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## Age-dependent effects of neonatal methamphetamine exposure on spatial learning

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### Abstract

Neonatal rats exposed to (+)-methamphetamine (MA) display spatial learning and reference memory deficits in the Morris water maze. In separate experiments the emergence and permanence of these effects were determined. Twenty litters were used in each experiment, and two male/female pairs/litter received saline or MA (5 mg/kg four times a day) on postnatal days (P) 11–20. In experiment 1, one MA and one saline pair from each litter began testing on either P30 or P40, whereas in experiment 2, testing began on P180 or P360. Animals received trials in a straight swimming channel and then in the Morris maze (acquisition, reversal, and reduced platform phases). In both experiments, MA-treated groups showed impaired learning in the platform trials and impaired reference memory in the probe trials, which were largely independent of age. The P30 and P40 MA impairments were seen on acquisition and reduced platform trials but not on reversal. In the probe trials, MA effects were seen during all phases. The P180 and P360 MA-induced deficits were seen in all phases of the platform trials. In probe trials, deficits were only seen during the reversal and reduced platform phases. The results demonstrate that neonatal MA treatment induces spatial learning and reference memory deficits that emerge early and persist until at least 1 year of age, suggesting permanence.

### Keywords

cognition; development; developmental effects; methamphetamine; Morris water maze; rats; reference memory; spatial learning

### Introduction

(+)-Methamphetamine (MA) is a widely abused drug, especially among adolescents and young adults. Annual US prevalence rates from 1999 to 2005 have varied from 2.5 to 4.7% for high school seniors (Johnston *et al.*, 2006a), and from 2.4 to 2.8% for those between 19 and 28 years of age (Johnston *et al.*, 2006b). By comparison, annual prevalence rates for cocaine are 4.8–6.2% for high school seniors and 5.4–7.1% for 19–28 year olds. For women, rates of drug use are highest among those of reproductive age (Day *et al.*, 1993). Such widespread use inevitably leads to significant numbers of children being exposed prenatally. The prenatal effects of cocaine have been well documented (e.g. Richardson *et al.*, 1996a, b; Lester *et al.*, 1998). By contrast, little information is available on the effects of prenatal MA exposure.

Considerable information is available about the effects of MA on the mature brain (e.g. Wilson *et al.*, 1996; Mszczynska *et al.*, 2004; Meredith *et al.*, 2005; Barr *et al.*, 2006), but much less

is known about its effects on the developing brain. Early studies of infants exposed to MA (Little *et al.*, 1988) or MA and cocaine found reduced birth weight, increased rates of prematurity, and intra-ventricular hemorrhage (Oro and Dixon, 1987; Dixon and Bejar, 1989). MA–cocaine exposed infants were also found to have increased signs of withdrawal, using the Finnegan rating scale (sleep disturbances, tremors, poor feeding, hypertonia, vomiting, increased sneezing, high-pitched cry, increased fist sucking, tachypnea, loose stool, fever, hyperreflexia, increased yawning, and excoriation) (Dixon, 1989). In addition, 78% of flash-evoked visual potentials were abnormal in MA-cocaine exposed infants. A later study found reduced visual recognition memory on the Fagan test of Infant Intelligence among retrospectively ascertained MA-exposed infants of 6–12 months of age (Struthers and Hansen, 1992).

More recently, reduced creatine and glutamate/glutamine ratios in prenatally MA-exposed children were found by magnetic resonance spectroscopy (Smith *et al.*, 2001). Magnetic resonance imaging identified volumetric reductions in the hippocampus and other forebrain areas in prenatally MA-exposed children (Chang *et al.*, 2004). In a large study, 13 808 pregnant women were screened. Of these, 7119 were eligible for the study, 1618 consented to participate, and 84 were identified as abusing MA. The MA-exposed infants had reduced birth weight and increased rates of being small for gestational age (Smith *et al.*, 2006). Fifty-six percent of the MA-abusing women used it during the first and second trimesters, whereas 44% used it throughout pregnancy (Smith *et al.*, 2006). This indicates that nearly half of all women who use MA during gestation have third-trimester exposure. In a study from Thailand, 47 prenatally MA-exposed and 49 MA-unexposed infants were examined; it was found that MA-exposed infants had reduced birth weight and head circumference, and higher rates of being small for gestational age. They also reported that MA-exposed infants showed increased agitation, vomiting, and tachypnea (Chomchai *et al.*, 2004).

In rats, we developed models of the effects of MA exposure during the early (Acuff-Smith *et al.*, 1995) and later (Vorhees *et al.*, 1994a, b) stages of brain development, analogous to the first and second half of human intrauterine brain development (West and Pierce, 1987; Bayer *et al.*, 1993; Rice and Barone, 2000; Clancy *et al.*, 2001, 2006, 2007; Herlenius and Lagercrantz, 2004). We found that (male and female) rats exposed to MA from P11 to P20 exhibit long-term spatial learning and memory deficits in the Morris water maze (MWM), whereas cued MWM performance and multiple T-maze learning in the Cincinnati water maze (CWM) were spared. Moreover, no learning impairments were seen after exposure on P1–10 (Vorhees *et al.*, 1994a, b, 2000). The effects of P11–20 MA exposure on spatial learning and reference memory in the MWM are also seen in ACI (Vorhees *et al.*, 1998) and Dark Agouti rats (Vorhees *et al.*, 1999), and have also been reported recently, after comparable exposure in mice (Acevedo *et al.*, 2007). In rats, these exposures caused no changes in MWM matching-to-sample performance, in which the platform was moved to new locations daily (Williams *et al.*, 2003b). Using the same exposure, that is from P11–20, with half of each litter receiving pretraining to a randomly placed platform and half receiving no pretraining, it was revealed that the MA-induced MWM deficits represent a compound impairment; pretrained rats no longer showed acquisition deficits, but still showed reversal deficits, whereas non-pretrained MA-exposed animals showed both acquisition and reversal deficits (Williams *et al.*, 2002).

Follow-up experiments showed that doses as low as 0.625 mg/kg × 4 doses/day on P11–20 induce MWM performance deficits, although higher doses are more effective (Williams *et al.*, 2004b). In addition, treatment of offspring on P11–15 or P16–20 caused MWM deficits only in the P11–15 exposed MA group, but not in the P16–20 MA group (Williams *et al.*, 2003a).

The mechanism by which P11–20 MA treatment affects brain development is not yet known. P11–20 MA treatment causes significant increases in the release of corticosterone (Williams *et al.*, 2000) and this effect is both age-dependent and previous drug experience-dependent. For example, we found a U-shaped response function, in which corticosterone release was higher 30 min after MA treatment on P1 and 3 than on P5, 7, 9, 11, or 13, and was higher again on P15, 17, and 19. When examined 105 min after dosing, corticosterone release showed a similar U-shaped response with highest responses on P1, followed by P3 and 5, attenuated responses on P7, 9, 11, and 13, and larger responses on P15, 17, and 19. If the drug was, however, given chronically (4 doses/day) for 4 days with one final dose on the 5th day, the 30-min drug  $\times$  age corticosterone response was no longer U-shaped but increased progressively with age. This was evident as the 30-min corticosterone levels showed smaller increases on P5 or 7 than after an acute dose, but were dramatically higher on P13, 15, 17, and 19 than after an acute dose. By contrast, 105-min corticosterone levels were lower on all these days than after an acute dose. This suggests that the attenuated corticosterone response on P7–15 seen after acute MA (Williams *et al.*, 2006) follows a pattern similar to that observed during the stress hyporesponsive period (Sapolsky and Meaney, 1986), that is, on P2–14 when stressors do not elicit an adult-like release of corticosterone. In contrast, chronic MA treatment modifies this pattern by inhibiting the early corticosterone response and amplifying the later response (after P15). Therefore, one effect of MA exposure during development is to induce an exaggerated stress-like corticosterone release, especially as levels are still elevated 18 h after the fourth dose (Schaefer *et al.*, 2006). What causes the prolonged rise in corticosterone or how it relates to the later cognitive deficits is not known.

MA treatment on P11–20 also results in long-term changes to dopaminergic systems. Rats treated with 10 mg/kg  $\times$  4/day MA on P11–20 and assessed at P90 show 10–15% reductions in neostriatal  $^3\text{H}$ -spiperone binding to D2-like receptors with no changes in affinity, 20% reductions in neostriatal protein kinase activity, and 15% reductions in neostriatal dopamine and DOPAC tissue concentrations (Crawford *et al.*, 2003). When MA is administered on P11, and dopamine and DOPAC are examined on P12, no differences in monoamine levels are observed compared with saline-treated animals (Schaefer *et al.*, 2006), indicating that chronic dosing is required to induce long-term neurochemical changes in young animals. These effects were found in both male and female animals.

Another aspect of modeling the long-term effects of early MA exposure is determining how soon the deficits emerge and how long they last. The latter is particularly important: if the effects are transient or dissipate with age, they might be of less significance than if they are permanent. In contrast, if the effects are long lasting, they may have more serious implications. Accordingly, the purpose of the present experiments was to ascertain whether the spatial learning deficits observed previously, after P11–20 MA exposure, emerge early (animals that began testing on P30 or P40) and whether they persist (animals that began testing on P180 or P360).

