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Analgesic and behavioral effects of amphetamine enantiomers, *p*-methoxyamphetamine and *n*-alkyl-*p*-methoxyamphetamine derivatives

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Abstract

The analgesic effects of (+)- and (−)-amphetamine (AMPH), (±)-*p*-methoxyamphetamine (MA), (±)-*N*-methyl-*p*-methoxyamphetamine (MMA) and (±)-*N*-ethyl-*p*-methoxyamphetamine (EMA) were compared using two different algesimetric tests in rats. In the formalin test, (+)-AMPH elicited significant antinociception at doses of 0.2, 2 and 8 mg/kg (i.p.); (−)-AMPH was active at 2 and 8 mg/kg, but not at 0.2 mg/kg; MA elicited very potent and long-lasting antinociception; MMA was less active than MA; EMA showed significant effects only at doses of 2 and 8 mg/kg. In the C-fiber evoked nociceptive reflex assay, i.v. (+)- and (−)-AMPH were ineffective, but the methoxy derivatives showed a similar pattern of action combining inhibitory and excitatory actions. To clarify apparent discrepancies between both algesimetric tests, some behavioral motor performance tests were carried out. These tests confirm the motor stimulatory properties of (+)-AMPH, not shared by the methoxylated amphetamine derivatives. The three methoxy derivatives elicited some stereotypies related to dopaminergic activation such as grooming behavior. (+)-AMPH was also the only drug to increase the acquisition of CARs while MA and EMA were without effect. Avoidance conditioning was seriously impaired in rats injected with MMA. This conditioned behavior can be related to the significant decrease of spontaneous motor activity observed with this drug. In conclusion, the introduction of a *para*-methoxy group strongly increases the analgesic effects of amphetamine without its stimulatory behavioral effects. The introduction of *N*-alkyl substituents decreases the analgesic potency of MA.

Keywords: Analgesia; Amphetamine enantiomers; *p*-Methoxyamphetamine; Formalin test; C-fiber reflex

1. Introduction

Analgesic actions of amphetamine (1-phenyl-2-amino-propane) were first reported by Burrill et al. (1944), and since then, a number of papers have examined the antinociceptive properties of the amphetamine enantiomers, alone (Altier and Stewart, 1998; Clarke and Franklin, 1992; Frussa-Filho et al., 1996; Morgan and Franklin, 1991; Tocco et al., 1985), or in combination with other drugs such as morphine (for a review, see Dalal and Melzack, 1998). The structural framework of 1-phenyl-2-amino-propane has served as a lead for very many compounds of pharmacological interest. Amphetamine itself (AMPH, Fig. 1A) is a sympathomimetic stimulant at nominal dosages and leads to a psychotomimetic syndrome in humans only at very high levels. On the other hand, ring substitution with methoxyl and related groups leads to psychotropic compounds which are usually described as hallucinogens or psychotomimetics (Shulgin and Shulgin, 1991), such as *para*-methoxyamphetamine (PMA, 4-methoxyamphetamine, MA, Fig. 1B) or 3,4-methylene-dioxyamphetamine (MDA).

Much less is known, however, about the effects of *N*-substitution on these compounds. The *N*-methylation of

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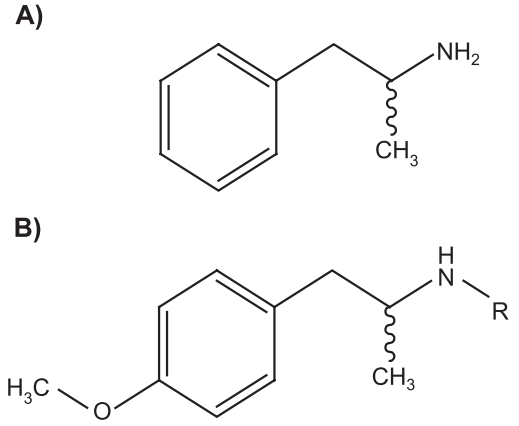

Fig. 1. Formulas of the compounds studied. (A) (+)-Amphetamine, CH_3 above plane of figure; (−)-amphetamine, CH_3 below plane of figure. (B) (±)-*p*-Methoxyamphetamine R=H; (±)-*N*-methyl-*p*-methoxyamphetamine R=CH_3; (±)-*N*-ethyl-*p*-methoxyamphetamine R=C_2H_5.

ring-unsubstituted amphetamine provides methamphetamine, which is similar to its parent compound in both potency and actions. Nevertheless, *N*-methylation of psychotomimetic ring-substituted amphetamine derivatives seems to quite generally reduce their hallucinogenic potency, an effect that is presumably related to loss of affinity for 5-HT_2 receptors (Glennon et al., 1994). In the particular case of MDA, however, this provides 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") in which not only are the hallucinogenic properties considerably weakened but other intense and subjectively different manifestations are accentuated (Davison and Parrott, 1997). MDMA has been viewed as the paradigm of a small group of drugs known collectively as entactogens (Nichols, 1986). Alternatively, the actions of MDMA have been considered to be intermediate between those of classical hallucinogens and stimulants, and the *N*-methylated analogue of MA (PMMA or MMA, Fig. 1B) has been proposed as a better training model for drug discrimination studies (Glennon et al., 1988, 1997).

Interestingly, MDMA and other *N*-substituted derivatives of MDA have analgesic or antinociceptive actions in mice and rats subjected to the hot plate, stretch reflex or tail flick tests (Braun et al., 1980; Crisp et al., 1989). One crucial conclusion of the studies in mice (Braun et al., 1980) was that increasing the bulk or the length of the *N*-substituent led to a decrease in central activity. This might be related to decreasing ability to elicit the release of dopamine and serotonin (O'Loisingh et al., 2001). Another effect of ring-substituted amphetamine derivatives which decreases with *N*-alkylation is the selective, reversible inhibition of monoamine oxidase A (Scorza et al., 1997; Hurtado-Guzmán et al., 2003), which might indirectly affect the availability of monoamine neurotransmitters.

For this reason, we have tested MA and its *N*-methyl- (MMA) and *N*-ethyl (EMA) derivatives (Fig. 1B) in order to determine if in this family of compounds *N*-substitution also affects the analgesic or antinociceptive properties which MA might exhibit. To this end, we have used two different algesimetric tests: the formalin test, which is a model of persistent pain performed in freely moving rats where pain and analgesia may be observed in the context of the animal's ongoing behavior, and the C-fiber reflex as a model of intermittent acute pain performed in anesthetized rats that is eminently suitable for evaluating the effects of putative central analgesic drugs because it does not involve inflammatory processes. We also characterized the in vivo actions of amphetamine and its derivatives in models in which dopaminergic mechanisms are strongly implicated, such as spontaneous motor activity and acquisition of conditioned avoidance responses (CARs; for a review, see Missale et al., 1998) to allow a better analysis of the results and clarify discrepancies between the two algesimetric tests.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats weighing 250–300 g were housed six per cage in Plexiglas cages in an animal room at 21±2 °C, under a 12:12 light/dark cycle (lights on from 08:00 to 20:00 h) with free access to water and commercial food pellets. To avoid sex differences in pain threshold (Cruz et al., 1996) and in the motor effects of AMPH (Forgie and Stewart, 1994; Díaz-Véliz et al., 1994), in the formalin test and in the behavioral studies only male rats were used, but in the C-fiber reflex algesimetric test, performed in anestethized animals, both male and female rats were used. After each experiment the rats, which were used only once, were killed with an overdose of urethane (10 g/kg i.p.) according to ethical guidelines of the International Association for the Study on Pain (The Committee for Research and Ethical Issues of I.A.S.P., 1980). The experimental protocols were approved by the Faculty of Medicine Ethics Committee.

