Neuro Detective©
INTERNATIONAL
A Contract Research Network Of Neuroscientists

Test of the Specificity of COMPOUND X in Amelioration of Cognitive Loss

DATE

This study was conducted under terms of a Services Agreement between NeuroDetective Inc. and COMPANY, entitled *Test of Compound X in Amelioration of Cognitive Loss and Induction of Neuron Sprouting in Aged Female Rats*, dated xxxx.

Objective

This study tested whether COMPOUND X, a compound active at YYYY receptor sites, can improve spatial memory in late middle-aged or aged female rats. The study consisted of two separate experiments, the first administering COMPOUND X to aged (30-31 month old) female rats, and the second administering COMPOUND X to late middle-aged (19-20 month old) female rats. The first experiment tested COMPOUND X's ability to reduce existing cognitive deficits, while the second experiment tested COMPOUND X's ability to prevent the development of age-related cognitive deficits.

Subjects and Surgery

Exp. I

COMPOUND X-containing pellets (100mg, formulated by Innovative Research of America) were implanted subcutaneously in the dorsal fascia of the neck in thirteen 27-30 month old female Sprague-Dawley rats under ketamine/xylazine anesthesia. COMPOUND X placebo pellets of the same size and source were similarly implanted in ten rats. The placebo animals corresponded with their COMPOUND X counterparts in respect to strain, gender and age. With an average weight of approximately 320g over the course of this study, each COMPOUND X rat received a dose of approximately 5.2mg/kg/day of that compound, calculated from the release rate for these pellets as supplied by the formulating company.

Fifty-five to sixty days following initial implantation of the COMPOUND X pellets, new pellets were implanted, following instructions from the client that the compound is fully released from these pellets by 60 days. Each animal therefore received two implantations of either COMPOUND X or placebo pellets. Total dosing time was 45 days until the beginning of the place learning experiment, 70 days until the beginning of the place memory experiment.

Exp. II

COMPOUND X pellets (100 mg, as above) were implanted subcutaneously in the dorsal fascia of the neck in thirteen female 13-14 month old Sprague-Dawley rats under ketamine/xylazine anesthesia. COMPOUND X placebo pellets of the same size and source were similarly implanted in thirteen rats that corresponded to their COMPOUND X counterparts with respect to strain, gender and age. The calculated release rate of the drug was 5.2mg/kg/day.

As the time over which the compound was released was 60 days (according to information supplied by the client), dosing nominally ceased before the beginning of behavior testing. Specifically, there was a five-day period

between the end of dosing and the beginning of testing for place learning, and a sixteen-day post-dosing delay period prior to place memory testing.

Testing Procedure

Place learning

The animals were tested in a polypropylene pool 182 cm in diameter and 76 cm deep. The pool was filled with water to a depth of 34 cm. and maintained at a temperature of 22°C. The water was made refractory to light by the addition of a dilute solution of white tempura paint (Washable Poster Paint, Palmer Paint Products). A white Plexiglas platform 12.5 cm square is located 1 cm under the surface of the water in the center of one quadrant of the pool, and is not visible to the rat when swimming.

On each trial the rat is held by the torso behind the shoulders and gently placed into the pool facing the wall. On the first day, the rat is given one habituation trial, in which it is allowed to swim for 60 sec. On subsequent testing days, the rat is similarly placed in the pool and allowed to swim until it finds and stands upon the hidden platform, where it remains for 10 sec. and is then removed from the pool. If the rat does not find the platform after 60 sec has elapsed, it is removed from the pool. Starting point for each trial is randomly selected from one of the four cardinal compass points in the pool (north, south, east, west), without allowing a sequential repeat. For the testing period each rat is given 4 trials a day for 8 days for a total of 32 trials. Inter-trial interval is 10-12 minutes. Principal measures are latency (sec) and distance, or swim-path length (cm), to find the platform. For data analysis, the means of each rat's performance on each 4 trial block are used for ANOVA.

