.

Membrane protein concentrations were determined using Peterson's (1977) modification of the Lowry method.

Receptor binding was determined as described in Johanning et al. (1992). With respect to the 5-HT_{1A} receptor, B_{max} for [^{3}H]8-OH-DPAT binding was determined at 20 °C, in a final volume of 300 μl of Buffer 1 (including 5-mM $MgCl_2$), containing 50-μl membrane suspension and [^{3}H]8-OH-DPAT at one of six concentrations between 0.1 and 2.0 nM. Specific binding was determined by using 1-μM buspirone as the displacing agent. With respect to the 5-$HT_{1B/D}$ receptor, B_{max} for [^{3}H]5-HT binding was determined at 0 °C, in a final volume of 500 μl of Buffer 1 (including 50-nM 8-OH-DPAT, 5-mM ascorbic acid, 10-μM pargyline), containing 50-μl membrane suspension and [^{3}H]5-HT at 1 of 10 concentrations between 0.1 and 6.0 nM. Specific binding was determined by using 1-μM RU24969 as the displacing agent. With respect to the 5-HT_{2A} receptor, B_{max} for [^{3}H]Ketanserine binding was determined at 20 °C, in a final volume of 300 μl of Buffer 1 (including 5-mM $MgCl_2$), containing 50-μl membrane suspension and [^{3}H]Ketanserine at one of six concentrations between 0.25 and 4.0 nM. Specific binding was determined by using 10-μM mianserin as the displacing agent. With respect to the beta-adrenergic receptors, B_{max} for [^{3}H]DHAP (dihydroalprenolol) binding was determined at 20 °C, in a final volume of 300 μl of Buffer 1 (including 5-mM $MgCl_2$), containing 50-μl membrane suspension and [^{3}H]DHAP at one of six concentrations between 0.1 and 1.5 nM. Specific binding was determined by using 1-μM propranolol as the displacing agent. With respect to the dopamine D_2 receptor,