2.2. Drugs

All drugs were dissolved in saline (NaCl 0.9%) and injected in a volume of 2 ml/kg. (−)-AMPH sulfate was purchased from Sterling-Winthrop Research Institute (New York, NY), and (+)-AMPH sulfate and NaCl were from Sigma (St. Louis, MO). The (±)-methoxy derivatives were synthesized in our laboratories by standard procedures: MA was prepared by lithium aluminum hydride reduction of 1-(4-methoxyphenyl)-2-nitropropene; MA was converted to its *N*-ethoxycarbonyl and *N*-acetyl derivatives by reaction with the corresponding acid chlorides, and the resulting amides were reduced to MMA and EMA, respectively, with lithium aluminum hydride. The methoxy derivatives were converted into their hydrochlorides and administered as such.

2.3. Formalin test

Each rat was placed in a Plexiglas cage (30×30×30 cm), in a soundproof room for 45 min before receiving an injection of formalin. An injection of 50 μl of sterile 5% formalin solution was then administered under the skin of the dorsal surface of the right forepaw and the pain responses were rated for 30 min. Saline or drugs were injected intraperitoneally (i.p.) 30 min before the formalin injection. Numerical values were assigned to the pain responses according to the rating scale for rats described by Dubuisson and Dennis (1977). The rating score is as follows: (0) both forepaws are placed on the floor and weight is evenly distributed; during locomotion, there is no discernible favoring of the uninjected paw; (1) the injected paw rests lightly on the floor and during locomotion there is an obvious limp; (2) the injected paw is elevated, and not in contact with any surface; (3) the injected paw is licked, bitten or shaken, while the uninjected paw is not. Rats were observed individually and the evolution of pain scores under each condition was recorded continuously over 30 min each time when changed. Numerical ratings of pain were calculated from the following formula:

$$\text{Pain Score} = \frac{T_1 + 2T_2 + 3T_3}{180}$$

where T_1, T_2 and T_3 are the times (in seconds) spent in states 1, 2 or 3, respectively, during 180 s (3-min block) in graphs 2–6A. A similar procedure was used to evaluate the overall pain score during the whole observation period (30 min), shown in Table 1.

Table 1
Algesimetric evaluations in the formalin test in rats

Group	Dose, mg kg^{-1}	Pain score, mean±S.E.M. (n)
Baseline without formalin		0.411±0.059 (15)
Saline control		2.304±0.044 (12)$^{\#}$
(+)-AMPH	0.2	1.661±0.282 (4)*
	2.0	0.260±0.131 (6)*
	8.0	0.282±0.119 (6)*
(−)-AMPH	0.2	2.143±0.053 (4)
	2.0	1.142±0.168 (6)*,†
	8.0	1.159±0.143 (6)*,†
MA	0.2	0.831±0.072 (4)*,†
	2.0	0.376±0.070 (5)*
	8.0	0.038±0.033 (4)$^{\#}$,*
MMA	0.2	1.704±0.140 (4)*,‡
	2.0	1.376±0.163 (5)*,‡
	8.0	0.760±0.101 (4)*,‡
EMA	0.2	2.190±0.088 (4)‡,§
	2.0	1.850±0.160 (4)*,‡
	8.0	1.642±0.094 (4)*,‡,§

Levels of significance calculated using a non-parametric analysis (Mann–Whitney rank sum test) are as follows:
* $p<0.05$ compared with saline control.
$^{\#}$ $p<0.05$ compared with baseline without formalin.
† $p<0.05$ compared with the same dose of (+)-AMPH.
‡ $p<0.05$ compared with the same dose of MA.
§ $p<0.05$ compared with the same dose of MMA.

2.4. C-fiber algesimetric test

The general procedure was essentially similar to that described previously (Falinower et al., 1994). Male and female rats were anesthetized with urethane (1 g/kg i.p.); this dose of urethane produces a deep and long-lasting hypnotic effect and no changes in heart rate in response to noxious stimulation. Body temperature was maintained at 37±0.5 °C by means of a homeothermic blanket system. Saline or drugs were injected intravenously. Electrophysiological recordings of C-fiber reflex activity elicited by electrical stimulation of the receptive field of the sural nerve were made in the ipsilateral biceps femoris muscle. For this purpose, a pair of non-insulated platinum–iridium needle electrodes were inserted subcutaneously (s.c.) in the medial part of the fourth and the lateral part of the fifth toe. Electromyographic (EMG) responses were recorded with another pair of non-insulated Pt–Ir needles, which were inserted through the skin into the biceps femoris muscle. The electrical stimuli were single-square shocks of 2 ms duration and were delivered once every 10 s (0.17 Hz) from a Grass S44 stimulator. The stimulus intensities and the EMG responses were fed to an oscilloscope for continuous monitoring and to a computerized system for on-line digitalization. The digitalized EMG responses were full-wave rectified and the C-fiber evoked responses were integrated within a time window from 150 to 450 ms after the stimulus onset. The integrated individual reflex responses were plotted against time to allow the study of their time courses. All individual experiments began with a control period during which the characteristics of the reflex were determined; 20–30 min after beginning the application of 15 V stimuli to the sural nerve, stable reflex responses with minimal spontaneous fluctuations were achieved. This preliminary finding was regarded as a prerequisite to start the pharmacological procedures. Following the stabilization period (at least 20 min), constant stimulation (3× threshold voltage) was applied. During the first 10 min, the stability of EMG responses was checked. The mean of 60 successive C-fiber reflex responses corresponding to the 10-min period preceding the drug injection was taken as the mean control value. Each individual EMG response at this intensity of stimulation was expressed as a percentage of this mean control value. The results for each individual animal were expressed ultimately as the means of 18 successive responses, which corresponded to a 3-min period in the procedure. Electrical stimulation was applied throughout a 30-min period following drug injection. A graph of the time course of the EMG response was drawn in order to evaluate the drug action at constant stimulation, allowing the area under the curve representing the effect observed during 30 min to be calculated. The results were then expressed in terms of percentage changes of the area under the curve

(%AUC), where 100% represented complete blockade of the C-fiber EMG signal during 30 min. The C-fiber reflex experiments were performed in animals that were anesthetized with urethane and subsequently sacrificed with an overdose of the same drug. For this purpose, an intravenous route was preferred from the beginning to allow a rapid onset of action with 100% bioavailability.

2.5. Behavioral studies

Saline or drugs were injected i.p. 30 min before behavioral tests. The rats were submitted to behavioral experiments by using a fixed design: spontaneous motor activity followed by conditioned avoidance training.

2.5.1. Spontaneous motor activity

Each animal was individually placed in a Plexiglas cage (30×30×30 cm). The floor of the cage was an activity platform (Lafayette Instruments, Lafayette, IN) connected to an electromechanical counter. To avoid the influence of disturbing noises, the platform was placed in a soundproof chamber and the observations were made through a closed TV circuit. Spontaneous motor activity was recorded every 5 min during a 30-min period and was expressed as counts. Simultaneously, the number of times each animal reared and the time (s) spent in grooming behaviour were also recorded. Scores were generated from live observations and video sequences were used for subsequent reanalysis.

2.5.2. Active avoidance conditioning

Each rat was individually placed in a two-way shuttle box (Lafayette Instruments) composed of two stainless-steel modular testing units. Each unit was equipped with an 18-bar insulated shock grid floor, two 28-V DC lights and a tone generator (Mallory Sonalert 2800 Hz, Lafayette Instruments). Electric shocks were provided to the grid floor by a Master shock supply (Lafayette Instruments). The rats were trained over 50 trials, after a 5-min period of habituation. The trial consisted of the presentation of a tone that after 5 s was overlapped with a 0.20-mA foot-shock until the animal escaped to the opposite chamber, with maximum shock duration of 10 s. A conditioned avoidance response was defined as a crossing to the opposite chamber within the first 5 s (tone alone).