Twenty-four hrs after the last test trial, the hidden platform is removed, and the animals are placed in the pool again and allowed to swim for 60 seconds. Starting point is randomized for the two cardinal compass points furthest away from the previous platform location. Three measures are taken: Whishaw's corridor measure (per cent time and distance spent within a defined direct-line corridor between the starting point and the target location [former location of the hidden platform]; number of platform crossings (crossings of the previous location of the platform); and per cent time and distance spent within the vicinity of the former platform location (defined as a circle with its center on the target location and a radius of the distance between the target location and the edge of the pool). These measures indicate the strength of the rat's memory for the target location, in decreasing order.

All data collection is automated, using a video camera that records all animal movements. Associated software performs image analysis (Smart-Track, San Diego Instruments).

Place memory

The same water tank was used as with the place learning task, with the same general testing method. This test began at least 5 days after completion of the place-learning task for each animal. In the place-memory task the hidden platform was moved to one of 4 new locations. Beginning in the afternoon of the first day, the platform was moved to a new quadrant location (relative to the location used for place learning) and the starting point for the animal was at one of the two cardinal compass points furthest away from this new platform location. The animal was allowed 2 minutes to find the platform. The animal was re-tested after a 60 second delay (which began when the animal first climbed onto the platform and included the 10 sec time when the animal was allowed to remain on the platform). The animal was then re-tested again after an 18-hr. delay, i.e. in the morning of the next day. On each of these three trials (Initial, 60-sec Delay, 18-hr Delay) the animal started from the same location and the hidden platform remained in the same location. In the afternoon following the 18-hour delay trial, the location of the hidden platform was changed and the sequence began again. If the animal failed to find the platform within 2 minutes on the initial trial, the animal was placed on the platform for 15 seconds and that particular pairing of the starting location/target location was repeated at the end of the sequence (described below). The 60-sec and 18-hr delay trials were given only when the animal found the new platform location (initial trial) within 2 minutes.

Each animal was tested once at each of 4 possible starting location/target location pairings. The first three target locations were in the center of one of the quadrants, randomly determined for each animal but without repeat and with the constraint that only the three quadrants not used in the Place Learning test were used. The fourth target location was at one of the four cardinal compass points (randomly determined), at the same distance from the pool wall as in the center-quadrant locations. When the target was in the center of a quadrant, the starting point was always one of the two cardinal compass points furthest away from the target, with each starting location occurring equally often for each group of animals. When the target location was at a cardinal compass point, the starting point was one of the two adjacent compass points, with each starting location occurring equally often for each group of animals.

As with place learning, the data analysed were latency to find the platform and swim path length, using the same software.

T-Maze

To assess the effect of COMPOUND X on attention, the discrete-trial version of the T-maze task was used (DiCamillo et al, 1998). Two different reward rules were utilized, rewarded alternation (delayed non-match to sample [DNMS]) and rewarded repetition (delayed match to sample [DMS]). In Exp. I and II the animals first learned delayed non-match, followed by delayed match, while the reverse order of learning occurred in Exp. III. The animals in Exp. I and II began testing either 45-60 days or 30 days (respectively) following pellet

implantation, while the animals in Exp. III began testing at 100 days following their first pellet implantation.

Rewarded Alternation (DNMS)

Before testing began, the rats were food restricted to 85% of their pre-test weight, acclimated to the maze and trained to eat food pellets out of trays placed at the ends of each arm of the T. The maze was T-shaped and constructed of clear acrylic. It consisted of a start box at the base of the T, the stem, and two arms at the top of the T. The start box and arms were both accessible to and from the stem through opaque guillotine doors. The width of all portions of the maze was 26cm; start box length was 28cm, stem length was 90cm, and arm length was 28 cm. Across the entrances to the arms of the T, in front of the guillotine doors and blocking the animal's view of both the door and the food well at the end of each arm, were attached pieces of black felt. Thus in order to enter either arm of the T, the animal had to push aside the piece of black felt. A food well was attached to the end of each arm of the T, 6cm above the floor.

The animal was placed in the start box at the base of the stem of the T, the door to the box opened and the animal allowed to traverse the stem to the crossed arms of the T, where it was allowed to enter one or the other of the arms. Although the entrances to each of the arms of the T were "blocked" by the black felt, animals quickly learn to nose the felt aside and enter the arms.