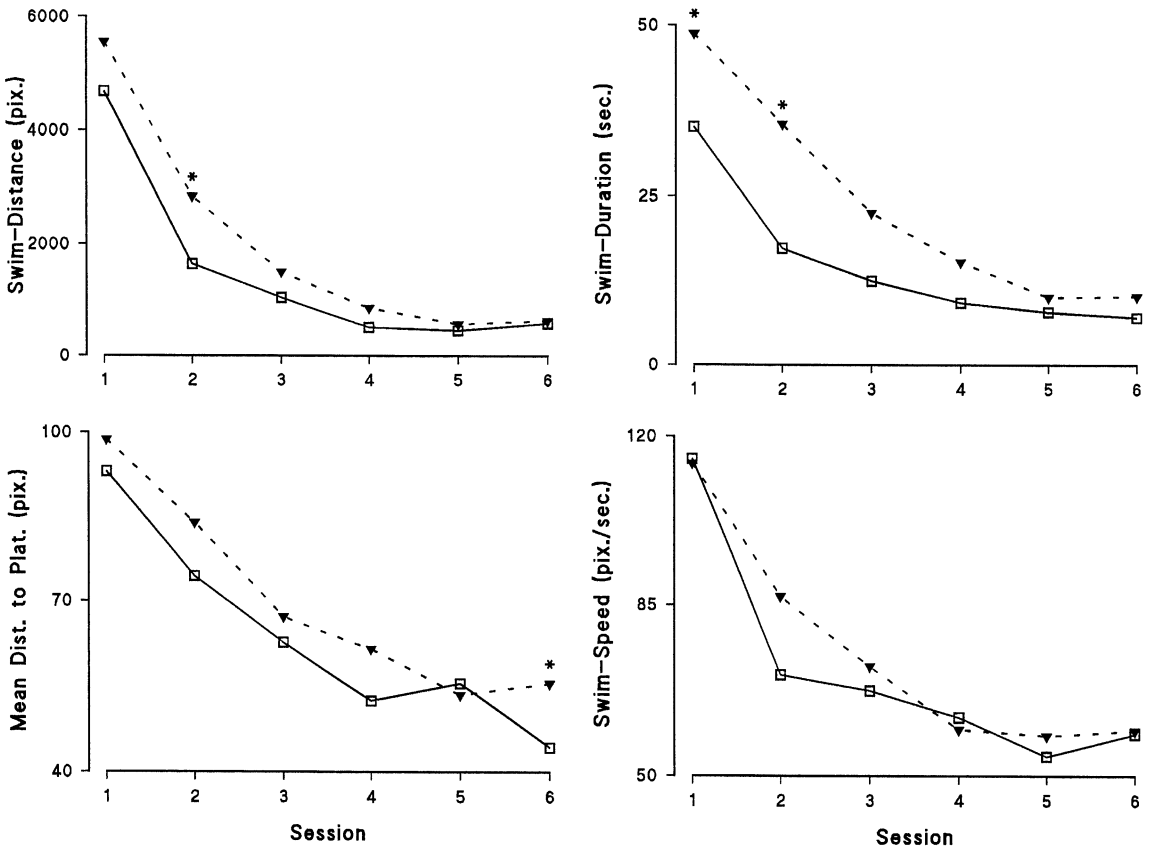

Fig. 7. Place learning (early test, Experiment 3). Performance of the two dosage-defined experimental groups (symbols as indicated for Fig. 1) on the six sessions of the place learning acquisition period. Values are given as medians. * Significantly ($P<.05$) different from the vehicle-injected control group.

B_{max} for [^{3}H]Racloprid binding was determined at 20 °C, in a final volume of 300 μl of Buffer 1, containing 100-μl membrane suspension and [^{3}H]Racloprid at one of six concentrations between 0.1 and 3.0 nM. Specific binding was determined by using 1-μM 6,7-ADTN as the displacing agent.

2.3.2. Statistical analysis

The biochemical data were subjected to parametric statistical analysis. The outcome of the receptor assays from Experiment 1 (in which four dosage-defined experimental groups were studied) were initially analysed using a one-way ANOVA (Winer, 1962). Whenever the ANOVA demonstrated significant group differences, individual groups were compared using the Newman–Keuls method (Winer, 1962). The outcome of the expanded analysis of the 5-HT_{2A} and beta-adrenergic receptors (in only two groups of animals, but after "postimipramine" pauses of 60 and 96 h) was analysed by comparison between imipramine- and saline-injected groups by *t* tests (Winer, 1962). While a two-tailed *t* test was selected for the beta-adrenergic receptor, one-tailed tests of receptor down-regulation were applied to the 5-HT_{2A} receptor. The 5-HT_{2A} receptor must, on the basis of the results of Johanning et al. (1992), be expected to be either down-regulated or unaffected. Statistical comparisons between the outcome of receptor analysis for the 5-HT_{2A} and beta-adrenergic receptors, after a postimipramine pause of 24 h in saline-injected animals and animals treated with the imipramine dosage selected for Experiments 3–4, had already been conducted as part of the ANOVA in Experiment 1. It was, however, decided to ease the comparison between these results and the corresponding receptor analysis, after longer postimipramine pauses, by reanalysing data from these two receptors in the two relevant dosage-defined groups from Experiment 1 by *t* tests comparable to those conducted on the biochemical results from subsequent experiments.

3. Results

3.1. Behaviour

3.1.1. Open field

3.1.1.1. Early test (Experiment 4). The results obtained in the open-field test (as well as significant group differences) are illustrated in Figs. 1 and 2. Both the locomotion and