2.6. Statistical analysis

The results were expressed as means±S.E.M. for a number (n) of animals as indicated in the tables. An analysis of variance (ANOVA) by calculation of Fischer's F factor was used for examine treatment as a variable, considering the three doses assayed and saline as control. In the formalin test, ANOVA was followed by a Mann–Whitney rank sum test for non-parametric analysis. In the C-reflex, ANOVA was followed by Tukey's post hoc test. The percentages calculated for the C-fiber reflex and shown in the graphs and the tables were transformed by means of an angular transformation (arcsin) to make the variances more uniform. Such a transformation is considered to produce more reliable results when proportional data are subjected to statistical analysis (Zar, 1974). Behavioral results were analyzed using one-factor repeated measures ANOVA, followed by post hoc Newman–Keuls's multiple comparison test, when appropriate. In all cases, alpha was set at 0.05.

3. Results

3.1. Formalin test

Pain rating curves for successive 3-min blocks are shown in Figs. 2–6A. Overall pain scores for the whole 30-min observation period are shown in Table 1. The pain-free baseline was evaluated in 15 rats during 30 min before formalin injection but only the latter 9 min (−10 to 0 min time period) are shown in the figures. During this period, the rats showed some tendency to explore the box and the average rating (0.41±0.06, n=15) was due entirely to rearing and grooming. The formalin injection (performed at zero time in the figures) caused a noticeable withdrawal of the injected paw characterized by shaking, licking and biting; locomotion was performed raising the injected paw or there was an obvious limp. The pain scores of rats from the saline condition were significantly higher than those from the pain-free baseline condition (p<0.001).

3.1.1. (+)-Amphetamine

The lowest dose of this compound (0.2 mg/kg i.p.) elicited weak but significant (p<0.05) antinociceptive activity, which is reflected in both the time course (see Fig. 2A) and the pain score (see Table 1). Furthermore, when doses were increased to 2 and 8 mg/kg, complete suppression of the aversive behavior evoked by the s.c. injection of 5% formaldehyde solution was achieved. It may be seen in Fig. 2A that the curves for the two higher doses show continuity with the pain-free baseline. In addition, Table 1 shows that the pain scores for 2 or 8 mg/kg and the pain-free baseline are similar and significantly lower than that for control saline-treated rats (p<0.001). It is obvious from these results that the maximum effect is reached with 2 mg/kg of (+)-AMPH. However, a clear dose–response relationship was established (F(3,24)=95.7, p<0.001).

3.1.2. (−)-Amphetamine

The dose of 0.2 mg/kg i.p. of (−)-AMPH was devoid of antinociceptive activity, both with regard to the pain score shown in Table 1 and the time course (Fig. 3A), which were similar to those of the saline control group. In contrast, 2 mg/kg induced a significant reduction in pain scores for the whole 30-min period; regarding the time course, a highly significant peak of antinociceptive activity was observed 9–

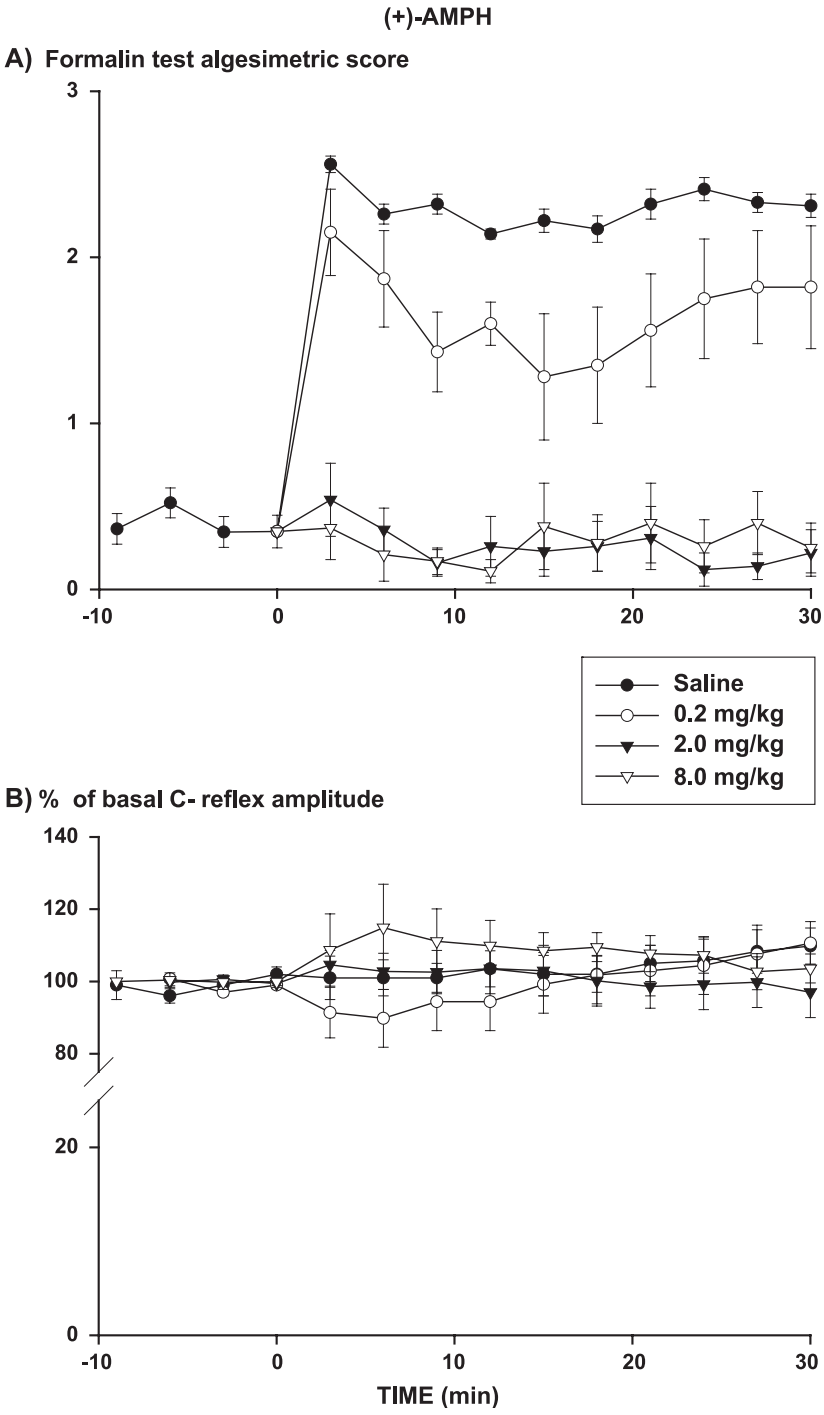

Fig. 2. Time courses of the effects of (+)-amphetamine ((+)-AMPH) in the formalin test (A) and in the C-fiber reflex (B). The doses assayed were 8 (open triangles), 2 (closed triangles) and 0.2 (open circles) mg kg^{-1}. Saline solution, used as control, was injected in a volume of 2 ml/kg (closed circles). The points represent the mean±S.E.M. (*n* as indicated in Tables 1 and 2).