## Methods

### Subjects

Sprague–Dawley CD IGS rats weighing 251–275 g (males) and 151–175 g (females) were obtained from Charles River (Raleigh, North Carolina, USA) and acclimatized for a minimum of 2 weeks before breeding. Detection of a sperm plug was designated embryonic day 0 (E0). Before parturition, gravid female rats were transferred to polycarbonate cages. Starting on E21, female rats were checked for the presence of a litter; birth was counted as P0 (postnatal day 0). On P1, litters were randomly culled to four male and four female rats. On P11, offspring were uniquely identified (ear-punched). Food and water were provided *ad libitum* in rooms where ambient temperature was controlled at  $21 \pm 1^\circ\text{C}$  and humidity at  $50 \pm 10\%$ . The vivarium

is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), and the protocol was approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Research Foundation. Dams were separated from their litters on P28. Thereafter, same-sex pairs from the same litters were housed together, with one treated and one control animal in the same cage.

### **Methamphetamine and treatments**

Two experiments were performed. For each experiment, 20 litters were used. In each experiment, half of the male and half of the female animals in each litter received MA and the remaining half received saline (Sal): hence two male and two female rats per litter received MA and two male and two female rats per litter received Sal (split-litter design). (+)-Methamphetamine HCl (MA) was obtained from the National Institute on Drug Abuse (purity > 95%). The dose of MA (calculated as the free base) was 5 mg/kg body weight and was given subcutaneously four times a day at 2-h intervals on P11–20. Control animals were administered an equivalent volume of saline (3 ml/kg).

The two experiments were identical in the treatment aspects and differed only in the test ages of the offspring. In each experiment, half of the MA-treated and half of the Sal-treated offspring in each litter were tested at each of two separate ages. In experiment 1, half of each litter (i.e. one male and one female MA-treated pair and one male and one female Sal-treated pair) was tested beginning on either P30 or P40. In experiment 2, half of each litter was tested beginning on either P180 or P360.

### **Body weights**

Body weights were obtained before each dose on P11–20; however, only two weights from each day were used in the analysis of body weights during dosing (before the first and last doses). Prewaning and adult weights were obtained once weekly beginning on P21 and continued through P91 in the first experiment, and through P238 (for the P180 test group) or P392 (for the P360 test group) in the second experiment.

### **Behavioral assessments**

Straight channel swimming (latency to reach an escape ladder) was assessed in a 15 × 244 cm channel with 20 cm high sides filled to a depth of 10 cm with water on four trials (2-min limit/trial). This procedure was used to assess swimming ability and motivation to escape from water and to teach the animals that escape was possible.

MWM hidden platform trials were conducted in three phases, each consisting of 4 trials/day on each of 5 consecutive days, as described previously (Vorhees and Williams, 2006). The apparatus was a 210-cm (diameter) black circular stainless-steel water tank with 51-cm high walls filled to a depth of 30 cm with water (temperature  $21 \pm 1^\circ\text{C}$ ). Phase I (acquisition) used a platform of area  $10 \times 10$  cm submerged 2 cm below the water. Phase II (reversal) used the same platform, but it was positioned diagonally in the opposite quadrant. Phase III (reduced) used a smaller  $5 \times 5$  cm platform placed back in the original quadrant location. For all trials, rats were given a 2-min trial limit and 15-s intertrial interval (ITI) on the platform. If an animal did not find the platform within 2 min, it was placed on the platform. Platform positions within each phase were counterbalanced (e.g. for acquisition, half of the animals had the platform in the SE and half in the NW quadrant). Start positions were arranged such that those adjacent to the quadrant containing the platform were excluded. Rats were placed in the water facing the wall. Performance was recorded automatically using a video-tracking system (Polytrack; San Diego Instruments, San Diego, California, USA). Dependent variables were path length, cumulative distance, latency, and angle of first bearing to the goal.

MWM probe trials were conducted on the 6th day of each phase. Each probe trial was 30 s with the platform removed. Start positions on probe trials were from a location 180° to the goal, which was not one of the four learning trial start locations. Dependent variables on probe trials were angle of first bearing, average distance to the target site, and percent time spent in the target quadrant.

## Statistics

Body-weight data were analyzed by analysis of variance (ANOVA) for repeated measures using the general linear model (SAS Proc GLM, SAS Institute, Cary, North Carolina, USA) with treatment Group, Age of testing, and Sex as within-litter factors; therefore litter was the statistical unit in these analyses. Variance–covariance matrices were tested for sphericity. In cases where matrices were significantly nonspherical, the Greenhouse–Geisser adjusted degrees of freedom and *F* ratios were used. Significant interactions with treatment were further analyzed using simple-effect ANOVAs on each day. As litter effects are an important source of variance in developmental studies, behavioral data were analyzed using a mixed general linear-model ANOVA (SAS Proc Mixed, SAS Institute). In this model, litter was a block factor. With litter accounted for in the block factor, treatment Group, Sex, platform Position, and test Age were between-group factors. Day was a repeated-measure factor. Variance–covariance models were selected based on best-fit statistics for each dependent variable. The models were either compound symmetry or first-order autoregressive. Significant interactions were further analyzed on each day using the slice-ANOVA option within Proc Mixed, which, as there were only two groups at each test age, was functionally equivalent to a *t*-test. For clarity of presentation, only significant Group (MA or Sal) main effects or interactions are provided with *F* ratios. Significance was set at  $P \leq 0.05$ .

## Results

### Experiment 1

**Mortality**—No animal died in experiment 1.

**Body weight**—Body weights obtained during treatment showed that there were significant effects of MA treatment. There was a significant main effect of Group [ $F(1,19) = 462.21$ ,  $P < 0.001$ ], and significant interactions of Group  $\times$  Day [ $F(9,171) = 436.20$ ,  $P < 0.001$ ], Group  $\times$  Time of Day [ $F(1,19) = 6.60$ ,  $P < 0.02$ ], and Group  $\times$  Day  $\times$  Time of Day [ $F(9,171) = 11.43$ ,  $P < 0.001$ ]. Further analyses of the interactions on each day and at each time of day revealed that the groups did not differ on P11 before the first morning injection, but were different in the afternoon before the second injection. Group differences were found on all other days and times from P12 through P20. In all cases, MA treatment significantly reduced body weight compared with Sal treatment. The body weight taken closest to the beginning of testing is shown in Table 1. No interactions were observed between Sex and Group.

Animals were weighed one day after the end of treatment (P21) and on the day they were separated from their dams (P28). An ANOVA on these body weights showed a significant Group main effect [ $F(1,19) = 264.84$ ,  $P < 0.001$ ]; no other main effects or interactions were significant. After weaning, animals continued to be weighed weekly until the end of the experiment (P35-91). An ANOVA of these adolescent through adult body weights showed no treatment main effect. The main effects of Age and Sex were significant as expected ( $P < 0.001$ ). The Group  $\times$  Age interaction showed a nonsignificant trend ( $P < 0.07$ ).

**Straight channel**—Straight channel swimming times were unaffected by MA treatment regardless of test age group (P30 or P40), indicating no differences in swimming ability between the groups. There were no interactions with Sex.



**Morris water maze**—Four primary measures of performance were used to assess learning: latency, path length, cumulative distance from the platform, and first bearing (i.e. the angle of deviation from a direct line to the platform center, based on average directional heading during the first 13 cm of movement at the beginning of each trial). We have previously demonstrated that latency, path length, and cumulative distance are highly correlated with each other ( $r > 0.93$ ), whereas first bearing, because it reflects initial performance rather than the entire trial, is less correlated with the three other measures ( $r = 0.41\text{--}0.57$ ) (Vorhees and Williams, 2006). Accordingly, we use path length for illustrative purposes and describe the latency and cumulative distance effects. We use first bearing to reflect the animal's initial sense of where to go. A third measure we use to clarify certain trends in data is peripheral swim time (thigmotaxis). This has been shown to be useful in dissecting the effects of other treatments (Cain and Saucier, 1996; Cain *et al.*, 1996) and we have previously shown its value in rats neonatally treated with MA (Williams *et al.*, 2002). On probe trials, we use average distance from the platform, number of platform site crossings, first bearing, and percent time and distance in the target quadrant. Average distance and first bearing are used for illustrative purposes.

*Phase 1, acquisition* The Group  $\times$  Day interaction on path length was significant [ $F(4,408) = 2.78, P < 0.05$ ], but the main effect of Group and Group  $\times$  Age interaction was not; there were no interactions with Sex. Predicted pairwise group comparisons at each age, based on the Group  $\times$  Age interaction on average distance, showed that the MA-treated P40 group was farther from the platform site than the Sal-treated P40 controls ( $P < 0.05$ ). The learning curves at each age and combined across ages for path length are illustrated in Fig. 1. For latency, the Group main effect was significant [ $F(1,126) = 7.47, P < 0.01$ ], as was the Group  $\times$  Day interaction [ $F(4,401) = 2.56, P < 0.05$ ]. For cumulative distance, the main effect of Group fell short of significance, as did the Group  $\times$  Day or Group  $\times$  Age interactions (both  $P < 0.06$ ). The Group main effect was significant for first bearing [ $F(1,119) = 23.18, P < 0.001$ ], and is illustrated in Fig. 2a. As can be seen, when the data are combined across test ages, the MA-treated group had larger average first bearings than Sal-treated controls. A follow-up analysis of time in the periphery confirmed that MA-treated offspring spent more time around the edge of the pool than Sal-treated controls [ $F(1,138) = 12.77, P < 0.001$ ]; Fig. 2b).

On the probe trial, there was a significant Group  $\times$  Age interaction on average distance [ $F(1,119) = 3.91, P < 0.05$ ], a significant Group main effect on first bearing [ $F(1,119) = 10.87, P < 0.002$ ], and a significant Group  $\times$  Age interaction on percent distance in the target quadrant [ $F(1,119) = 3.99, P < 0.05$ ]. No significant interactions were observed between Sex and Group. Group comparisons on percent distance in the target quadrant showed a deficit in the MA-treated P30 group compared with the P30 Sal-treated group ( $P < 0.05$ ; not shown). ANOVAs on platform site crossings and percent time in the target quadrant were not significant.