Following acclimation, the animals received a series of paired-run trials. Each trial consisted of, first, a forced run and then a choice run. On the forced run, one arm of the T was blocked by the guillotine door, behind the black felt. In the food well of the open arm were three pellets (45mg each, Noyes) selected from a random assortment of chocolate, banana and peanut butter-flavored reward pellets. On the choice run, the doors to both arms were open, with the food pellets available only in the arm opposite the side of the forced run. There was a 90 sec delay between the forced and choice runs, during which the animal was in a holding cage adjacent to the base of the stem. (Throughout training in Exp. I and II the animals had also been shaped to return to the start box and climb into the adjacent holding cage after receiving their pellets on the forced run.) At the end of the 90 sec inter-run delay a bell sounded, cueing the animal to climb from the holding cage back into the start box. This particular shaping procedure was used to minimize handling of the animal within the trial. (In Exp. III the animals were only trained to return to the start box after receiving their pellets from the forced run, at which point the animal was placed by the experimenter into the holding cage. At the end of the inter-trial delay, the animal was returned by the experimenter to the start box.)

Upon completion of the choice run, the animal was returned to its home cage. The inter-trial interval was 15-20 min. Removing the animal for this extended time between trials was intended to emphasize the discreteness of each trial, in order to minimize any effect of response patterning.

A self-correction procedure was also used; a trial in which the animal entered the unrewarded arm (an "error") on the choice run was repeated until the animal entered the rewarded arm, with a maximum of three sequential errors allowed. Outside of this constraint, the side of the forced arm was randomly determined for each trial, with no more than three consecutive forces to a particular side.

Animals received 5 trials per day until reaching a criterion of 8/10 trials correct over discrete 2-day blocks, or until 60 trials had occurred (50 trials in Exp. I). Entries were scored correct or incorrect (according to the DNMS reward rule) when all four paws entered the arm. Between the forced and choice runs of a trial, both the floor of the maze at the intersection of the stem and the arms (the choice point) as well as the felt covering the entrances to the arms, were sprayed with a deodorizing disinfectant (Quatricide PV), to minimize odor cues.

Rewarded Repetition (DMS)

Animals were tested with the rewarded repetition rule either three-five days after reaching criterion on rewarded alternation (Exp. I and II) or before being tested on rewarded alternation (Exp. III). All aspects of the testing procedure, other than the changed reward rule, were identical to those for rewarded alternation learning, with the exception that, in Exp. I only, testing continued until a criterion of 4/5 correct was reached on one day.

Results

Place learning

Exp. I

These animals were tested in place learning at 29-30 mos. of age, following 45 days of COMPOUND X dosing. The results were equivalent with both the latency and swim-path length measures; only the more common latency data are presented in what follows.

As expected, overall ANOVA was highly significant, $F(14) = 3.05$, $*p = 0.0004$, reflecting more rapid learning by the young animals. Post-hoc Fischer's LSD tests showed that this significant effect was in fact due solely to young animals learning faster than either group of old animals ($p<0.05$). The COMPOUND X treated animals were equally slow in their learning rate as placebo animals (Fig. 1). In the probe trial all groups of animals spent equivalent percentages of time in the vicinity of the former target location, and made equivalent numbers of platform crossings (data shown in Appendix).

Exp. II

These animals were tested in place learning at 19 mos. of age, following six months of daily COMPOUND X treatment (app. 5.2mg/kg/day). As with Exp. I, the results with both the latency and swim-path length measures were equivalent; only the more common latency data are presented here.

As expected for 19 mos. Sprague-Dawley females, there was no significant impairment in place learning, compared to 7-9 mos. animals, and (not surprisingly therefore) no COMPOUND X effect, F (14) = 1.09, p = 0.36 (Fig. 2). There were similarly no significant effects in the probe trial (data shown in Appendix).

Place memory

Exp. I

The aged (30-31 mos) females were significantly slower than their young controls only at the short delay (60 sec); there were no significant group differences in the initial or long delay trials (Fig. 3). Similarly aged females treated for 70 days with COMPOUND X were significantly faster than the placebo animals at the short delay, though also significantly slower than the young controls. In the overall repeated measures ANOVA, only the interaction was significant, $F(4) = 4.882$, $p < 0.003$, reflecting the fact that all groups were significantly different from each other at the 60 sec delay ($p < 0.01$ by Fischer's LSD test), but on no other trial.