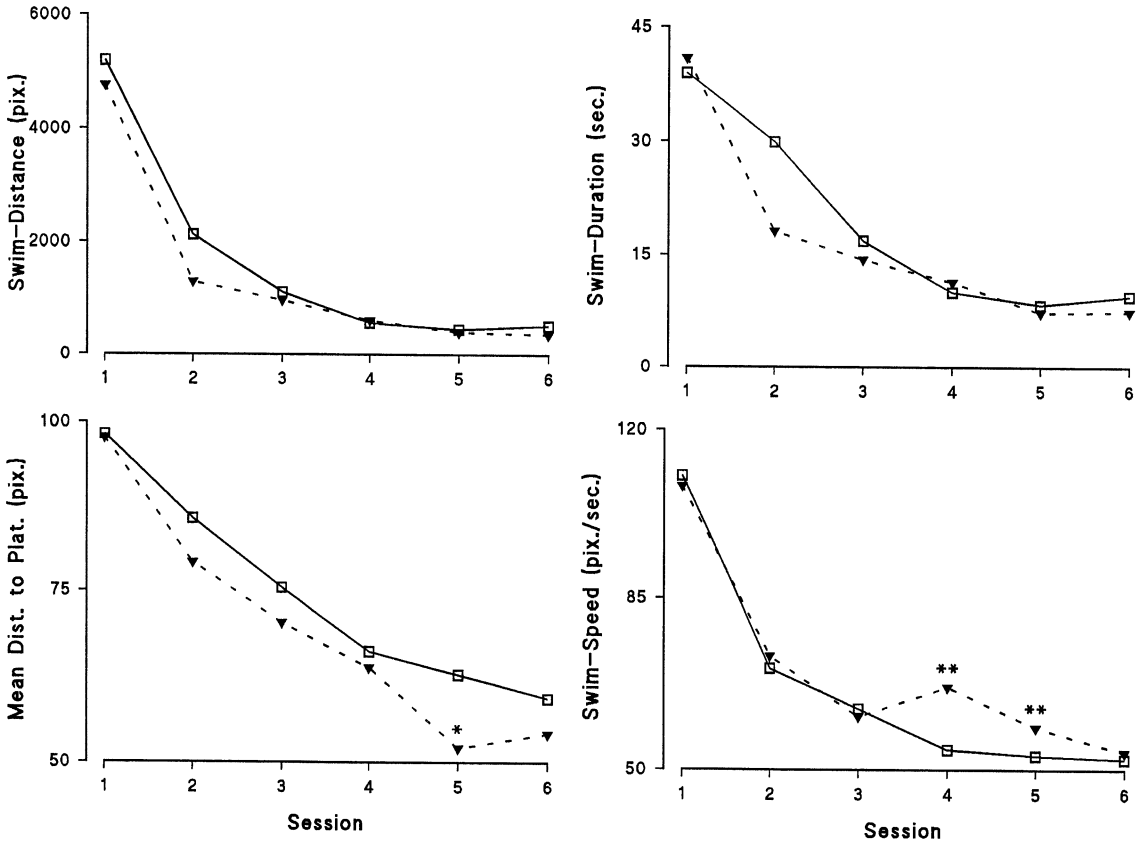

Fig. 8. Place learning (late test, Experiment 2). Performance of the two dosage-defined experimental groups (symbols as indicated for Fig. 1) on the six sessions of the place learning acquisition period. Values are given as medians. * Significantly ($P<.05$) different from the vehicle-injected control group. ** Significantly ($P<.01$) different from the vehicle-injected control group.

exploration of the imipramine-treated group were significantly reduced throughout the session. The habituation patterns of both locomotion and exploration in the imipramine-treated animals, however, were parallel to those of the vehicle-injected control group.

3.1.1.2. Late test (Experiment 1). The results obtained in the open-field test (including significant differences between individual experimental groups) are illustrated in Figs. 3 and 4. The group receiving imipramine in the dosage of 10 mg/kg body weight per day differed, only marginally, from the vehicle-injected control group. In contrast, highly significant changes in locomotion and, especially, exploration were seen in the two groups receiving imipramine in the dosages of 20 and 30 mg/kg body weight per day, respectively—the group given 20 mg/kg body weight per day showing the most pronounced effects. At the outset of the session, the level of locomotion was similar in all groups, but the two groups receiving the higher dosages of imipramine subsequently developed a lower level of locomotion (overhabituated). The pattern of imipramine-associated changes observed in exploration indicated two phases of drug-associated modifications: an initial hyperexploration followed by an overhabituation (both the higher dosages of imipramine being associated with significantly increased exploration during the first 6-min period, while subsequently showing significantly reduced levels of exploration from the third 6-min period onwards).