*Phase 2, reversal* No significant main effects or interactions were observed on reversal trials for path length (Fig. 3), latency, or cumulative distance, and no interactions with Sex. A significant interaction was observed of Group  $\times$  Age  $\times$  Day [ $F(4,462) = 2.80, P < 0.05$ ; Fig. 4a and b] on first bearing and a significant Group main effect, [ $F(1,133) = 13.02, P < 0.001$ ; Fig. 4c]. There were no significant Group effects on time in the periphery. On the probe trial, there were no significant Group main effects on average distance, site crossings, percent time, or distance in the target quadrant, but there was a significant Group  $\times$  Sex effect on first bearing [ $F(1,133) = 6.38, P < 0.02$ ]. This was attributable to a significantly increased first bearing among MA-treated male rats averaged across test ages (Fig. 5).

*Phase 3, reduced* On the third phase of MWM, when a smaller, relocated platform was used, there were no significant Group main effects on path length, latency, or cumulative distance, but there were significant Group  $\times$  Age  $\times$  Day interactions on path length [ $F(4,424) = 3.60$ ,

$P < 0.01$ ], latency [ $F(4,424) = 2.85, P < 0.05$ ], and cumulative distance [ $F(4,423) = 3.73, P < 0.01$ ]. No significant interactions between Group and Sex were observed. For path length, the Group  $\times$  Age  $\times$  Day interaction is illustrated in Fig. 6a and b. As can be seen, the MA-treated P40 group had significantly longer path lengths on the first day of testing. A significant Group main effect was also seen on first bearing [ $F(1,126) = 5.27, P < 0.05$ ; Fig. 6c], and a significant Group  $\times$  Sex  $\times$  Day  $\times$  Age interaction [ $F(4,425) = 2.76, P < 0.05$ ]. Converging evidence that the MA-treated animals at both P30 and P40 were impaired was provided by a follow-up ANOVA on time in the periphery, in which a significant Group main effect was observed [ $F(1,126) = 4.86, P < 0.05$ ]. As can be seen in Fig. 6d, the MA-treated group spent approximately 30% more time in the periphery than Sal-treated controls. On the probe trial, average distance, site crossings, and quadrant analyses showed no significant Group main effects, but there was a significant Group main effect on first bearing [ $F(1,126) = 4.51, P < 0.05$ ]. As can be seen in Fig. 7, the MA-treated animals were farther off course than the Sal-treated animals. There were no significant interactions between Group and Sex.

## Experiment 2

**Mortality**—No mortality occurred during dosing (P11–20) or during the week thereafter (P21–28). There were no deaths among the 40 Sal-treated female or the 40 Sal-treated male rats between P35 and P175. There was one death among the 40 MA-treated female rats during this time and no deaths among the 40 MA-treated male rats. Among the subgroups retained for testing beginning on P360, one death occurred in each of the four groups (Table 1) between P175 and P357; hence the  $N$  in each cell at this test age was 19. In addition, one of the 19 animals in the Sal female subgroup died before completing all three phases of the MWM and its data for the incomplete phase were excluded.

**Body weight**—An ANOVA on body weights recorded during dosing on P11–20 showed a significant Group main effect [ $F(1,19) = 464.91, P < 0.001$ ], and significant interactions of Group  $\times$  Day [ $F(9,171) = 423.89, P < 0.001$ ], and Group  $\times$  Day  $\times$  Time of Day [ $F(9,171) = 10.44, P < 0.001$ ]. Further analyses on each day and at each time of day showed that there were no body weight differences on P11 before the first injection. Differences were significant before the last injection of the afternoon on P11 ( $P < 0.05$ ) and for all remaining days and times (P12–20). No significant interactions between Group and Sex were observed.

An ANOVA on P21 and P28 body weights showed a significant Group main effect [ $F(1,19) = 389.32, P < 0.001$ ], and significant interactions of Group  $\times$  Age [ $F(1,19) = 5.80, P < 0.05$ ], Group  $\times$  Week [ $F(1,19) = 5.25, P < 0.05$ ], and Group  $\times$  Sex  $\times$  Week [ $F(1,19) = 5.69, P < 0.05$ ]. When further analyzed for the Group  $\times$  Age interaction, Group was significant for both age groups. When the Group  $\times$  Sex  $\times$  Week interaction was further analyzed, there were group differences among male and female rats at both P21 and P28. The interaction arose because the magnitude of the MA effect was slightly different for each sex at these two ages, but the pattern of differences was the same.

After P28, all animals were weighed weekly until P238, which was after the subgroup that began testing on P180 completed their testing. At that point they were killed and only the subgroup scheduled for testing beginning on P360 were retained and weighed weekly until after the end of their testing on P392. An omnibus ANOVA on the P35–238 body weights showed no significant main effect of Group, but a significant Group  $\times$  Week interaction was obtained [ $F(29,551) = 4.15, P < 0.02$ ]. When the interaction was analyzed, there were significant group differences on P35, 42, 49, and 56, but from P63 onward the MA groups recovered such that no significant differences remained. In the subgroup that continued on to P360, no significant Group main effects or interactions were seen. Body weights at the age

nearest to the age of testing are shown in Table 1. At no point were there any significant interactions between Group and Sex.

**Morris water maze—Phase 1, acquisition** The main effect of Group on path length (Fig. 8a and b) was not significant but there was a significant Group  $\times$  Day interaction  $F(4,431) = 2.55$ ,  $P < 0.05$ . As can be seen in Fig. 8c, the MA-treated animals had longer path lengths on day 2 of acquisition than did the Sal-treated animals. This same interaction (Group  $\times$  Day) was also significant for cumulative distance,  $F(4,427) = 2.84$ ,  $P < 0.05$ , but fell short of significance for latency ( $P < 0.09$ ). There was a significant Group main effect on first bearing [ $F(1,126) = 10.16$ ,  $P < 0.002$ ], as well as a significant Group  $\times$  Age interaction [ $F(1,126) = 4.53$ ,  $P < 0.05$ ]; both of these effects reflected the fact that the MA-treated animals were farther off course than the Sal-treated animals and that this was more pronounced for the P180 group compared with those tested at P360. No significant interactions of Group  $\times$  Sex were obtained.

On the probe trial there was a significant Group main effect on first bearing [ $F(1,114) = 6.00$ ,  $P < 0.02$ ], which is illustrated in Fig. 9. The first bearing data show that the MA-treated group was significantly more off course than the Sal-treated group across both ages combined. There were no significant Group effects on average distance, site crossings, or quadrant time or distance, and no Group  $\times$  Sex interactions.

*Phase 2, reversal* The main effect of Group was significant for path length [ $F(1,119) = 5.87$ ,  $P < 0.02$ ]. None of the interactions was significant. As can be seen in Fig. 10, the MA-treated group had longer path lengths across ages. Although, visually, Fig. 10 suggests that the P360 group contributed more to this effect than the P180 age group, none of the interactions was significant. The main effect of Group was also significant for latency [ $F(1,118) = 9.72$ ,  $P < 0.005$ ], and cumulative distance [ $F(1,118) = 5.44$ ,  $P < 0.05$ ]. A significant Group main effect was also observed on first bearing [ $F(1,119) = 18.32$ ,  $P < 0.001$  (not shown)]. All of these effects confirmed what was found for path length.

On the probe trial, there were significant main effects of Group on average distance [ $F(1,118) = 19.12$ ,  $P < 0.001$ ], site crossings [ $F(1,118) = 5.00$ ,  $P < 0.05$ ], and percent time [ $F(1,118) = 5.55$ ,  $P < 0.02$ ], and percent distance in the target quadrant [ $F(1,118) = 6.07$ ,  $P < 0.02$ ], but the effect on first bearing fell short of significance ( $P < 0.07$ ). The effect on average distance is shown in Fig. 11, and as can be seen, the MA-treated group was farther from the platform site when averaged across test ages than the Sal-treated group. The interaction of Group  $\times$  Sex was not significant.

*Phase 3, reduced* During the final phase of testing, when the platform was relocated to another quadrant and a smaller platform was used, there were significant main effects of Group on all measures: path length [ $F(1,126) = 6.33$ ,  $P < 0.02$ ]; latency [ $F(1,127) = 11.53$ ,  $P < 0.001$ ]; cumulative distance [ $F(1,126) = 7.31$ ,  $P < 0.01$ ]; and first bearing [ $F(1,125) = 25.02$ ,  $P < 0.001$ ]. In addition, the Group  $\times$  Age interaction was significant for latency [ $F(1,127) = 7.46$ ,  $P < 0.01$ ], but not for path length ( $P < 0.07$ ), cumulative distance ( $P < 0.07$ ), or first bearing ( $P < 0.08$ ). The main effect of Group and the nonsignificant Group  $\times$  Age interaction are illustrated in Fig. 12a–c. The primary contribution to the main effect of Group came from the longer path lengths of the MA-treated group at P360 compared with the MA-treated group at P180. There was also a significant Group  $\times$  Sex interaction for path length [ $F(1,127) = 4.35$ ,  $P < 0.05$ ], and cumulative distance [ $F(1,127) = 4.52$ ,  $P < 0.05$ ], but not for latency ( $P > 0.10$ ), or first bearing ( $P < 0.09$ ). The interactions with Sex were attributable to significant impairments in path length and cumulative distance among female compared to male rats at both ages.