21 min after the formalin injection. Interestingly, 8 mg/kg of (−)-AMPH did not induce greater changes in pain rating scores than 2 mg/kg, and the time courses of both doses were similar, indicating that a ceiling effect had been reached (see Fig. 3A). When compared with its enantiomer, (−)-AMPH showed weaker analgesic activity (significance in Table 1).

3.1.3. p-Methoxyamphetamine

When administered in doses ranging from 0.2 to 8 mg/kg i.p., MA produced significant antinociceptive effects (Fig. 4A). It must be noted that the pain score with 8 mg/kg is nearly zero and significantly lower than the baseline value without formalin ($p<0.05$), reflecting the relative immobility of the rats with this dose, which were akinetic

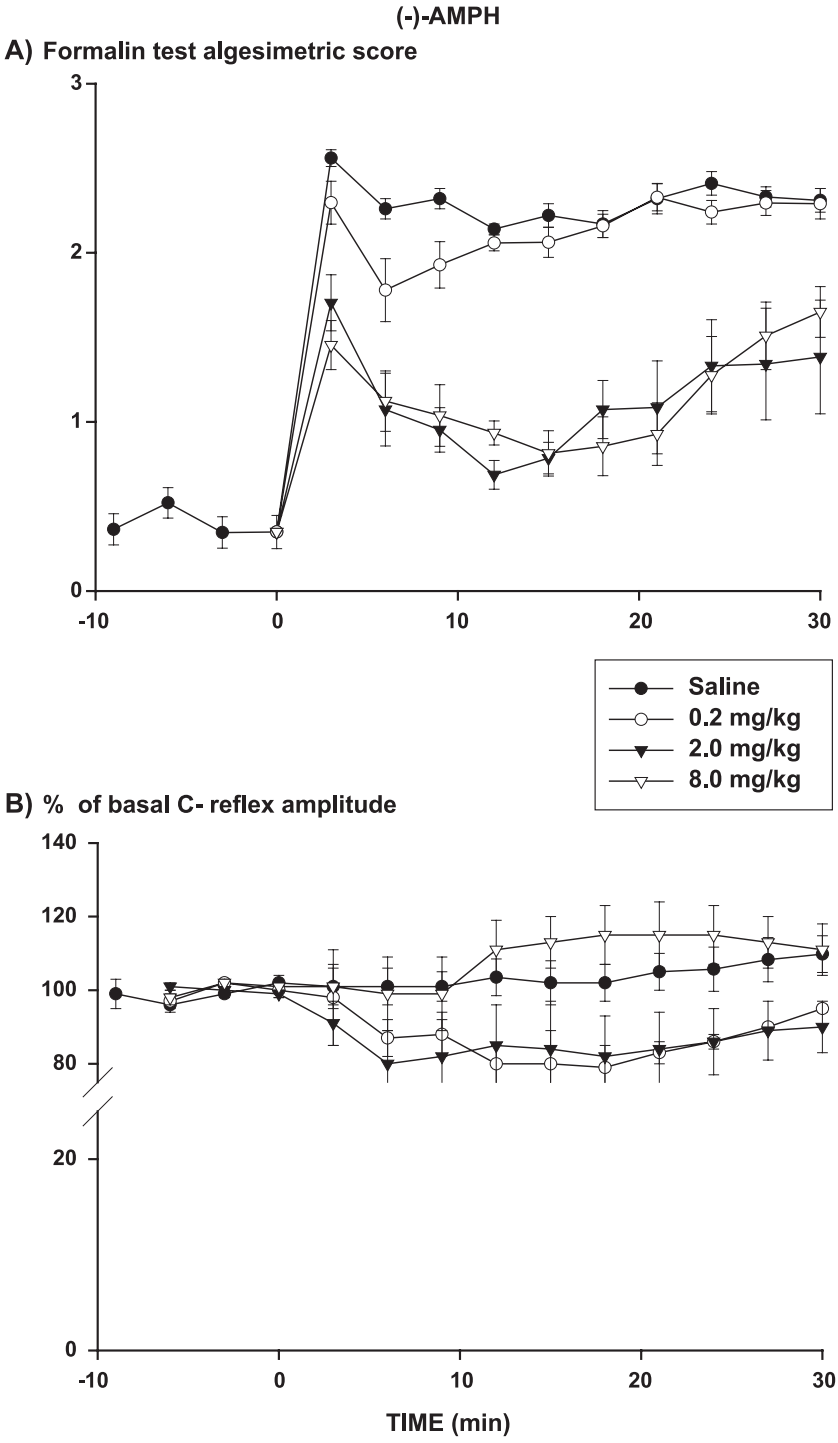

Fig. 3. Time courses of the effects of (−)-amphetamine ((−)-AMPH) in the formalin test (A) and in the C-fiber reflex (B). The doses assayed were 8 (open triangles), 2 (closed triangles) and 0.2 (open circles) mg kg^{-1}. Saline solution, used as control, was injected in a volume of 2 ml/kg (closed circles). The points represent the mean±S.E.M. (*n* as indicated in Tables 1 and 2).

without grooming or exploratory activity. Also, during their brief and rare motions, the injected paw was placed normally on the floor. The pain scores of the groups treated with 0.2 and 2 mg/kg were lower than the saline control value, showing a clear antinociceptive activity of MA in this test (see Table 1). The graphic expression of the time course in Fig. 4A shows that the antinociceptive effects of 8 and 2 mg/kg of MA were rapidly installed after the formalin injection and did not change during the whole 30 min observation period. However, the time course for the group treated with 0.2 mg/kg was more variable because the strongest antinociceptive effect was only attained after 12 min, although all points of this curve lie significantly below the control curve.