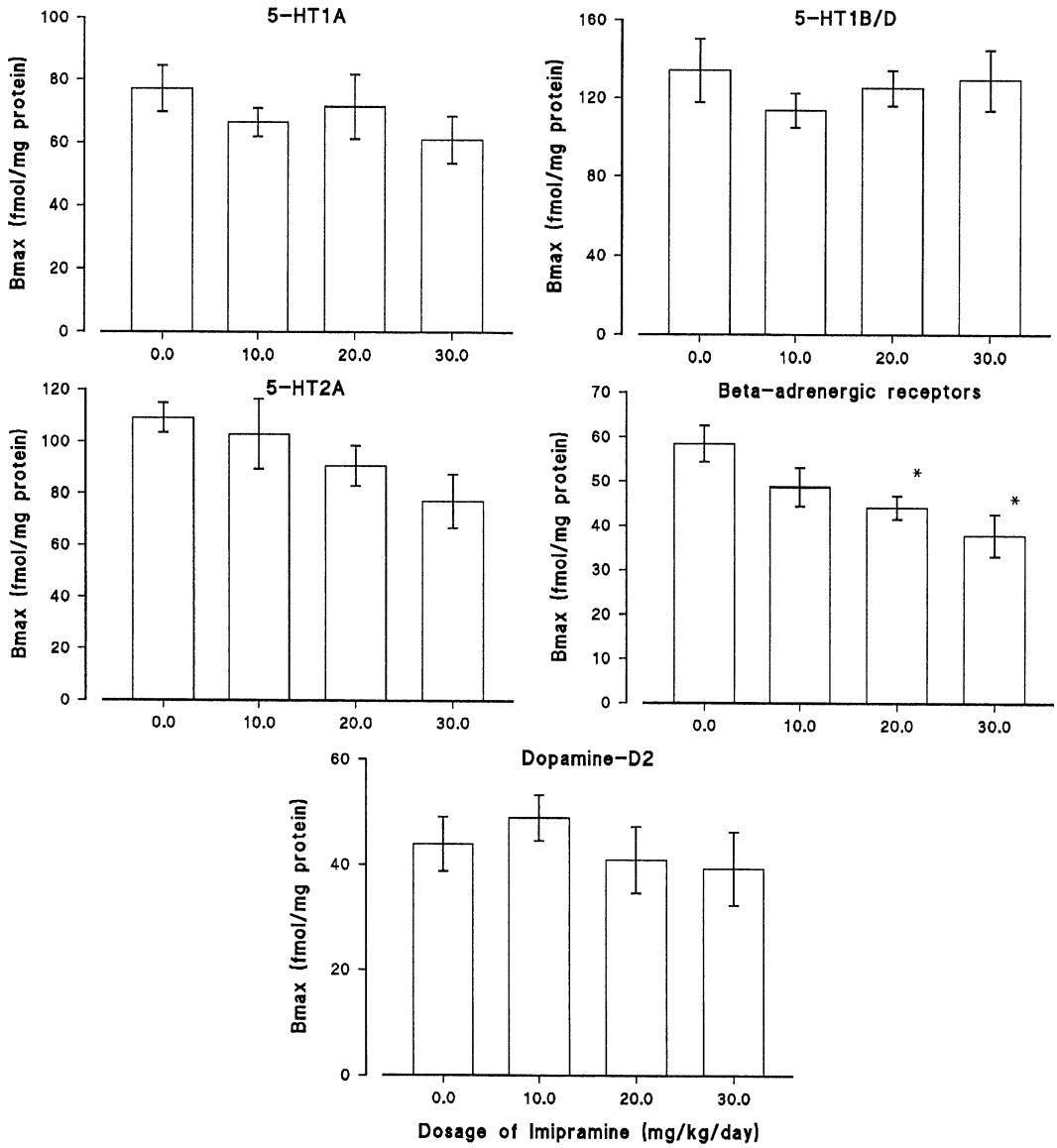

Fig. 9. Receptor assays (Experiment 1). Results of the receptor assays for each of the four dosage-defined experimental groups (0.0, 10.0, 20.0, and 30.0 mg/kg body weight per day of imipramine). Values (expressed as femtomole per milligram of protein) are given as means with S.D. * Significantly ($P < .05$) different from the vehicle-injected control group.