On the probe trial, there were significant Group main effects on average distance [ $F(1,119) = 10.09$ ,  $P < 0.002$ ], and site crossings [ $F(1,119) = 5.32$ ,  $P < 0.05$ ], but not on first bearing, or



percent time or distance in the target quadrant. The effect on average distance is shown in Fig. 13. As can be seen, the MA-treated group was farther off course in swimming to the platform site than the Sal-treated group, when averaged across test ages. There were no interactions between Group and Sex.

## Discussion

The present experiment showed the following: (i) MWM spatial learning and reference memory deficits we had shown previously after P11–20 MA treatment and assessed at P60–90 are also observed at other ages (both earlier and later); (ii) P11–20 MA-induced MWM learning and memory deficits emerge early after the cessation of treatment and are present when testing begins as early as P30 or P40; (iii) there is little difference in the effect of MA whether testing begins on P30 or P40; (iv) P11–20 MA-induced MWM learning and memory deficits are long lasting and evident in groups tested beginning at P180 or P360; and (v) there is little difference in the effect of MA whether testing begins on P180 or P360. In the latter case, although the MA effects are obtained at both of these ages, the effects seem to be more pronounced at P360 than at P180, although the interaction was not significant. Whether this is an indication that the deficit worsens very slightly (but not significantly) with age or is the product of a less than perfect sampling distribution of treated animals at the two ages is unclear. Another possibility is that this difference is related to the size of the animals in relation to the platform. At P180, male rats had difficulty climbing onto the platform. This was especially noticeable during the reduced trial phase when the platform was 75% smaller. P180 animals climbed onto the platform but often fell off, forcing them to relocate the platform (although the trial had officially ended). At P360, the animals were even larger and could not stand on the smaller platform and merely held onto it to end the trial. It might be that falling off the platform among the P180 animals interfered with their learning. Examination of Fig 12a shows that saline-treated animals' learning was progressing well on days 1 and 2 but deteriorated on day 3. From day 3 onward the saline-treated group's performance resembled that of the MA-treated group. A predicted line for the saline-treated group based on their days 1 and 2 and extrapolated to days 3–5 or based on the actual performance of the P360 saline-treated group would be well below that of the MA-treated group, suggesting that some factor disrupted control performance on the reduced platform trials.

The finding that neonatal MA treatment results in spatial learning and reference memory deficits in the MWM suggests that MA can affect hippocampal development. Many experiments using lesions, NMDA antagonists, and gene targeting lead to deficits in MWM platform and probe trial performance (see Brandeis *et al.*, 1989; D'Hooge and de Deyn, 2001; Burgess *et al.*, 2002; Morris *et al.*, 2003 for reviews). Consistent with this, we have shown that animals exposed to MA from P11–20 exhibit decreased spine density in the hippocampus (Williams *et al.*, 2004a). Although treatments that disrupt hippocampal function reliably disrupt MWM performance, treatments that disrupt MWM performance are not necessarily restricted to the hippocampus, because injury to other brain regions can also result in impaired MWM performance (Brandeis *et al.*, 1989).

We have shown here and previously that P11–20 MA treatment does not impair swimming speed (Vorhees *et al.*, 2000; Williams *et al.*, 2003b, 2004b). Moreover, path length and cumulative distance measures are insensitive to differences in swimming speed and these indices clearly showed impairments of spatial learning and memory. Although it is the case that MA-treated offspring exhibit task-specific as well as spatial impairments during the acquisition phase, task-specific effects do not contribute to impairments during reversal (Williams *et al.*, 2002). We have also shown that MA-treated offspring do not show impairments on early trials on day 1 of testing, indicating that they do not have preexisting performance differences (Vorhees *et al.*, 2000). Increased thigmotaxis during the acquisition

phase can be attributed to lack of acquiring task-specific skill learning (Williams *et al.*, 2002), as seen in the present experiment, but increased thigmotaxis during the reduced platform phase represents a very different behavior. From observing the swim paths of animals during this phase of testing, it is evident that MA-treated offspring enter the periphery only after they fail to find the platform where it had been located previously. The reason MA-treated offspring spend more time in the periphery of the maze on these trials is because they adapt more slowly to the new location of the platform than controls.

Drug studies in animals present complex problems for determining appropriate doses. Ideally, one would have physiologically-based pharmacokinetic data but such data are difficult to obtain. Pharmacokinetic data have been obtained in adult (Cho *et al.*, 2001), neonatal (Cappon and Vorhees, 2001), and fetal rats (Acuff-Smith *et al.*, 1995), but human fetal data have not been available. Another problem is species differences in metabolism (De La Torre and Farre, 2004). Human pharmacokinetic data come from low-dose experiments whereas abuse-related intake can only be extrapolated, with the assumption of linear pharmacokinetics, from levels in individuals arrested by police. In a recent report based on 105 arrestees suspected of drug intoxication, for example, the authors assumed that the users took the drug intravenously in four evenly spaced doses per day for 4 consecutive days (Melega *et al.*, 2007). This model yielded an estimated median dose of 52 mg/dose, leading to an estimated 260 mg/dose for a high-dose user. On a weight-adjusted basis, these doses would (for a woman weighing 60 kg) be 0.87 mg/kg for a median user and 4.33 mg/kg for a high-dose user. These are similar to the doses used in the present work. Also, as rats metabolize MA more rapidly than humans (elimination half-life in humans is ~ 12 h whereas in rats it is ~ 1 h)(Cho *et al.*, 2001) total exposure is higher in humans than in rats unless rats are dosed more often.

The situation is further complicated because we are modeling prenatal exposure and little is known about the doses pregnant MA users take. To model the second half of human intrauterine brain development, rats have to be exposed postnatally (Clancy *et al.*, 2007). This eliminates the placenta and might change the dynamics of the exposure. MA readily crosses the placenta and reaches equilibrium with maternal serum, but exposure is relatively prolonged because the rate of clearance from the fetal compartment is slower (Burchfield *et al.*, 1991).

Another approach to extrapolating doses is by interspecies scaling (Mordenti and Cappell, 1989). Body size influences physiological processes including metabolic rate, oxygen consumption, CO<sub>2</sub> clearance, and there is a systematic relationship between body mass multiplied by an empirically determined scaling coefficient raised to the power 0.67 to 0.75 and these physiological processes (West *et al.*, 2002). The validity of scaling formulas has been widely demonstrated for processes ranging from cellular to organismal levels and among different species from tree shrews to elephants. Pharmaceutical applications of this general principle have been used to extrapolate from doses obtained in laboratory animal studies to those used in initial human trials. Based on doses in the present study, the formula  $\text{dose}_{\text{human}} = \text{dose}_{\text{rat}} \times [(\text{weight}_{\text{human}}/\text{weight}_{\text{rat}})^{0.75}]$  suggests that the dose used in rats in the present experiment is at the lower end of the human abuse spectrum.

Some concerns have, nevertheless, been raised about the use of allometric dose scaling (De La Torre and Farre, 2004; Baumann *et al.*, 2007). For example, De La Torre and Farre (2004) note that species differences in the metabolism of  $\pm$  3,4-methylenedioxymethamphetamine caused by differences in cytochrome P450 result in an active principal metabolite, methylenedioxymethamphetamine, in rats but to a relatively inactive principal metabolite, dihydroxymethamphetamine, in humans. In addition, De La Torre and Farre (2004) note that, following doses > 10 mg/kg in adult rats, brain concentrations of the parent drug appear to rise nonlinearly. Our previous work, however, suggests that this nonlinearity might not occur in neonatal rats treated with MA (Cappon and Vorhees, 2001). Baumann *et al.* (2007) recently

raised concerns with allometric scaling for extensively metabolized agents such as amphetamines and argued instead for a pharmacologically based approach. They noted, for example, that the neurochemical and physiological actions of  $\pm$  3,4-methylenedioxymethamphetamine occur at doses of 0.3–2.5 mg/kg in rats and at a dose of ~1.5 mg/kg in humans. It is, however, worth questioning the relevance of pharmacological endpoints for understanding intake in drug-abusing populations. For example, low doses of MA do not produce reductions in brain monoamines but it has been shown that MA users exhibit large reductions in caudate-putamen and globus pallidus DA postmortem (Wilson *et al.*, 1996; Moszczynska *et al.*, 2004), supporting the use of high doses of MA in laboratory studies.

For studies in neonatal animals, such as reported here, the issue of dose relevance remains controversial. Certainly, it would be helpful to analyze cord blood, meconium, or hair from women using MA near the time of delivery. Until such data are available, choosing doses for laboratory studies remains necessarily imprecise; however, it should be noted that we have published data previously on the effects of P11–20 MA using 4 doses/day of 5 mg/kg/day, as used here, and also using several lower doses (i.e. 5.0, 2.5, 1.25, or 0.625 mg/kg). The long-term effects of MA on MWM learning even at these lower doses were similar to those reported here (Williams *et al.*, 2004b).