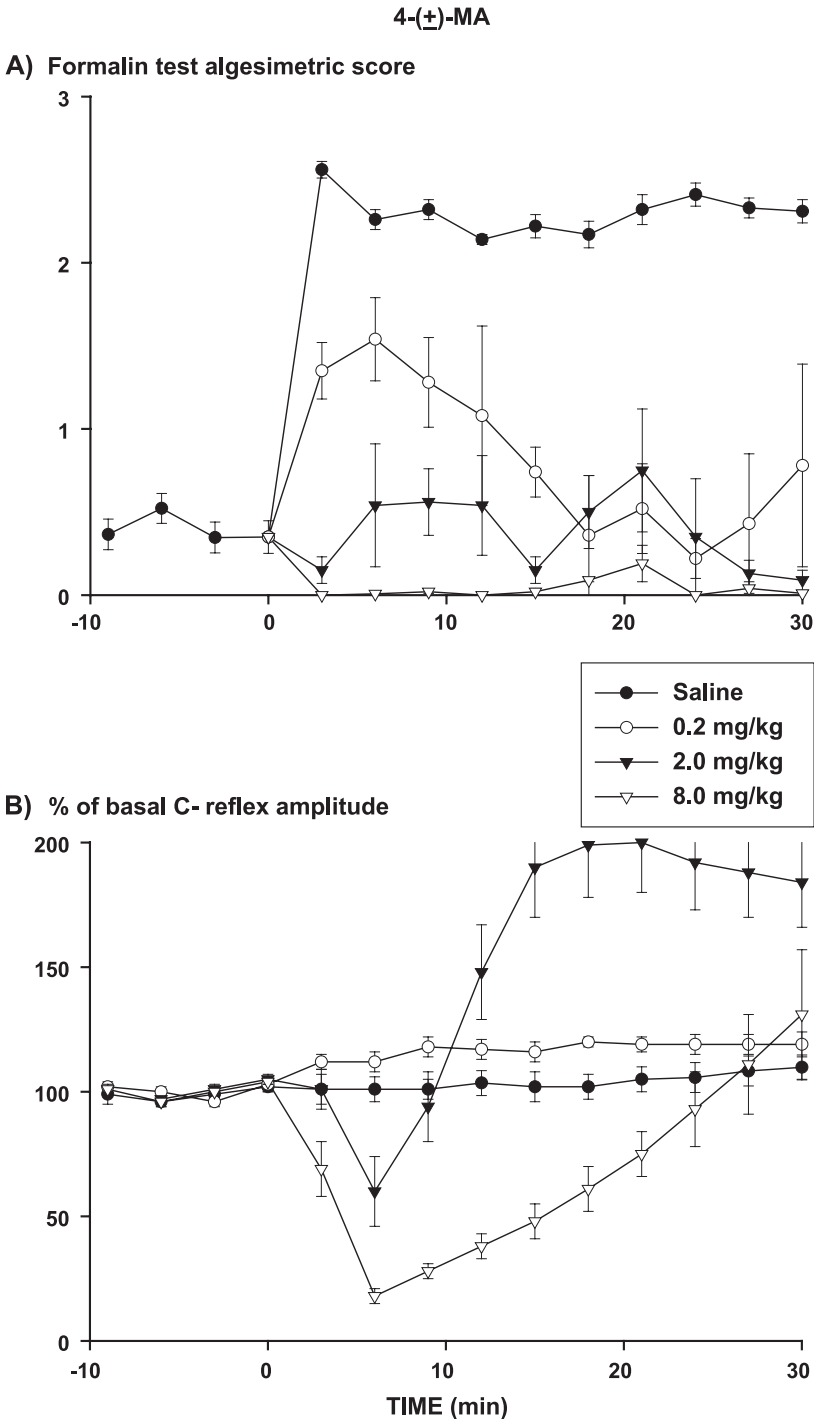

Fig. 4. Time courses of the effects of (±)-*p*-methoxyamphetamine ((±)-MA) in the formalin test (A) and in the C-fiber reflex (B). The doses assayed were 8 (open triangles), 2 (closed triangles) and 0.2 (open circles) mg kg^{-1}. Saline solution, used as control, was injected in a volume of 2 ml/kg (closed circles). The points represent the mean±S.E.M. (*n* as indicated in Tables 1 and 2).

3.1.4. N-Methyl-p-methoxyamphetamine (MMA)

This substance produced a significant dose-related antinociceptive effect ($F(3,21)=325$, $p<0.001$). Moreover, with the highest dose of this compound (8 mg/kg i.p.), the rats still exhibited exploratory behavior during their displacements, establishing a clear difference with the groups treated with MA (significance in Table 1). Fig. 5A shows the time course of the MMA profile. It can be seen that with the lowest dose (0.2 mg/kg), a progressive antinociceptive effect was observed during the initial half-period, and then a nociceptive rebound was seen from min 18 until the end; with 2 mg/kg, the profile was rather similar with a less pronounced rebound. In contrast, 8 mg/kg produced significant antinociception from the beginning until minute 30, with the

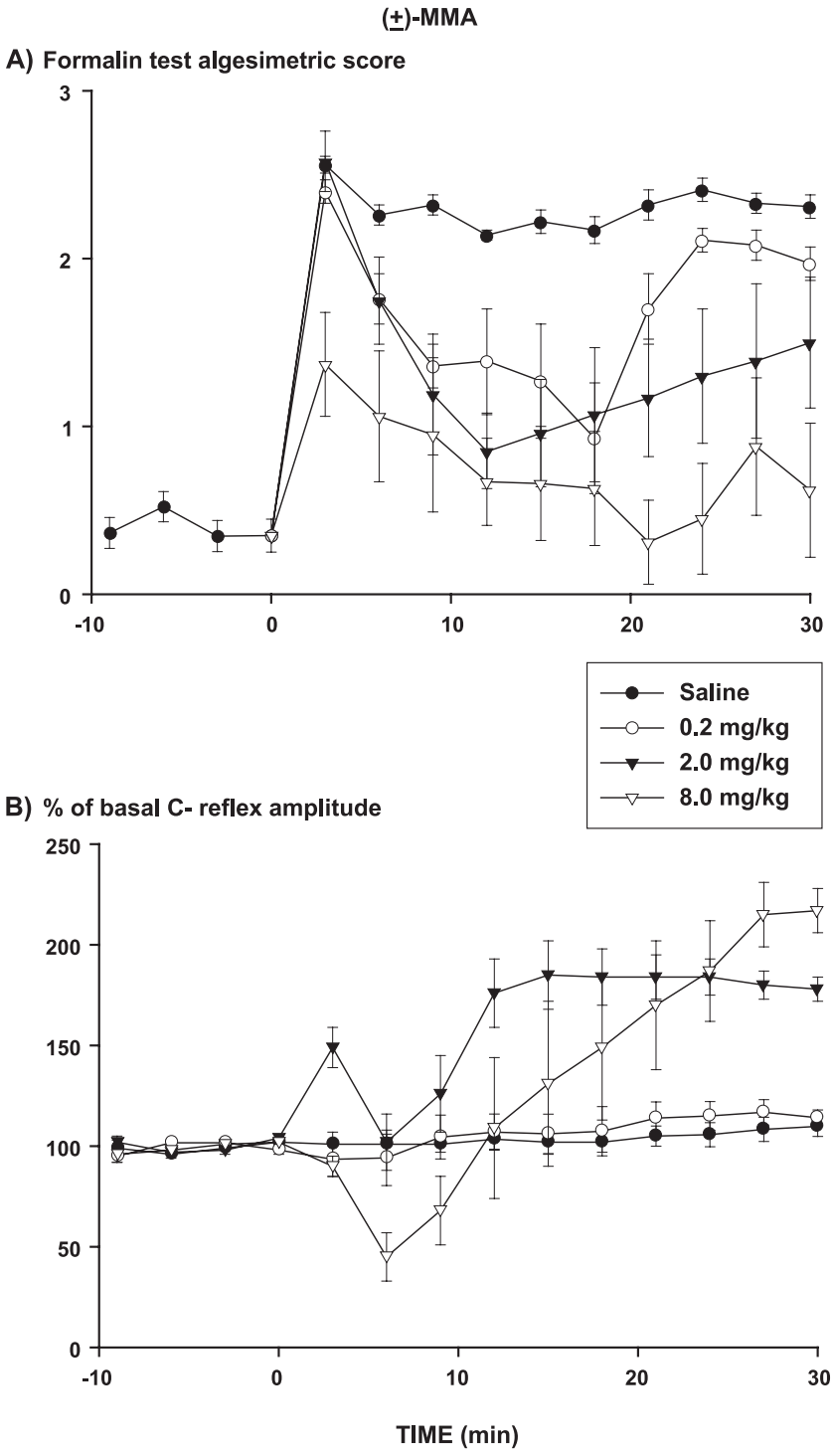

Fig. 5. Time courses of the effects of (±)-*N*-methyl-*p*-methoxyamphetamine ((±)-MMA) in the formalin test (A) and in the C-fiber reflex (B). The doses assayed were 8 (open triangles), 2 (closed triangles) and 0.2 (open circles) mg kg^{-1}. Saline solution, used as control, was injected in a volume of 2 ml/kg (closed circles). The points represent the mean±S.E.M. (*n* as indicated in Tables 1 and 2).

pain scores remaining constant from 6 to 24 min, to finish with a slightly higher value at the end.