Exp. II

The late middle-aged females (19-20 mos) were also significantly slower than their young controls only at the short delay (Fig. 4). Similarly aged females treated with COMPOUND X for 6 mos prior to testing were also significantly faster than placebo animals at the short delay. In the repeated measures ANOVA there was a significant group effect, $F(2) = 5.587$, $p < 0.006$, reflecting overall faster performance by the young controls. Post-hoc Fischer's LSD tests showed the COMPOUND X group to be significantly faster than the placebo group at the 60 sec delay, and the placebo group significantly slower than the young controls at that delay, $p < 0.05$. The difference between the COMPOUND X and young control groups at the short delay just missed significance, $p > .05$.

Rewarded Alternation

Exp. I

During rewarded alternation testing one old placebo animal and one old untreated animal died. Also, one animal in each group did not reach performance criterion within 50 trials, and their data are not included. There

were also no differences between the two old control groups (Placebo and Untreated) and so their data were combined for statistical analysis.
The 26-28 month old animals receiving COMPOUND X made half the number of errors while learning this DNMS task as did the similarly aged animals in the Placebo (Untreated) group, a difference that was statistically significant (Table 1A).

Rewarded Repetition

Exp. I

Again there were no differences between the two old control groups (Placebo and Untreated), and so their data were combined for statistical analysis. Both the old COMPOUND X treated and old control animals committed more errors than the untreated young animals in this DMS version of the T-maze task, however the group differences were not statistically significant in an overall ANOVA, F (3, 20) = 1.15, $p = 0.35$. A comparison of all old animals grouped together regardless of treatment condition vs. the young animals also missed statistical significance at $p < 0.09$. The absence of a significant age effect with this rule of T-maze learning appeared to result from poorer than usual performance by all animals, especially the young ones, compared to other studies in our laboratory (Table 1B).

Rewarded Alternation

Exp. II

The significantly more rapid learning of the alternation rule of T maze learning (DNMS) by old animals receiving COMPOUND X, compared to old controls, was repeated in a second experiment with a different set of animals (Table 2A). Bartlett's test for equal variances showed a large and significant difference among the groups in this experiment, mostly due to 2 old Placebo animals that performed exceptionally well (B [corrected] = 13.72, $p < 0.002$), leading to the choice of non-parametric analysis.

Rewarded Repetition

Exp. II

Again each group of old animals committed more errors than the young animals with the DMS rule of T-maze learning, but in this experiment the difference between young and combined old animals (regardless of treatment) reached statistical significance, t (18) = 2.5, $p < 0.03$. Following an overall ANOVA of all groups, only the COMPOUND X/young Untreated comparison was significant (Table 2B).

Thus in two experiments, 30-45 days treatment with COMPOUND X improved learning of a DNMS task by old animals, but did not improve learning of the reverse rule (DMS) by the same animals. In both experiments the animals learned DNMS before DMS.

Rewarded Repetition

Exp. III

When animals were first trained in the T-maze with the DMS rule, the results were similar in that COPMPOUND X did not improve learning by the aged females (Table 3A). Overall the aged females committed significantly more errors than young controls (overall ANOVA: F (2) = 3.48, p = 0.0503; post-hoc grouping of COMPOUND X and placebo groups vs. untreated young controls, t (16) = 2.51, p = 0.03).

Rewarded Alternation

Exp. III

In contrast to the results of both Exp. I and II, in which the animals learned rewarded alternation first, there was no significant COMPOUND X effect when the rewarded alternation task followed the rewarded repetition task, F (2) = 0.3, p = 0.74 (Table 3B).

Together with the results of Exp. I and II, these data indicate that COMPOUND X improves initial learning by aged female rats that is coincident with the animals' pre-existing tendency (alternation). COMPOUND X does not improve learning the more difficult, reverse behavior (note the higher absolute number of errors committed during initial learning of rewarded repetition [Exp. III] compared to initial learning of rewarded alternation ([Exp. I and II]).