3.1.2. Object recognition

3.1.2.1. Early test (Experiment 4). The results obtained in the object recognition test (as well as significant group differences) are illustrated in Fig. 5. The imipramine-treated group demonstrated a significantly reduced object discrimination (reflected in both the D1 and D2 measures of discrimination), while having levels of exploration (E1 and E2), as well as habituation (H1 and H2) similar to those of the vehicle-injected control group.

3.1.2.2. Late test (Experiment 3). The results obtained in the object recognition test (as well as significant group differences) are illustrated in Fig. 6. The imipramine-treated group demonstrated normal levels of object discrimination (reflected in both D1 and D2), while having significantly reduced levels of habituation of exploration (as reflected in H1 and H2). On the measure of exploration, during the acquisition phase of the experiment (E1), the level of exploration demonstrated by the imipramine-treated group was significantly reduced.

3.1.3. Place learning

3.1.3.1. Early test (Experiment 3). Aspects of the results obtained from the two experimental groups during the six sessions of the place learning procedure (including significant group differences) are illustrated in Fig. 7. Additionally, significant group differences were found on the percentage swim time in the outer maze centred annulus (the imipramine-injected group having the higher value) on Sessions 1 ($P<.001$), 2 ($P<.05$), and 3 ($P<.05$), as well as on the mean distance to platform on Session 6 ($P<.05$) (the imipramine-injected group having the higher value). The pattern of results demonstrate that the imipramine-injected group initially had a significantly impaired quality of place learning (primarily reflected in the two "quality parameters" swim distance and duration), which, however, with continued training, managed to reach a normal level of proficiency.

3.1.3.2. Late test (Experiment 2). Aspects of the results obtained from the two experimental groups during the six sessions of the place learning procedure (including significant group differences) are illustrated in Fig. 8. As primarily reflected in the two "quality parameters" swim distance and duration, the imipramine-treated group demonstrated a place learning, which was as proficient as that of the vehicle-injected control group.

3.2. Receptor assays

3.2.1. Experiment 1

The results are illustrated in Fig. 9. The receptor assays showed that none of the imipramine dosages significantly affected the number of 5-HT_{1A}, 5-$HT_{1B/D}$, 5-HT_{2A}, or dopaminergic D_2 receptors present in brain membrane homogenates. The beta-adrenergic receptors were significantly down-regulated by imipramine in the dosages 20 and 30 mg/kg body weight per day for 15 days ($P<.05$).

3.2.2. Experiments 2–4

The results are illustrated in Fig. 10. The results showed that the down-regulation of the beta-adrenergic receptors observed after 15 days of treatment with imipramine in the dosage 20 mg/kg body weight per day did not outlast the first 24 h after the final injection of the drug. On the other hand, the use of hypothesis-guided statistics allows us to reveal a slight reduction in the number of 5-HT_{2A} receptors, which remains detectable up to 96 h after the last imipramine injection.