3.1.5. N-Ethyl-p-methoxyamphetamine (EMA)

This compound showed the weakest activity of the three methoxy derivatives in the formalin test. It did not produce any clear or obvious behavioral alterations in the rats subjected to the nociceptive stimulus, although following the evaluation proposed by Dubuisson and Dennis (1977) a significant dose-related antinociceptive effect emerged by ANOVA for the treatment ($F(3,20)=14.2$, $p<0.001$), in spite of the non-significance at the lower dose (see Table 1). In

fact, the time course of the lowest dose is similar to that of saline, but 2 and 8 mg/kg i.p. produced significant antinociceptive effects in the 30-min period ($p<0.001$). In Fig. 6A, it can be seen that the curve corresponding to 0.2 mg/kg of EMA may be superimposed on the saline curve; in contrast, several time points of the 2 and 8 mg/kg curves were statistically different from saline points.

3.2. Nociceptive C-fiber reflex

Saline administration (2 ml/kg i.v.) had no effect on either the amplitude or the duration of the reflex. As may be seen in Figs. 2–6B, there were only minor variations in the amplitude during the 30-min recording period, and consequently the % variation in AUC was approximately 0.

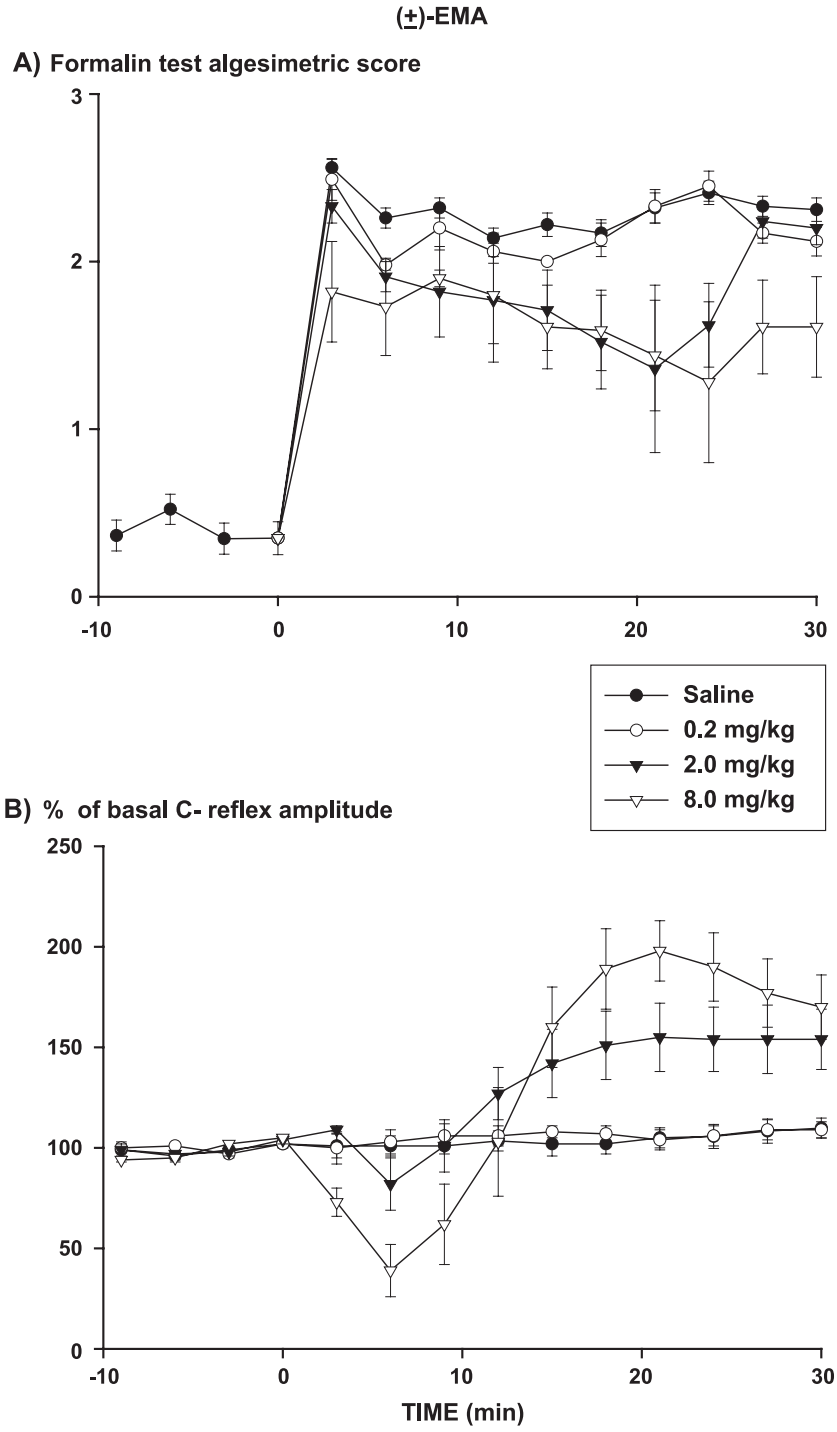

Fig. 6. Time courses of the effects of (±)-*N*-ethyl-*p*-methoxyamphetamine ((±)-EMA) in the formalin test (A) and in the C-fiber reflex (B). The doses assayed were 8 (open triangles), 2 (closed triangles) and 0.2 (open circles) mg kg^{-1}. Saline solution, used as control, was injected in a volume of 2 ml/kg (closed circles). The points represent the mean±S.E.M. (*n* as indicated in Tables 1 and 2).

3.2.1. (+)-Amphetamine

The i.v. administration of (+)-AMPH at the highest dose (8 mg/kg) produced severe disturbances in the autonomic equilibrium of the rat, as revealed by changes in the cardiac and respiratory frequencies and massive salivation. Also, it was noted that the rats woke up and performed some aversive movements, indicating a decrease in the depth of anesthesia, in spite of the usually stable condition achieved with 1 mg/kg urethane. Thus, an increase in the amplitude and duration of the C-fiber reflex was observed, as shown in Fig. 2B and in Table 2, as a negative value of %AUC which, however, did not differ significantly from that obtained with saline, reflecting the lack of antinociceptive activity. With 2 and 0.2 mg/kg, the autonomic disturbances were less pronounced and only minor changes in anesthetic stability were observed. Regarding the reflex characteristics, a non-significant decrease (~15%) was observed in both its amplitude and its duration; these effects, however, do not seem to be dose-related judging from the similarity in the %AUC for both dose levels ($F(3.14)=0.37$, $p=0.8$).

3.2.2. (−)-Amphetamine

No changes were observed in the amplitude or duration of the C-fiber evoked EMG signal after the administration of 8, 2 or 0.2 mg/kg i.v. of this enantiomer (Fig. 3B); thus, when calculating %AUC, values were close to zero and similar to those obtained with saline ($F(3.14)=2.55$, $p=0.1$), reflecting the lack of antinociceptive activity as evaluated by this methodology. Regarding other effects, the lower dose (0.2 mg/kg) did not induce any visible changes in the autonomic responses; with 2 mg/kg only mydriasis and brief (5 min) changes in respiratory frequency were observed but after the administration of 8 mg/kg of (−)-AMPH, increases in respiratory frequency, salivation, mydriasis and piloerection were seen.