## Acknowledgments

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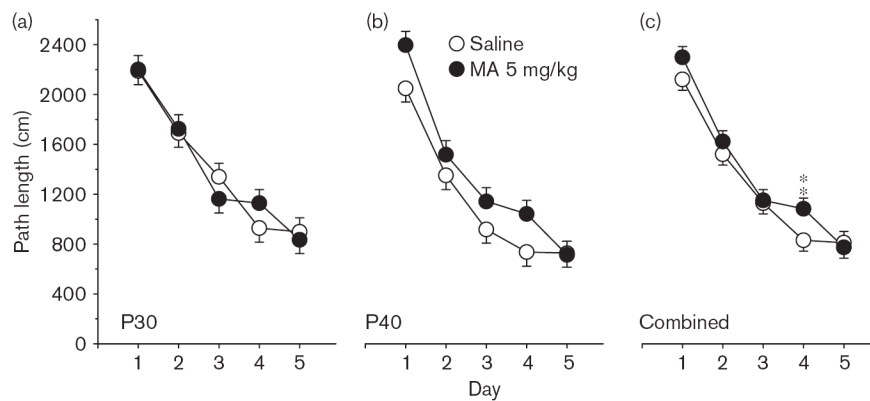
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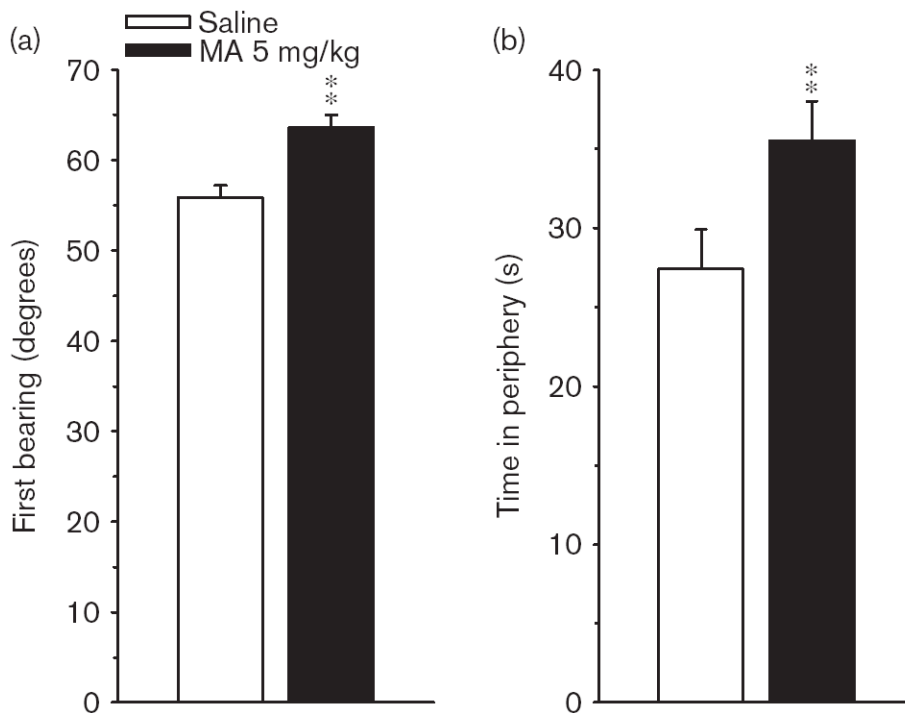
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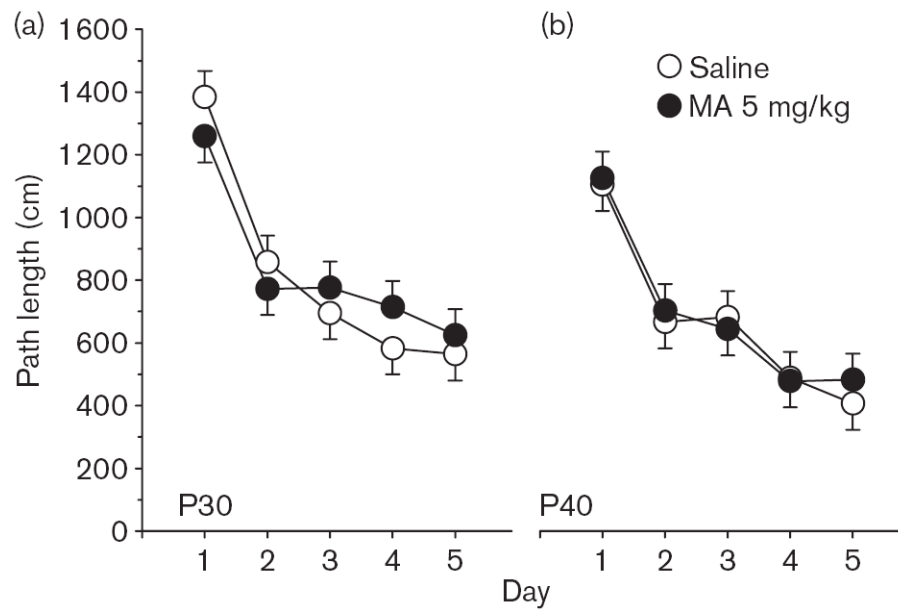
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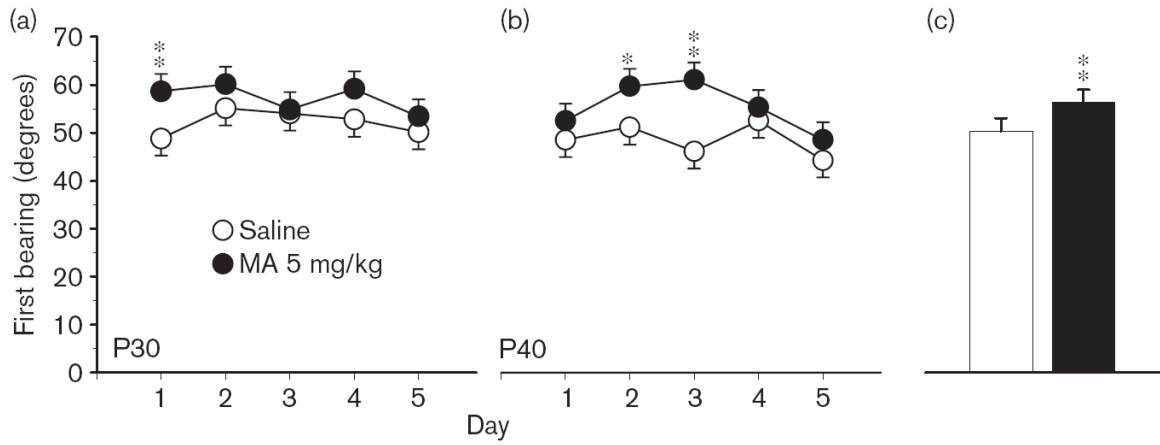
**Fig. 1.** Experiment 1. (P30, P40) Morris water maze, acquisition phase, platform trials for path length: (a), path length (cm) averaged across 4 trials/day and across sexes for the P30 groups. (b) Same as in panel (a) except that data are for the P40 groups. (c) Same as in (a) except that data are the combined averages of the P30 and P40 groups. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \*\* $P < 0.01$  vs. saline.



**Fig. 2.** Experiment 1. (P30, P40) Morris water maze, acquisition phase, platform trials. (a) Angle of first bearing to the platform site measured in the first 13 cm at the start of each trial averaged across 4 trials/day for 5 days for male and female rats. (b) Time in the periphery of the maze (outer annulus) on platform trials averaged as in (a). Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times per day on P11–20. \*\* $P < 0.01$ .

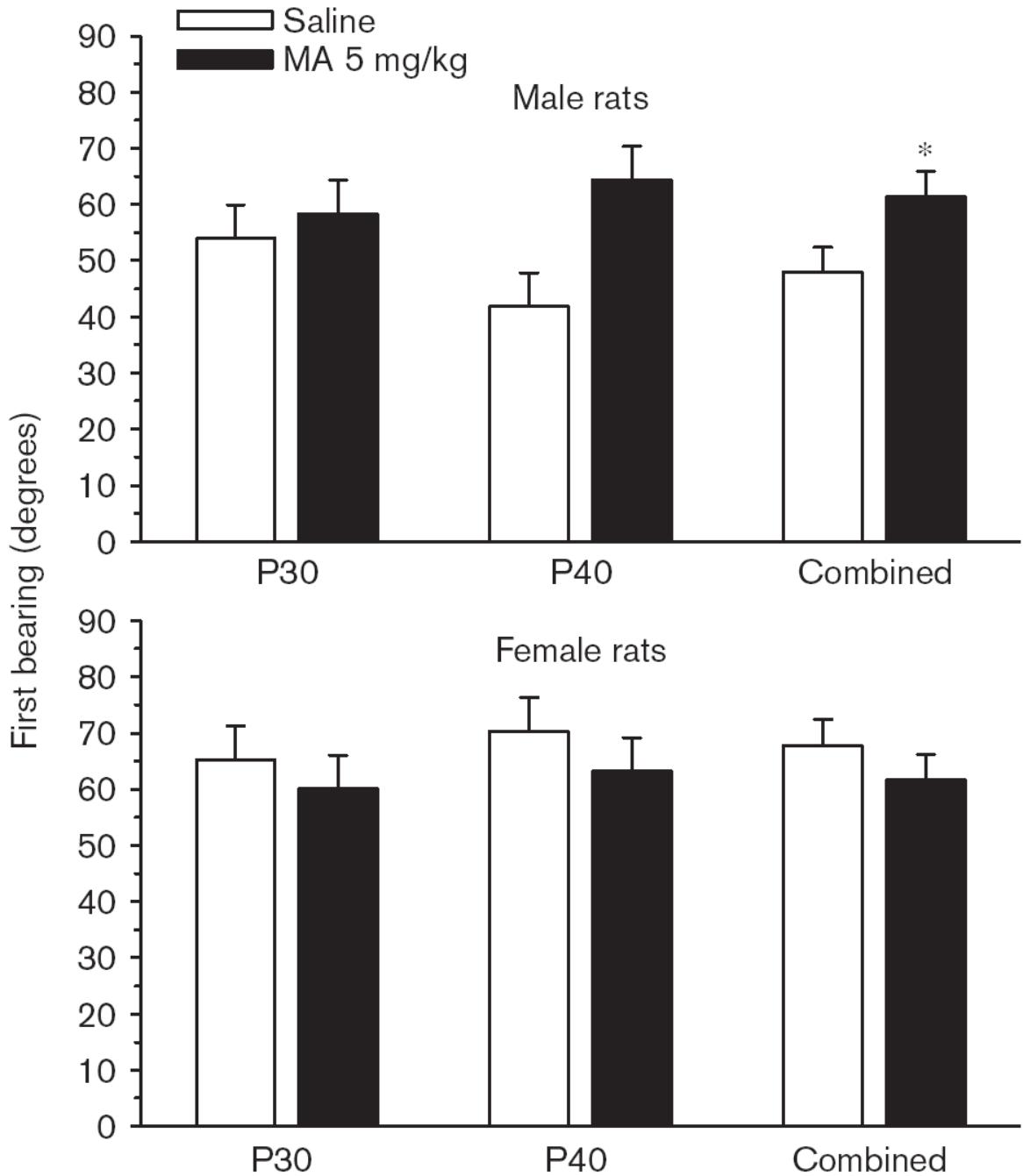


**Fig. 3.** Experiment 1. (P30, P40) Morris water maze, reversal phase, platform trials for path length: (a) Path length (cm) averaged across 4 trials/day and across sexes for the P30 groups. (b) Same as in (a) except as applicable to the P40 groups. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20.