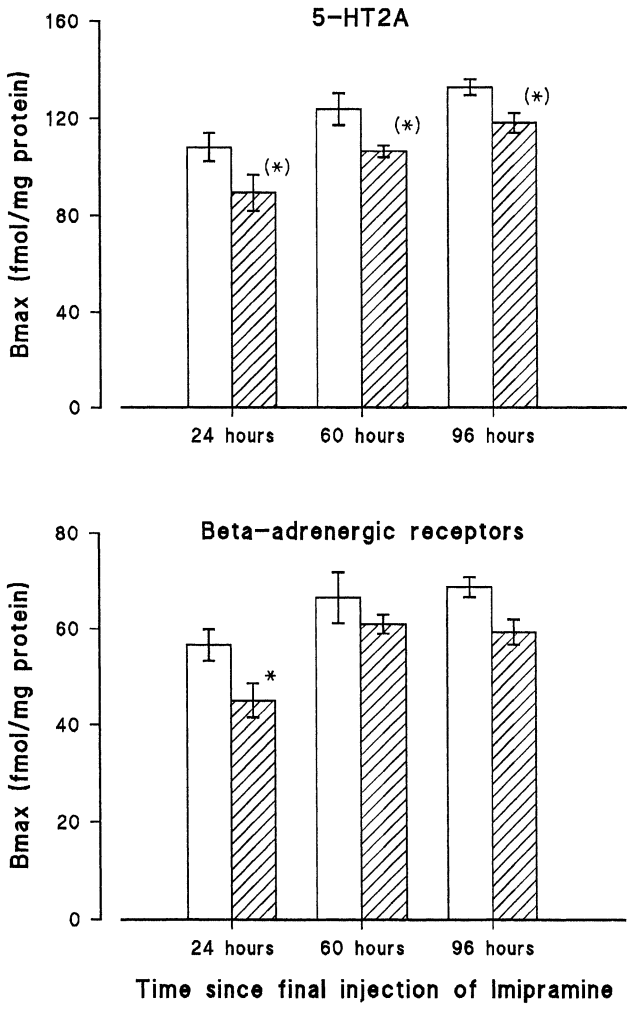

Fig. 10. Receptor assays (Experiments 2–4). Results from imipramine- (shaded bars represent imipramine in the dosage of 20 mg/kg body weight per day) and vehicle-injected control (open bars) groups assayed 24, 60, or 96 h after the final injection of imipramine. Values (expressed as femtomole per milligram of protein) are given as means with S.D. * Significantly ($P<.05$) different from the vehicle-injected control group assayed after the same posttreatment pause. (*)Significantly ($P<.05$, one-tailed) different from the vehicle-injected control group assayed after the same posttreatment pause.

4. Discussion

Previously, we (Mogensen et al., 1994) have found the effects of chronic administration of imipramine (20 mg/kg body weight per day for 15 days) on exploration measured in late tests to manifest themselves as an initial hyperexploration followed by an overhabituation. In Experiment 1 of the present communication, exactly such a pattern of behavioural changes was observed in the open field (Fig. 4)—thereby showing that the imipramine-associated behavioural changes originally (Mogensen et al., 1994) demonstrated in the vertical hole board can also be found in different experimental setups. Furthermore, Experiment 3 of the present paper demonstrated an imipramine-associated change in the "habituation of exploration" parameters (and, to a certain extent, also the "exploration" parameters) of the late-test administered object recognition test (Fig. 6). It may be noted that in the late test of exploration (Experiment 1), even "locomotion" seemed to reflect a certain level of imipramine-associated overhabituation (Fig. 3). It should, however, be remembered that the open-field locomotion test is rather sensitive to modifications in the exploratory tendencies of the animal and, consequently, modifications within the locomotion parameter could be secondary to exploratory changes.

The overall hypothesis of the present study was that associative types of learning would primarily or exclusively be impaired in early tests, while the nonassociative learning type habituation of exploration would primarily or exclusively be modified in late tests. As is summarized in Table 1, the behavioural results are in complete agreement with this prediction. As already mentioned, late tests of habituation of exploration (in Experiments 1 and 3) demonstrated imipramine-associated exploratory changes most easily explained as a modified pattern of habituation of exploration. When exploration by imipramine-treated rats was examined in early tests (Experiment 4), the results either indicated normal levels of exploration and habituation of exploration (in the object recognition test—Fig. 5) or a generally reduced level of exploration (and locomotion) (in the open-field test—Fig. 2) without an indication of modified patterns of habituation of exploration—when compared to the habituation pattern displayed by the vehicle-injected control group. Consequently, it seemed that while habituation of exploration was consistently modified in late tests, no such modifications could be observed in early tests. In early tests, however, the associative place learning (Experiment 3—Fig. 7), as well as the object recognition (Experiment 4—Fig. 5), were clearly impaired by the administration of imipramine. In late tests, neither place learning (Experiment 2—Fig. 8) nor object recognition (Experiment 3—Fig. 6) displayed any imipramine-associated impairments.