Table 2
Percentage of variation of the area under the curve in the C-fiber reflex algesimetric procedure

Group	Saline, 2 ml kg^{-1}	0.2 mg kg^{-1}	2 mg kg^{-1}	8 mg kg^{-1}
Saline	−4±5 (4)			
(+)-AMPH		13±3 (4)	15±9 (5)	−9±7 (5)
(−)-AMPH		0±7 (5)	−1±6 (5)	−4±6 (4)
MA		−17±3 (4)†	−56±14 (5)†,*	33±11 (4)†,*
MMA		−7±9 (4)	−65±10 (4)†,*	38±22 (4)‡
EMA		−6±4 (4)	33±11 (4)†,‡,§	50±23 (4)†,‡

Values are expressed as the mean±S.E.M. for a number (n) of animals indicated between brackets. Positive values represent net antinociceptive effects in the whole 30-min period; negative values indicate excitatory effects. For statistical purposes, data were transformed to arcsin function and then subjected to ANOVA followed by Tukey's post hoc test for single comparisons between groups.
Levels of significance are as follows:
* $p<0.05$ compared with saline control.
† $p<0.05$ compared with same dose of (+)-AMPH.
‡ $p<0.05$ compared with same dose of MA.
§ $p<0.05$ compared with same dose of MMA.

3.2.3. MA and derivatives

The three methoxylated compounds caused large changes in the C-fiber reflex, but the effect on the area under the curves was minimized because of the combination of inhibitory and excitatory effects that this data expression does not discriminate, during the 30-min period; for this reason, we chose to analyze the time course of the curves shown in graphs 4–6B instead of the area under the curves. In general, the time course profiles were similar for all the MA analogues, but quantitative variations were seen. In fact, with doses of 8 mg/kg i.v. of all three derivatives, a biphasic effect was observed which began with a marked inhibition of the C-fiber reflex lasting 21 min for MA and 6–9 min for the *N*-alkyl derivatives; then the reflex activities recovered to the control values (100%) in the case of MA while, with MMA and EMA, activation (in excess of 200%) was commonly observed after the initial inhibitory phase (see Figs. 4–6B). Doses of 2 mg/kg i.v. produced a similar time course, with slight differences reflecting the different potencies of the three compounds. For all of them, a less pronounced inhibitory phase and a longer excitatory phase were observed than with 8 mg/kg. At 2 mg/kg, MA was the only compound showing depression of reflex activity (i.e., antinociception) immediately after injection, while the *N*-alkyl derivatives did not show this. On the other hand, the activating effects of MA and MMA were similar and more intense than the effects of the *N*-ethyl derivative (see Figs. 4–6B). Doses of 0.2 mg/kg i.v. produced activation (~15%, $p>0.05$) of the reflex activity when MA was used. This activating effect of MA was constant throughout the whole 30 min observation period (see Fig. 4B and Table 2). The *N*-methyl and *N*-ethyl derivative-treated animals did not show significant differences from saline-treated controls (see Figs. 5 and 6B). Table 2 summarizes the overall effect of each substance for 30 min (%AUC), showing that MA at 8 mg/kg was the only compound giving a positive value (33±11, $n=4$) corresponding to antinociceptive effects; on the contrary, the *N*-alkyl derivatives showed net negative values of the %AUC, reflecting the EMG activation induced by these drugs.

3.3. Behavioral studies

The behavioral changes induced by (+)-AMPH and the three racemic methoxy derivatives are summarized in Table 3. All three methoxy compounds were assayed at doses of 8 mg/kg i.p., while (+)-AMPH, the most active enantiomer with regard to motor performance over a wide range of doses (Maickel et al., 1982), was assayed at 2 mg/kg. Previous reports from our laboratory have shown that this dose induces stimulatory effect on motor activity with a maximal response on conditioned behavior (Mora and Díaz-Véliz, 1983).

3.3.1. Spontaneous motor activity

This parameter represents the overall motor behaviors exhibited by the rats throughout the 30-min observation

Table 3
Effects of (+)-amphetamine and three methoxy derivatives on spontaneous motor responses and CARs

Group	Motor activity [counts]	Rearing [events]	Grooming [s]	CARs [%]
Saline	880±81	46±5	356±26	34±6
(+)-AMPH	2430±197*	89±9*	128±15*	73±2*
MA	738±88†	36±4†	72±14*,†	33±6†
MMA	277±39*,‡	25±5*	163±20*,‡	3±6*,‡
EMA	572±60*,§	36±4	404±63‡,§	34±3§

The results are expressed as the mean±S.E.M. All compounds were administered in doses of 8 mg kg^{-1} (n=8) with the exception of (+)-AMPH which was administered at a dose of 2 mg kg^{-1} (n=15). The motor behavioral responses were observed over a 30-min period. CARs represent the percentage of conditioned avoidance responses for 50 trials. Data comparing the treatment effect were analyzed using one-factor repeated measures ANOVA, followed by post hoc Newman–Keuls's multiple comparison test, when appropriate.
Levels of significance are as follows:
* p<0.05 when compared with saline.
† p<0.05 when compared with (+)-AMPH.
‡ p<0.05 when compared with MA.
§ p<0.05 when compared with MMA.

period and is the best indicator to assess the central effects of drugs that modify motor performance. One-way ANOVA revealed a significant effect of the treatment (F(4,42)=40.13, p<0.0001) on spontaneous motor activity. Subsequent Newman–Keuls test indicated that in the (+)-AMPH-injected rats, motor activity was significantly increased (p<0.001) as compared with the saline controls. MA at a dose of 8 mg/kg i.p. had no significant effect on spontaneous motor activity, but both N-alkyl derivatives significantly decreased this behavior. However, when evaluating the depressive effects on motor activity elicited by the N-alkyl derivatives, only MMA, but not EMA, induced significant motor depression when compared with the parent compound MA.

3.3.2. Rearing

One-way ANOVA revealed a significant effect of the treatment (F(4,42)=16.15, p<0.0001) on rearing behavior. Subsequent Newman–Keuls test indicated that only (+)-AMPH significantly enhanced the number of rearings (p<0.001). The administration of MA and EMA was unable to induce any significant change in this behavior. By contrast, the N-methyl derivative significantly diminished the number of rearings as compared with saline-injected rats (p<0.05).

3.3.3. Grooming

One-way ANOVA revealed a significant effect of the treatment (F(4,42)=23.35, p<0.0001) on grooming behavior. Subsequent Newman–Keuls test indicated that (+)-AMPH, MA and MMA significantly decreased the time spent in grooming behavior (p<0.001). The three methoxy derivatives proved to have decreasing potencies in modifying this behavior with increasing size of the N-substituent.

3.3.4. Conditioned avoidance responses

One-way ANOVA revealed a significant effect of the treatment (F(4,42)=46.94, p<0.0001) on conditioning avoidance responses. Subsequent Newman–Keuls test indicated that (+)-AMPH was the only drug that produced significant enhancement of the acquisition of CARs (p<0.001). The administration of MA and EMA failed to induce significant changes on this behavior. However, after MMA administration, the acquisition of CARs was seriously impaired (p<0.001).

3.3.5. Other observations

Stereotyped behavior consisting of highly repetitive sniffing, licking and biting was observed in freely moving rats when injected with 8 mg/kg i.p. (+)-AMPH, but these signs were not observed even after high doses of the methoxy derivatives, in agreement with previous work (Tseng and Loh, 1974). All compounds, including (+)-AMPH, produced marked sympathomimetic signs such as piloerection, exophthalmus and increased respiratory rate.

4. Discussion

The most important findings of this work are: (1) MA and its N-methyl and N-ethyl derivatives exert dose-related antinociceptive activity in rats, as demonstrated in the formalin test; (2) the N-unsubstituted compound MA shows the highest potency; (3) N-substitution of MA with methyl and ethyl groups is associated with progressively weaker antinociceptive activity according to the chain length; and (4) all compounds tested clearly induce autonomic disturbances and alterations of normal behavior.