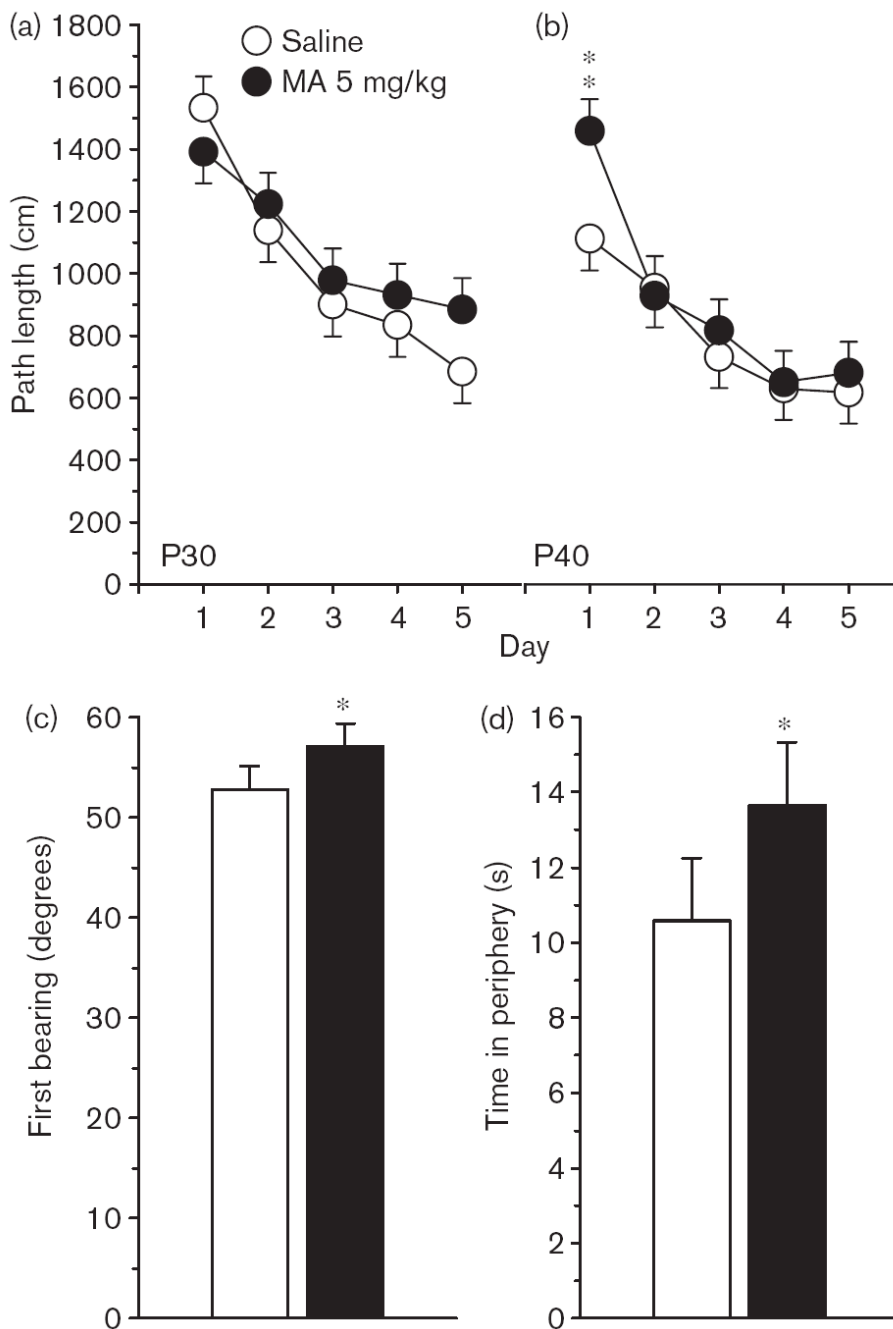
**Fig. 4.**

Experiment 1. (P30, P40) Morris water maze, reversal phase, platform trials for first bearing: (a) First bearing (degrees) for the P30 groups averaged across 4 trials/day and across sexes. (b) Same as in (a) except as applied to the P40 groups. (c) The average across trials, days, sexes, and test ages to show the main effect of Group. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \* $P < 0.05$  \*\* $P < 0.01$  vs. saline.