Table 1
Associative and nonassociative types of learning after chronic imipramine (20 mg/kg body weight per day for 15 days)

Early test		Late test	
Associative	Nonassociative	Associative	Nonassociative
Place learning in water maze	Habituation of exploration in open field	Place learning in water maze	Habituation of exploration in open field
Impaired (Experiment 3)	No change—reduced level of exploration (Experiment 4)	No impairment—minor modifications (Experiment 2)	Modified—initial hyperexploration followed by overhabituation (Experiment 1)
(Fig. 7)	(Figs. 1 and 2)	(Fig. 8)	(Figs. 3 and 4)
Discrimination in object recognition test	Habituation of exploration in object recognition test	Discrimination in object recognition test	Habituation of exploration in object recognition test
Impaired (Experiment 4)	No change (Experiment 4)	No impairment (Experiment 3)	Modified—reduced (Experiment 3)
(Fig. 5)	(Fig. 5)	(Fig. 6)	(Fig. 6)

Obviously, the present data only allow limited conclusions regarding effects of chronic imipramine on late tests of associative learning, since only two types of such learning have been studied. It does, however, appear interesting that while a series of highly dissimilar behavioural procedures have demonstrated closely related patterns of imipramine-associated change of habituation of exploration, two types of associative learning appear to be unimpaired by such an imipramine treatment.

As discussed previously (Mogensen et al., 1994) and in the Introduction, behavioural symptoms observed in late tests are, likely, mainly to reflect a pattern of receptor modifications initially provoked by the chronic administration of imipramine. Since such late tests are, at the earliest, initiated 24 h after discontinuation of imipramine administration, receptor regulations appear to be a more likely basis for the observed symptoms than a residual presence of the actual imipramine.

If, however, residual concentrations of imipramine should be considered the major factor provoking the late-test pattern of symptoms, it would be likely that the behavioural changes observed during early and late tests would primarily differ in magnitudes, rather than the actual pattern of behavioural modifications. One would assume that the higher imipramine concentration available during the early test would be associated with more pronounced (but essentially similar) symptoms, when compared to the results of late tests. The present demonstration of clearly contrasting patterns of behavioural changes in early and late tests, respectively, argues against the possibility that residual imipramine, per se, (rather than receptor regulations) would be the primary factor associated with the behavioural modifications observed in late tests. Although such an assumption appears to be likely, it must be remembered that certain pharmacological effects on behaviour do not reveal simple and straightforward dosage–response relationships, and that rather dissimilar patterns of effects may be seen after administration of various concentrations of a particular drug.

On the basis of the clear dissociation between the patterns of behavioural changes seen in the early and late tests of the present study, the most likely interpretation of the neurochemical basis of these symptoms is that while the modified habituation of exploration seen during late tests is a consequence of imipramine-provoked receptor regulations, the impaired associative learning observed in early tests primarily reflects the more direct consequences of the imipramine present in the brain at the moment of behavioural testing. Such consequences include (as mentioned in the Introduction) modifications of the availability of 5-HT and noradrenaline in the synaptic cleft, as well as antihistaminergic effects. It is, however, tempting to speculate that the presently observed effects on associative learning may primarily be associated with the known anticholinergic effects of imipramine (e.g., Bohman et al., 1982; Borbe and Zierenberg, 1985; El-Fakahany and Richelson, 1983; Rana et al., 1993; Richardson et al., 1984; Shaker et al., 1981; Snyder and Yamamura, 1977; Wachtel et al., 1988). Such a conclusion is supported by observations of impaired associative learning after administration of anticholinergic agents (e.g., Beninger et al., 1986, 1989; Brito et al., 1983; Hagan et al., 1987; Higashida and Ogawa, 1987; Kirk et al., 1988; Moran, 1993; Spangler et al., 1988; Spencer et al., 1985).