Conclusion

These data demonstrate that COMPOUND X treatment reduces the extent of an existing age-related impairment in the formation of new spatial memories (Exp. I), and prevents the development of an age-related impairment in the formation of new spatial memories (Exp. II). Both acute (45 days) and chronic (6 mos.) treatment with COMPOUND X did not affect learning of the basic place learning task, whether the animals had a deficit in the task relative to young controls (Fig. 1) or not (Fig. 2). Thus, COMPOUND X treatment did not affect either normal or impaired learning of this reference memory task.

However the same COMPOUND X treated animals located novel locations of the hidden platform (a working memory task) faster than similarly aged placebo animals, on the second (60 sec delay) trial. This was true for both the early old-age (19 mos) and advanced old age (30-31 mos) animals (Figs. 3, 4). Placebo animals at both these ages were slower to find the platform on the second trial compared to young controls. It may also be noteworthy that COMPOUND X's effect in Exp. II was seen beginning at least 16 days after the contents of the implanted COMPOUND X pellet had nominally been exhausted,

suggesting the drug may have produced a morphological or long-lasting biochemical change in the brain.

Compound X treatment improves learning. Aged Fischer/Norway females are only slightly impaired, if at all, in learning a spatial version of the delayed non-match to sample type of test, rewarded alternation in a T-maze. However, 30 days of COMPOUND X treatment (app. 4.8 mg/kg/day) improved DNMS learning in the T-maze by aged (26-28 mos.) rats, who committed half the number of errors as their age-matched controls before reaching criterion. When the reward rule was reversed (it became a DMS task), and the task became more difficult for these same animals, the COMPOUND X treatment was ineffective. In another experiment with different animals of the same age and strain, these effects were repeated with slightly longer COMPOUND X treatment (45 days). When aged Sprague-Dawley females (31-32 mos.) were tested on the DMS task initially, followed by the DNMS task, COMPOUND X treatment was again ineffective, in both tasks, indicating that COMPOUND X's positive effect on learning by aged animals is specific to the less difficult DNMS task when it is first being learned. When the animals have had some experience in the T-maze, COMPOUND X does not improve learning subsequent tasks, easy or difficult.

References

Brown, R, Gonzalez, C.R. & Kolb, B. (2000) (*a*) Nicotine improves Morris water task performance in rats given medial frontal cortex lesions. Pharmacology, Biochemistry and Behavior, 67, 473-478.

Brown, R.W., Gonzalez, C.L.R., Whishaw, I.Q., & Kolb, B. (2000) (*b*) Nicotine improvement of Morris water task performance after fornix lesions is blocked by mecamylamine. Behavior Brain Research, 119, 185-192.

DiCamillo, A.M., Neff, N.T., Carswell, S., & Haun, F.A. (1998) Chronic sparing of delayed alternation performance and choline acetyltransferase activity by CEP-1347/KT-7515 in rats with lesions of nucleus basalis magnocellularis. Neuroscience, 86, 473-483.

Fischer, W., Chen, K.S., Gage, F.H., & Bjorklund, A. (1991) Progressive decline in spatial learning and integrity of forebrain cholinergic neurons in rats during aging. Neurobiology of Aging, 13, 9-23.

Gage, F.H., Chen, K.S., Buzsaki, G. and Armstrong, D. (1988) Experimental approaches to age-related cognitive impairments. Neurobiology of Aging, 9: 645-655. Kolb, B., & Walkey, J. (1987) Behavioural and anatomical

studies of the posterior parietal cortex in the rat. Behavioural Brain Research, 23, 127-145.

Lindner, M.D. (1997) Reliability, distribution, and validity of age-related cognitive deficits in the Morris water maze. Neurobiology of Learning and Memory, 68: 203-220.

Lindner, M.D. and Schallert, T. (1988) Aging and atropine effects on spatial navigation in the Morris water task. Behavioral Neuroscience, 102: 621-634.