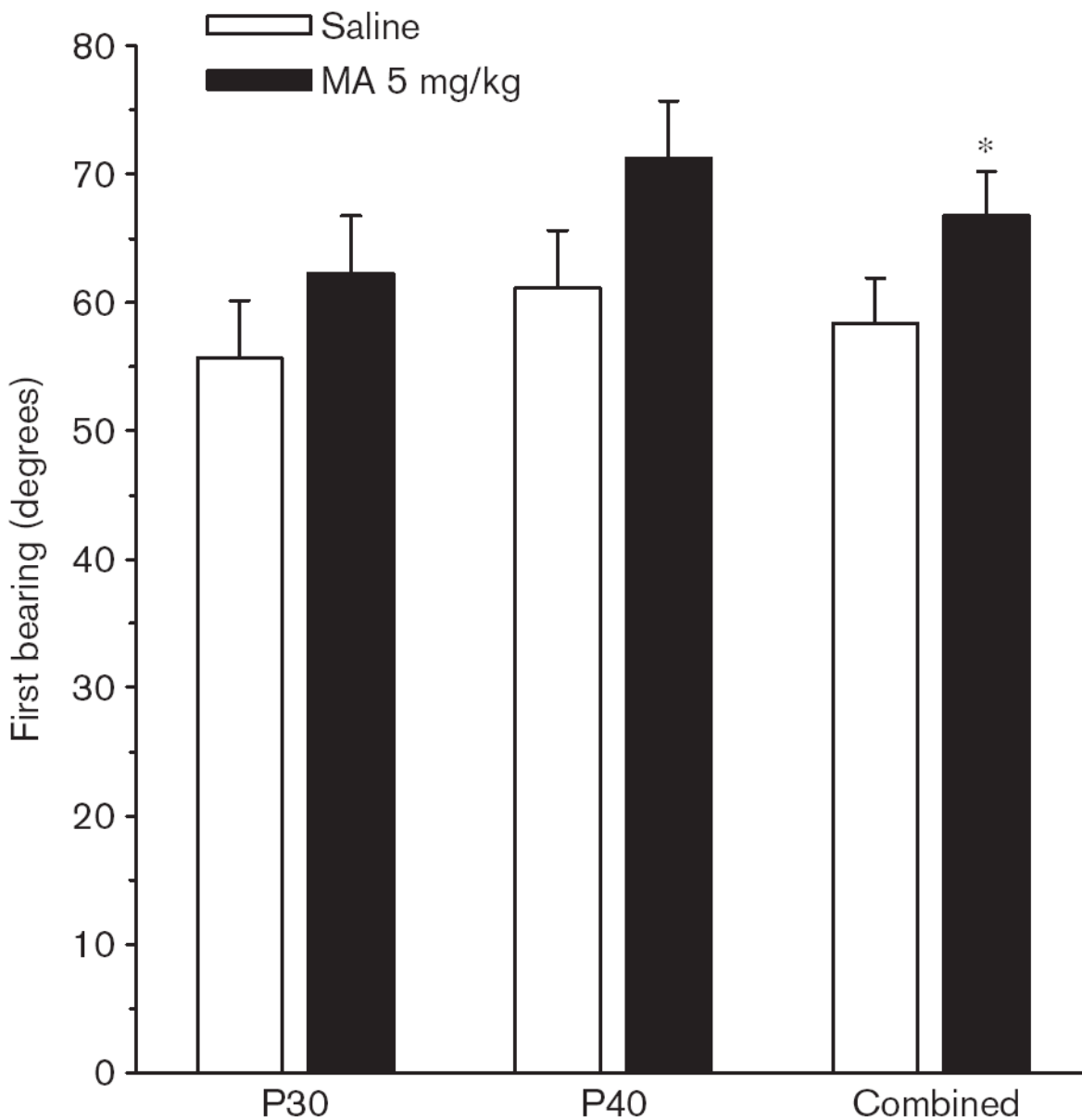




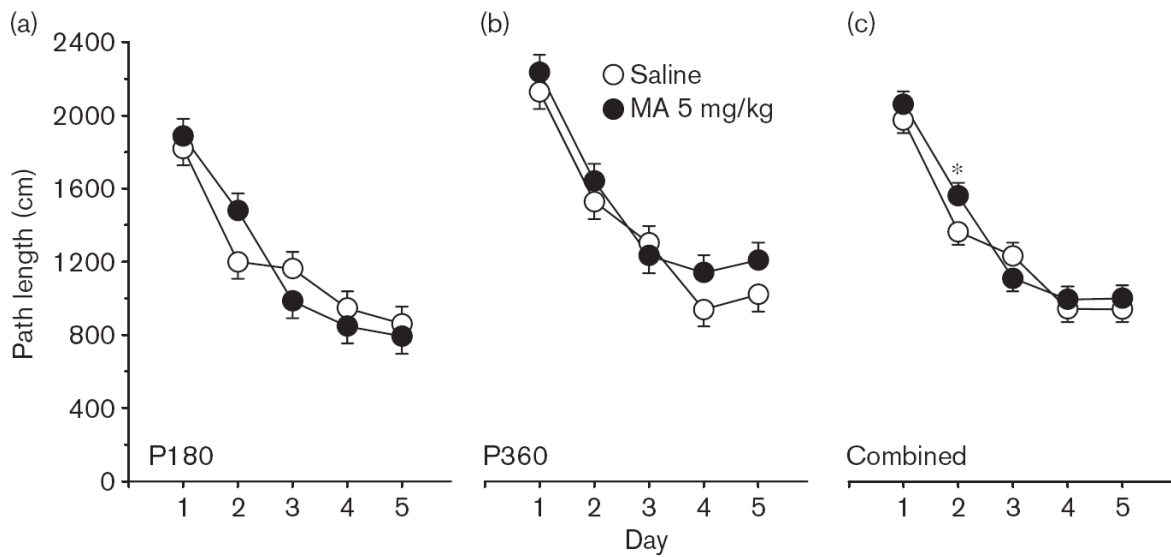
**Fig. 5.** Experiment 1. (P30, P40) Morris water maze, reversal phase, first bearing probe trial: Data are shown separately for male and female rats to reflect Group  $\times$  Sex interaction. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \* $P < 0.05$  vs. saline.



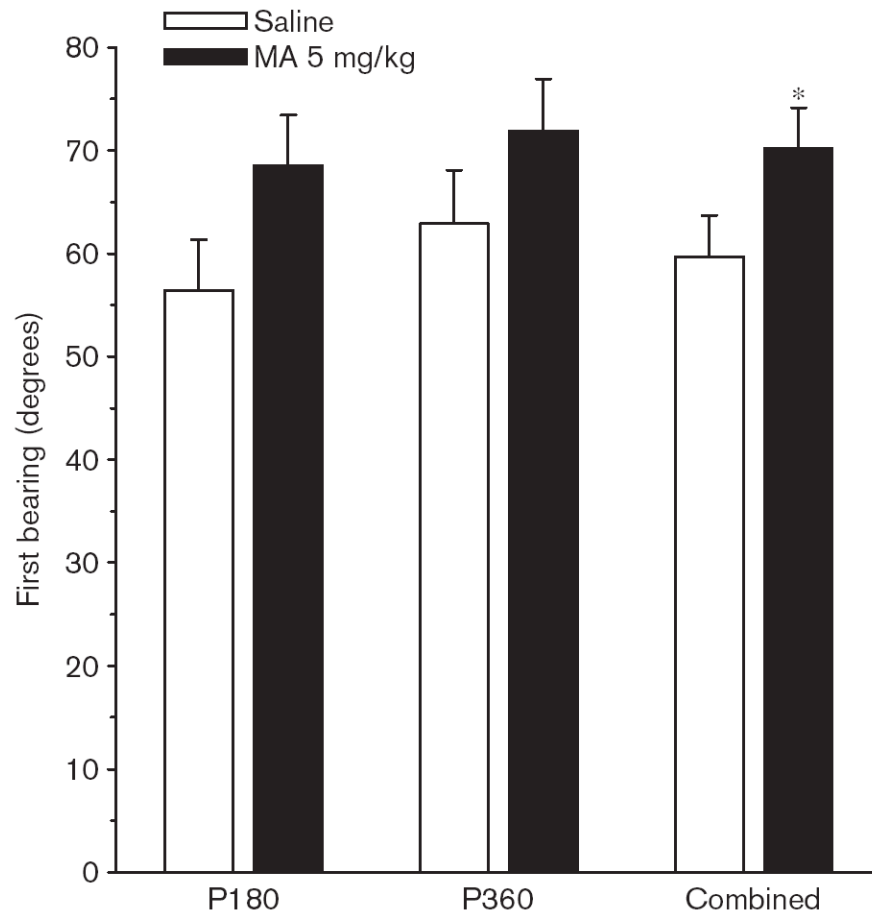
**Fig. 6.** Experiment 1. (P30, P40) Morris water maze, phase 3: Phase 3 was with the platform position being moved to a different quadrant from that during acquisition or reversal and with a smaller (5 × 5 cm) platform. (a) Path lengths averaged across 4 trials/day and across sexes for the P30 groups. (b) Path lengths as in (a) but for the P40 age groups. (c) First bearing averaged across trials, days, sexes, and ages to show the main effect of Group. (d) Time in the periphery (s) averaged across trials, days, sexes, and ages to show the main effect of Group. Data are means ± SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \* $P < 0.05$  \*\* $P < 0.01$  vs. saline.



**Fig. 7.** Experiment 1. (P30, P40) Morris water maze, phase 3, probe trial, first bearing: Data shown are for the P30 and P40 groups and for the combined averages of both test ages to show the main effect of Group. Data are averaged across sexes. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \* $P < 0.05$  vs. saline.

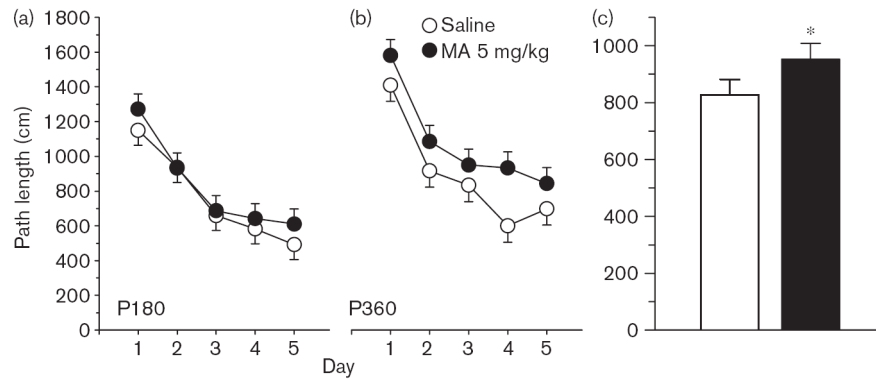
**Fig. 8.**

Experiment 2. (P180, P360) Morris water maze, acquisition phase, platform trials, path length: (a) Path length (cm) averaged across 4 trials/day and across sexes for the P180 groups. (b) Same as in (a) except as applied to the P360 groups. (c) Same as in (a) except that data are the combined averages of the P180 and P360 groups. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine. (MA) four times a day on P11–20. \* $P < 0.05$  vs. saline.