In the present study, the focus has exclusively been on early and late test consequences of chronic administration of imipramine. With respect to the neurochemical mechanisms mediating the behavioural results obtained in early tests, the present study does not allow a differentiation between the consequences of imipramine-provoked receptor regulations, the effects of the "acute" presence of imipramine in the brain, and potential interactions between these two factors. In future studies, it should be addressed in which way the presently examined behavioural tests are influenced by the acute administration of imipramine (given without a preceding chronic imipramine administration).

Since locomotion and exploration were both suppressed in the imipramine-treated group when tested in the early test version of the open-field test (Figs. 1 and 2), it might be speculated that apparent impairments of associative learning in the form of place learning and object recognition in early tests might be secondary to purely "motoric" disturbances during the early test phase. Such an interpretation is, however, contradicted by results obtained within the early tests of place learning and object recognition, respectively. While the early test of place learning demonstrated an initial—although transient—impairment of the quality of this associative learning process in the imipramine-treated group, the swim speeds of the same animals were never inferior to that of the vehicle-injected control group (Fig. 7). In the early test of object recognition—when imipramine-treated animals demonstrated a significantly impaired object recognition—the level of object exploration demonstrated by the imipramine-injected animals was not reduced, relative to the object exploration of the control group (Fig. 5). Consequently, it is unlikely that the impairments of associative learning seen in early tests of the imipramine-receiving group reflected "motoric", rather than cognitive changes.

The outcome of the receptor assays demonstrated that chronic treatment with imipramine in daily dosages of up to 30 mg/kg body weight did not affect the concentration of 5-HT_{1A}, 5-$HT_{1B/D}$, or dopaminergic D_2 receptors in whole brain homogenates. This does not exclude the possibility that these receptors might be regulated in individual brain regions. However, the method used in the present study cannot detect such region-specific changes. Beta-adrenergic receptor concentrations were significantly reduced at higher treatment dosages, while 5-HT_{2A} receptor concentrations were only marginally and nonsignificantly affected. This is generally in agreement with the literature (Charney et al., 1981). Experiments 2–4 confirmed those results. Here, the down-regulation of 5-HT_{2A} receptor concentration reached significance and lasted for up to 96 h, after the last imipramine injection, the latest time point studied. Beta-adrenergic receptor concentrations had already reached a level similar to that of the vehicle-treated control group by 60 h, after the final injection of the drug. This is an interesting observation and has to our knowledge not been reported before. These results support our conclusion that the effects of chronic imipramine treatment may vary with the interval between the last injection of the drug and the behavioural testing. It would be interesting in future studies to examine the regional distribution of these receptors and their regulation by imipramine treatment. This could shed light on the functional systems responsible for the behavioural changes observed at different times under and after imipramine treatment.

When considering the neural mechanisms mediating the modified pattern of habituation of exploration in late tests after chronic administration of imipramine (Mogensen et al., 1994 and Experiments 1 and 3 of the present study), it may be of relevance that we (Mogensen et al., 2003) recently have found that the habituation of exploratory activities in a vertical hole board are significantly modified by 5,7-dihydroxytryptamine-induced elimination of the brain's serotonergic systems, but only when tests were performed 14 days after the serotonergic depletion. When the same animals were tested 48 h after the neurotoxic lesion, normal patterns of habituation of exploration were found in the same behavioural test. Apparently, serotonin depletion induced receptor regulations—rather than the serotonergic depletion per se—and was responsible for the modifications of the habituation of exploration. Only future studies will be able to establish the degrees of similarities between the neural substrates of the habituation-associated changes seen after serotonergic depletion and chronic administration of imipramine, respectively. It should, however, be noted that in contrast to the previously (Mogensen et al., 1994) and presently reported pattern of hyperexploration, followed by overhabituation of exploration after chronic imipramine

treatments, neurotoxic lesions of the serotonergic systems (Mogensen et al., 2003) are associated with an underhabituation of exploration.

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