The literature records much evidence for the antinociceptive actions of amphetamine (Burrill et al., 1944; Tocco et al., 1985; Morgan and Franklin, 1991; Clarke and Franklin, 1992; Frussa-Filho et al., 1996; Connor et al., 2000). There are also some references to the antinociceptive activity of MDA and some of its N-substituted congeners such as MDMA (Braun et al., 1980; Crisp et al., 1989; Morley-Fletcher et al., 2002). No references were found, however, in relation to the antinociceptive actions of the simpler p-methoxyamphetamine and its N-alkyl-substituted analogues which were studied in this work. The p-methoxy derivatives seem to be more potent than at least MDA, because the latter compound only suppressed writhing and increased latencies in the hot plate and tail flick tests with doses in excess of 10 mg/kg (Braun et al., 1980) while MA and its N-alkyl derivatives, in this study, were clearly active at doses below 10 mg/kg i.p. in the formalin test. Nevertheless, MDMA, the N-methylated analogue of MDA, seems to be as potent as the p-methoxy derivatives because in the dose range of 1.5–6.0 mg/kg i.p., MDMA produced a dose-dependent elevation in hot-plate latency (Crisp et al., 1989) and was five times more potent than MDA to suppress stretch reflexes (Braun et al., 1980).

Our results in Tables 1 and 2 confirm that the amphetamine enantiomers show different antinociceptive actions (Tocco et al., 1985); in addition, they also exhibit different profiles of motor stimulatory activity and stereotypy. Single doses of (+)-AMPH in the 0.5–4.0 mg/kg range produced the expected dose-related increases in spontaneous motor activity, while (−)-AMPH (0.25–8.0 mg/kg) evoked an initial (0–15 min) decrease in activity followed by increased activity (30–60 min) of lesser magnitude than that elicited by the (+) isomer (Martin-Iverson et al., 1991). In algesimetric studies using the hot plate procedure or the tail flick test, it was clear that the effects of (−)-AMPH, while weak, were generally more intense and had a greater duration of action than those elicited by (+)-AMPH. However, when the writhing test was used, no differences were detected between the two isomers (Tocco et al., 1985). On the contrary, in our study using the formalin test, we found that (+)-AMPH was more potent than its counterpart regarding both the effect of the lowest dose or the maximal effects achieved with 2 and 8 mg/kg i.p. (see Table 1).

The formalin test has several advantages when compared with other traditional algesimetric tests because no restraint is placed on the rat, and therefore pain and analgesia may be observed in the context of the animal's ongoing behavior; however pain thresholds are not measured. Conversely, the nociceptive flexion reflex elicited by activation of C-fiber cutaneous afferents is eminently suitable for evaluating the effects of putative central analgesic drugs because it does not involve inflammatory processes; therefore, considering that the C-fiber reflex is regarded as a spinal nociceptive reflex which is only modulated by higher influences (Falinower et al., 1994) and that the antinociceptive effects of amphetamine depend to a great extent on the activation of the nucleus accumbens septi (Clarke and Franklin, 1992; Altier and Stewart, 1998) or other supraspinal sites where bulbo-spinal projections originate, it is reasonable to find antinociceptive activity by amphetamine and its analogues only in the formalin test. Furthermore, a sympathomimetic drug such as cocaine does not suppress spinal nociceptive reflexes (Pertovaara et al., 1988).

Spontaneous motor activity as measured in this work represents an overall screen of rat motor performance. While amphetamine-induced increases in motor activity are well documented (Maickel et al., 1982; Mora and Díaz-Véliz, 1983; Martin-Iverson et al., 1991; Díaz-Véliz et al., 1994), none of the methoxylated amphetamine analogues tested by us enhanced motor activity. These results are in agreement with those of Martin-Iverson et al. (1991), who were unable to demonstrate increases in motor activity by MA and 4-ethoxyamphetamine (4-EA) at doses of 32 mmol/kg (about 6.5 mg/kg for MA hydrochloride). Also, Tseng and Loh (1974) failed to demonstrate increases in motor activity caused by 3 and 10 mg/kg of MA, although when they used 30 mg/kg a significant increase in locomotion was observed. Likewise, Glennon et al. (1997) could not observe significant locomotor stimulation by MMA at doses of up to 30 mg/kg. In our experimental conditions, MMA injection induced a significant decrease of spontaneous motor activity.

Since spontaneous motor activity and active avoidance has been associated with activity of the central dopaminergic system, acquisition of avoidance responses could be altered by changes in motor activity. However, the present behavioral data suggest that the influence of some drugs on avoidance response were not necessarily consequence of equivalent changes in spontaneous motor activity. (+)-AMPH was the only drug to increase the acquisition of CARs, in agreement with the literature (Orsingher and Fulginiti, 1971; Mora and Díaz-Véliz, 1983), and this behavior can be a consequence of the hypermotility observed. On the other hand, in MMA-injected rats depressed motor activity was clearly accompanied by a significant decrease in avoidance conditioning. However, with EMA injection, both behaviors were dissociated. In fact, the impairment in motor activity was not accompanied by any change in avoidance conditioning.

Comparison of the behavioral effects of (+)-AMPH and its racemic methoxy derivatives is of interest because the introduction of a single methoxy group on the aromatic ring results in compounds with behavioral and biochemical effects which are substantially different from those of the parent compound. In particular, MA, but not (+)-AMPH, has been described as an only moderately potent hallucinogen in humans (Shulgin and Shulgin, 1991), but in an animal test system utilizing a CAR and analysis of reaction time designed to correlate with psychotropic activity in humans, MA was found to be highly potent as a disrupter of behavior, second only in this respect to lysergic acid diethylamide (Smythies et al., 1967), suggesting that species differences may be quite considerable.

In this study, all the substances studied exerted dose-related antinociceptive activity at least in the formalin test. This has been considered an essential criterion to demonstrate receptor-mediated pharmacological phenomena following the mass law (Kenakin, 1997). Thus, a preliminary conclusion of this work is that the amphetamine enantiomers, MA, and its *N*-alkyl derivatives, act as analgesics by interacting with macromolecular targets in neural tissue. The actions of amphetamine and monomethoxyamphetamines on the uptake, release and metabolism of catecholamines are well known (Hitzeman et al., 1971; Tseng et al., 1974; Tseng et al., 1976) and they provide a basis for the pharmacological effects of amphetamine-like compounds, including excitatory and motor effects. However, it has been demonstrated that MDMA administration to rats induces an acute and rapid release and depletion of 5-HT (for a review, see Green et al., 2003).

In any case, the amphetamine-like compounds may bear a mechanistic resemblance to antidepressant drugs which alleviate pain because of their ability to block the neuronal uptake of monoamines mediating the bulbo-spinal descending inhibitory system which modulates nociceptive input to the dorsal horns (Eschalier et al., 1994).

Another interesting hypothesis has been discussed by Braun et al. (1980). These authors suggest that the substances containing the phenylisopropylamine skeleton show some structural resemblance to morphine and the tyrosine moiety of the enkephalins. Thus, the analgesic action of MDMA and certain analogues might be due in part to their interactions with opioid receptors. Furthermore, Tocco et al. (1985), when using the hot plate test in rats, showed that the antinociceptive effects of (−)-AMPH were potentiated by morphine and slightly antagonized by naloxone. Moreover, it has been shown that the locomotor activity induced by AMPH is blocked by δ-opioid antagonists (Jones et al., 1993). From this point of view, and considering the high potency exhibited by MA, it would be interesting to test whether naloxone prevents or reverses the antinociceptive effects of this class of compounds. Experiments are under way to explore the possibility that an opioid mechanism participates in the actions of MA. Nevertheless, preliminary results did not shown any significant antagonism by naloxone in agreement with Frussa-Filho et al. (1996).

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