Figure Legends

Figure 1.
Latency (mean and standard error) to locate hidden platform (fixed location) in the Morris water task. $F(14) = 3.05$, $p < 0.0004$ (Group). $^{*} = p < 0.05$ vs. both Placebo and Compound X treated by *post hoc* Fischer's LSD tests.

Figure 2.
Latency (mean and standard error) to locate hidden platform (fixed location in the Morris water task. $F(14) = 1.09$, $p = 0.36$ (Group).

Figure 3.
Latency (mean and standard error) to locate hidden platform at four novel locations in the watermaze task, initially and at two delay times. $F(4) = 4.882$, $p < 0.003$ (Group x Trial). $^{**} = p < 0.001$ vs. 7-9 mos. Untreated, and (for Compound X treated) vs. 30-31 mos. Placebo, by *post hoc* Fischer's LSD tests.

Figure 4.
Latency (mean and standard error) to locate hidden platform at four novel locations in the watermaze task, initially and at two delay times. $F(2) = 5.587$, $p < 0.006$ (Group). $^{*} = p < 0.05$ vs. 8-10 mos. Untreated (for 19-20 mos. Placebo) and vs. Placebo (for Compound X treated),by *post-hoc* Fischer's LSD tests.

Table 1 (Experiment I)

Errors to Criterion, T-maze
(means ± s.e.m.)

		Placebo (26-28 mos)	COMPOUND X (26-28 mos)	Untreated (6-8 mos)
A.	Rewarded Alternation	15.3 ±1.6	6.0∗ ±1.2	11.0 ±1.8
B.	Rewarded Repetition	31.0 ±2.8	26.6 ±5.4	21.7 ±4.7

(A) -- ∗ = $p < 0.05$ vs. Placebo/Untreated by Fisher's LSD test following significant ANOVA, F (3,20) = 5.23, $p < 0.008$.
N = 8 (Placebo/Untreated), 5 (COMPOUND X) and 7 (Untreated).

Table 2 (Experiment II)

Errors to Criterion, T-maze
(means ± s.e.m.)

		Placebo (27-29 mos)	COMPOUND X (27-29 mos)	Untreated (6-8 mos)
A.	Rewarded Alternation	12.8 ±3.9	5.5∗ ±0.5	7.5 ±1.8
B.	Rewarded Repetition	23.8 ±5.0	29.5∗ ±3.5	17.7 ±1.2

(A) - ∗ = $P < 0.03$ vs. Placebo by Mann-Whitney test (U = 4.0) following significant ANOVA, Friedman (non-parametric) = 7.36, $p < 0.02$.
(B) - ∗ = $P < .05$ vs. Untreated (6 mos.) by Newman-Keuls test following significant overall ANOVA, F (2, 17) = 4.04, $p < 0.04$.
N = 8 (Untreated) and 6 (Placebo and COMPOUND X).

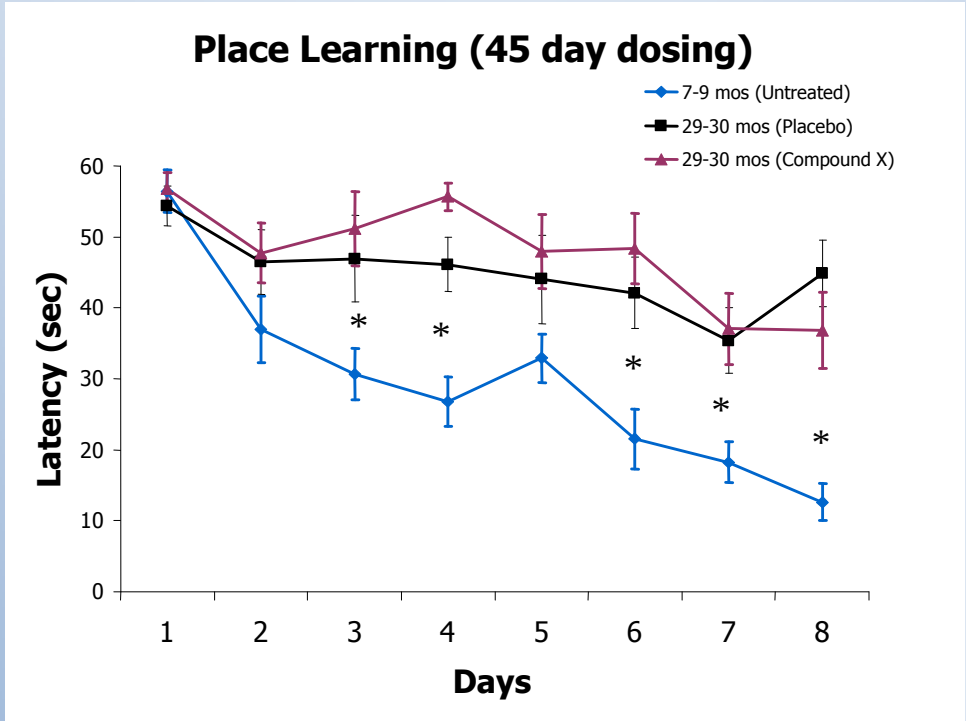
Table 3 (Experiment III)