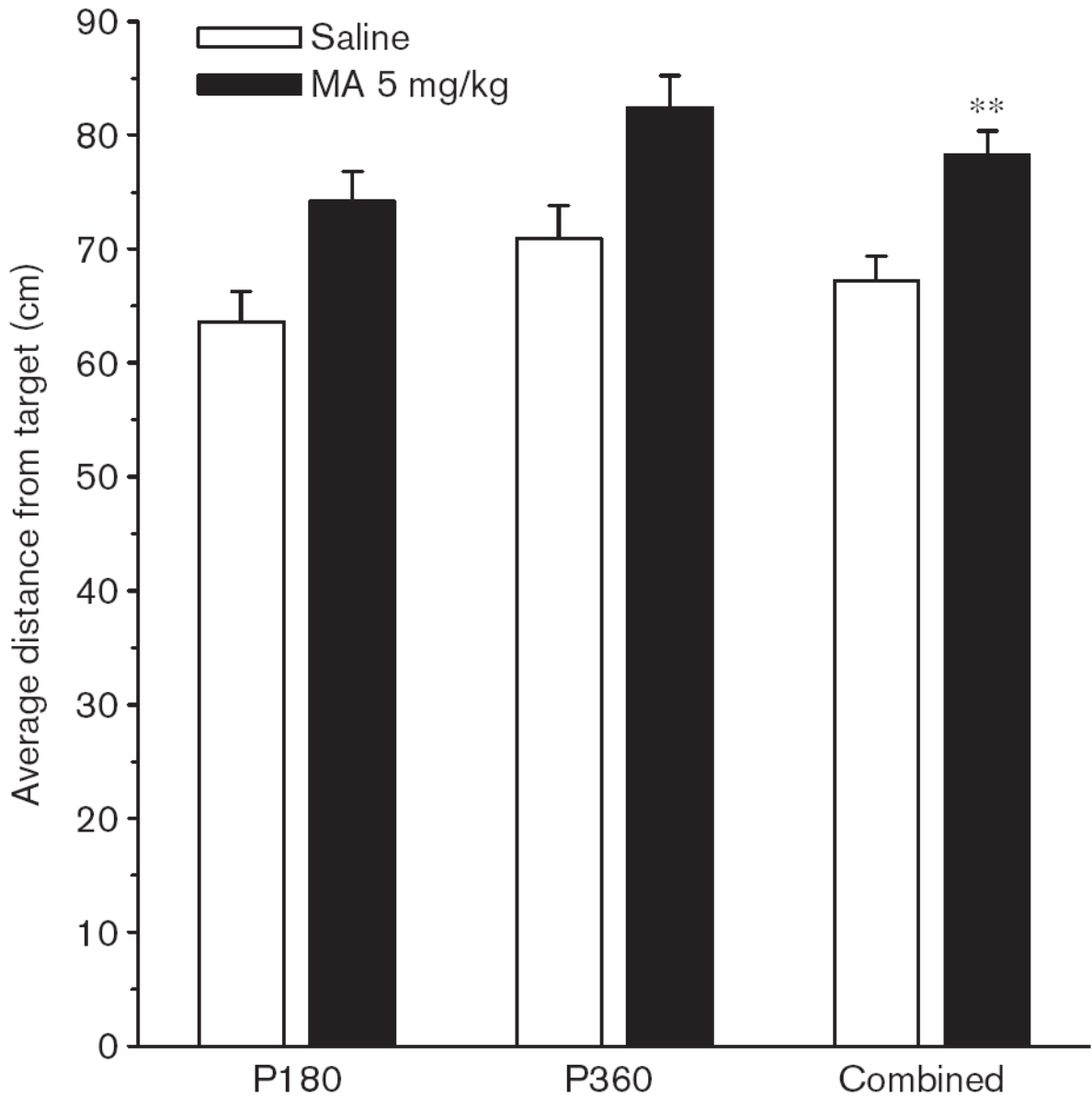


**Fig. 9.** Experiment 2. (P180, P360) Morris water maze, acquisition phase, probe trial, first bearing: First bearing, averaged across male and female rats, for the groups tested at P180, P360 or the combined average of both. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11-20. \* $P < 0.05$  vs. saline.

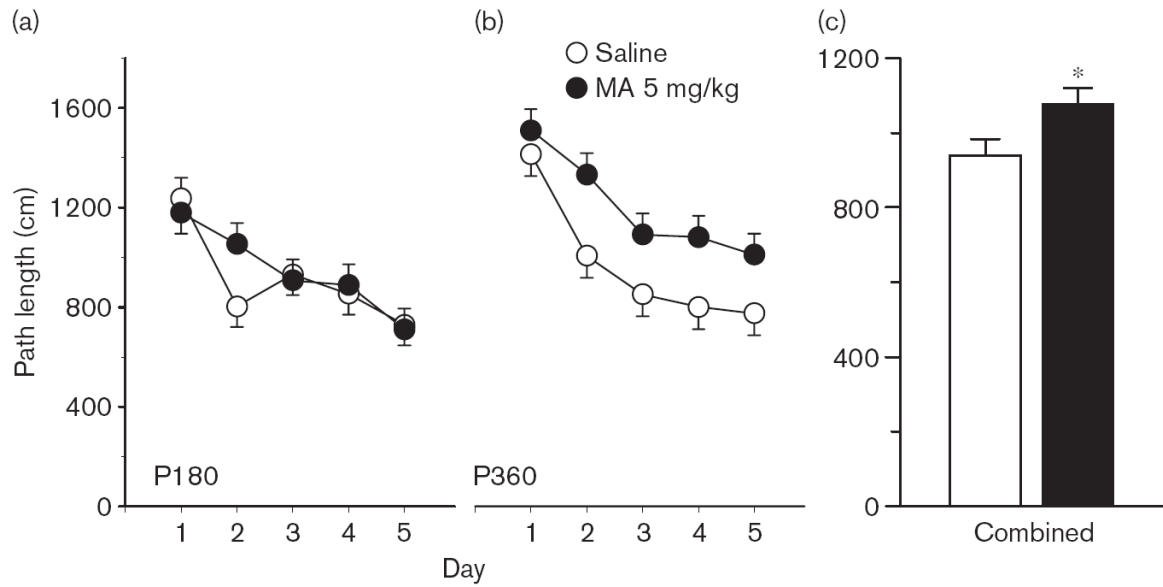


**Fig. 10.**

Experiment 2. (P180, P360) Morris water maze, reversal phase, platform trials, path length: (a) Path length (cm) averaged across 4 trials/day and across sexes for the P180 groups. (b) Same as in (a) except as applied to the P360 groups. (c) Same as in (a) except that data are the combined averages of the P180 and P360 groups, which are also averaged across days, to show the main effect of Group. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \* $P < 0.05$  vs. saline.

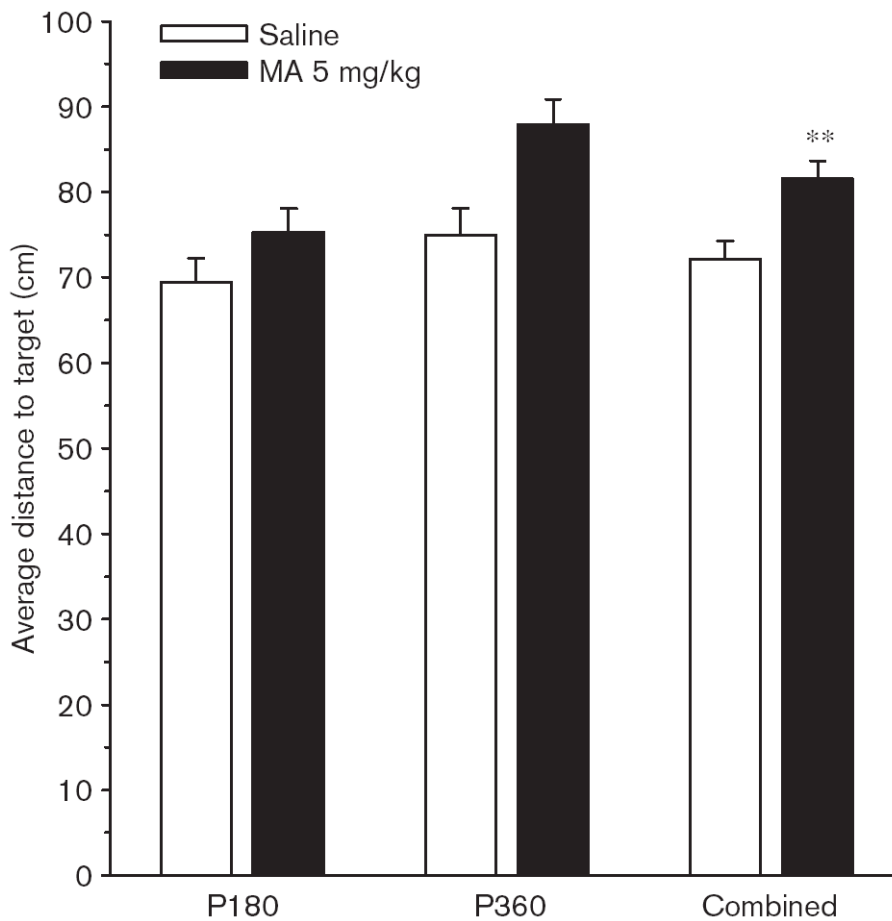


**Fig. 11.** Experiment 2. (P180, P360) Morris water maze, reversal phase, probe trial, average distance: Average distance (cm) is the mean distance from the platform site recorded every 55 ms during the probe trial. Data are shown for average distances averaged across male and female rats tested at P180 and P360 and for the combination of both ages. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \*\* $P < 0.01$  vs. saline.



**Fig. 12.**

Experiment 2. (P180, P360) Morris water maze, reduced phase (phase 3), platform trials, path length: (a) Path length (cm) averaged across 4 trials/day and across sexes for the P180 groups. (b) Same as in (a) except as applied to the P360 groups. (c) Same as in (a) except that data are the combined averages of the P180 and P360 groups, averaged across days to show the main effect of Group. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \* $P < 0.05$  vs. saline.



**Fig. 13.**

Experiment 2. (P180, P360) Morris water maze, reduced phase (phase 3), probe trial, average distance: Average distance (cm) is the mean distance from the platform site recorded every 55 ms. Data are shown as average distances averaged across male and female rats tested at P180 and P360 and across the combination of both ages. Data are means  $\pm$  SEM. Rats received 5 mg/kg methamphetamine (MA) four times a day on P11–20. \*\* $P < 0.01$  vs. saline.

Table 1

Body weights (mean  $\pm$  SEM) in grams (N)

Group	Sal	MA	Sal	MA
Test age		P30	P40	
Nearest weighing age		P28	P35	
Female	95.0 $\pm$ 2.1 (20)	83.7 $\pm$ 1.9* (20)	140.2 $\pm$ 2.8 (20)	131.0 $\pm$ 2.0* (20)
Male	103.7 $\pm$ 2.0 (20)	88.7 $\pm$ 2.3* (20)	163.9 $\pm$ 3.1 (20)	150.4 $\pm$ 2.8 (20)
Test age		P180	P360	
Nearest weighing age		P175	P357	
Female	342.3 $\pm$ 10.8 (20)	365.5 $\pm$ 10.7 (19)	498.7 $\pm$ 26.4 (19) <sup>a</sup>	503.5 $\pm$ 23.0 (19)
Male	692.6 $\pm$ 16.6 (20)	711.8 $\pm$ 19.7 (20)	841.1 $\pm$ 32.1 (19)	874.3 $\pm$ 27.3 (19)

\*  $P < 0.05$  vs. Sal. MA, methamphetamine; Sal, saline.<sup>a</sup> One additional animal in this group died before completing all phases of the Morris water maze and its data were excluded from the phase it did not complete.