Errors to Criterion, T-maze
(means ± s.e.m.)

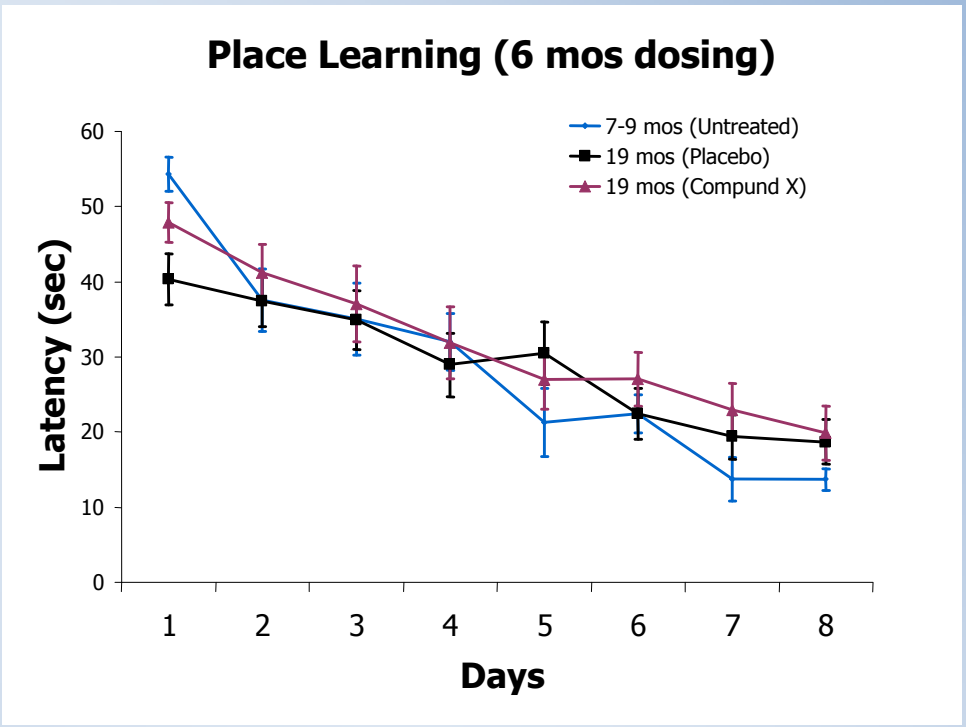
		Placebo (27-29 mos)	COMPOUND X (27-29 mos)	Untreated (6-8 mos)
A.	Rewarded Repetition	24.4∗ ±2.4	27.3∗ ±2.5	19.0 ±2.4
B.	Rewarded Alternation	12.14 ±3.7	12.25 ±2.4	15.0 ±3.5

(A) -- ∗ = $p < 0.03$, combined Placebo and COMPOUND X vs. Untreated, t (16) = 2.51, following overall ANOVA: F (2) = 3.48, p = 0.0503.
N = 8 (COMPOUND X and Untreated) and 7 (Placebo).

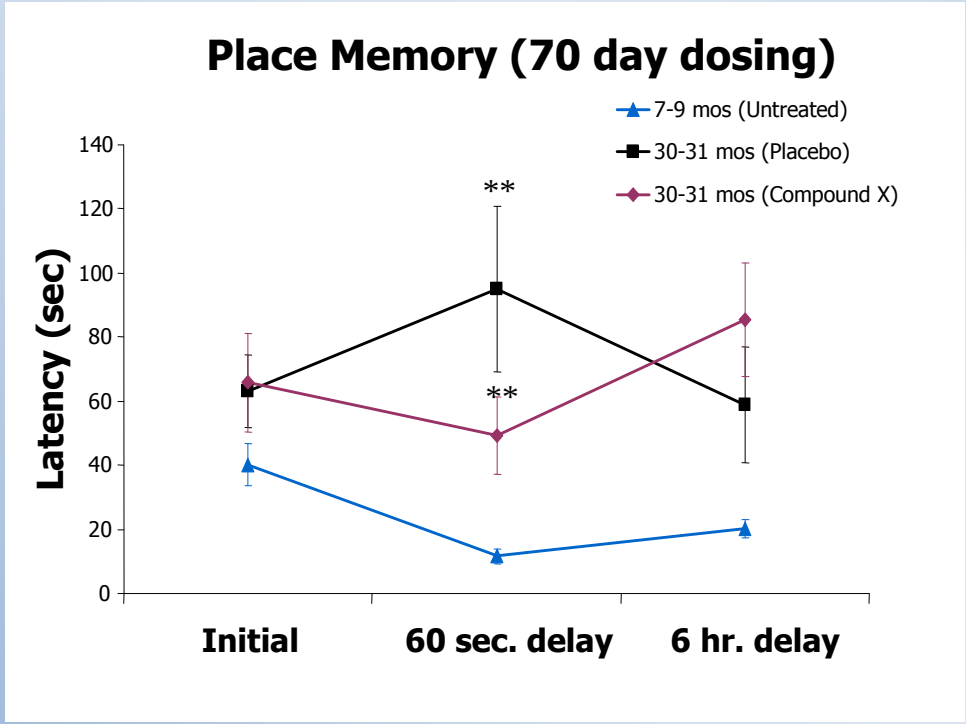
---------- **FIGURE 1** ----------



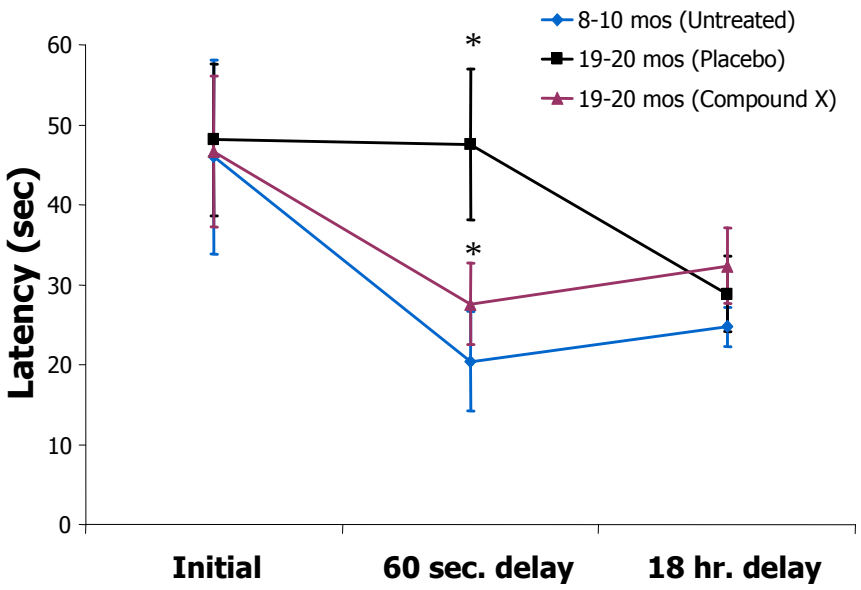
----------FIGURE 2 ----------



----------FIGURE 3 ----------



----------FIGURE 4 ----------



16 day period between end of dosing and beginning of testing.

For additional information, please contact us at:

PH: 1. 215. 536. 8757 / 8758
E: info@ndineuro.com