EXAMPLE

Test of *Compound X* in Scopolamine Impaired Rats

DATE

This study was conducted under the terms of a Services Agreement between NeuroDetective International and CLIENT, dated ….

1. Executive Summary

Purpose	This study compared two doses of "X" with the Alzheimer's disease therapeutic agent, donepezil HCl (Aricept®*), for effects on learning and memory using a scopolamine-induced cognitive impairment model in rats.
Design	Male Long-Evans rats (3 months of age) were administered either saline, donepezil (0.75 mg/kg), or "X" (3.0 mg/kg or 40.0 mg/kg) by oral gavage for 10 days prior to (and during the 5 days of) testing in a water maze task. The compounds were evaluated for their ability to reverse the amnestic effects of scopolamine (0.1 mg/kg) in this task.
Results	• Donepezil (0.75 mg/kg) and "X" (40 mg/kg) both appeared to reverse the effects of scopolamine in the last 2 days of hidden platform testing. "X" (40 mg/kg) treated animals also learned faster and more efficiently than donepezil treated animals during the middle portion of learning. • There were no treatment effects in the probe trials, which primarily assay memory, most likely because of the high (near maximum) level at which all animals learned this task by the fourth day of hidden platform testing. • Somewhat unexpectedly, the particular dose of scopolamine used tended to produce impairment in the animals' learning to swim to a visible platform. This effect was exacerbated by, and reached statistical significance when combined with, both doses of "X". This impairment may be indicative of altered visual acuity in these animals. • Notwithstanding the potential confounding effect described above, all treatment groups did learn the hidden platform task and performed the task quite efficiently by the fourth day of testing.

2. Methods

2.1 Subjects

Fifty male 3-month-old Long Evans rats (Harlan Sprague-Dawley, Inc.) were housed and tested in an AAALAC-approved research facility, under the direction of NDI personnel.

Test subjects were housed in pairs in polycarbonate cages with Bed-O-Cob® bedding in a temperature-controlled room (25°C) with a 12 hr light/dark cycle. Upon arrival, animals were provided with water and food (Teklad rodent feed® or Purina Rat Chow®) ad libitum. All rats were handled daily for a minimum of one week prior to behavioral testing. The procedures employed during this study were reviewed and approved by the relevant Animal Care and Use Committee (IACUC). The subjects used in the experimental groups were weighed prior to the experiment and the dose volume was based on the animals' individual body weights. Animals were observed for visible signs of adverse effects after each dose administration.

2.2 Drug administration

The animals were randomly assigned to one of 5 groups (n=10/group) and administered one daily dose of either of two doses of "X" (3 or 40 mg/kg), donepezil HCL (0.75 mg/kg), or vehicle (0.9% saline), via oral gavage (p.o.) beginning ten days prior to initiation of the water maze task. Daily dosing continued through the initial day of visible platform testing and the subsequent five days of water maze testing (i.e., on hidden platform test days as well as probe trial days). On both the single day of pre-testing (Visible Platform Test) as well as on each day of watermaze testing, thirty minutes prior to the first trial, 0.1 mg/kg of scopolamine hydrobromide was administered i.p. to four of the five groups (i.e., excluding the saline-saline group). Fifteen minutes after the scopolamine injection (or vehicle) each group was dosed with one of the test articles or vehicle (see Table 1 for an overview of the testing protocol). Test articles were prepared according to the supplied instructions, and aliquots of the solutions were stored (at -20°C). Test agents were freshly thawed at the time of use.

2.3 Procedures

In order to test and compare the effects of "X" on both acquisition and recall of spatial memories, the water maze hidden platform task was utilized. The rationale for using this test is based on published studies demonstrating that

the experimental paradigm of finding a platform hidden beneath the water surface in a swimming pool reveals spatial learning and memory capabilities of the rodent (e.g., Stewart and Morris, 1993; Sutherland et al., 1988). Scopolamine is known to impair performance in the water maze task, and blockage of scopolamine effects is known to reduce impairment in the water maze (e.g., Terry et al., 1999).

2.3.1 Testing apparatus

The water maze experiment was performed in a circular pool (diameter = 180 cm, height = 76 cm) made of black plastic. The pool was filled with water to a depth of 35 cm and the water was maintained at 25.0±1.0 °C. The pool was located in a large room with a number of extra-maze visual cues including geometric images (squares, triangles, circles etc.) hung on the wall, diffuse lighting, and black curtains used to hide the experimenter (visually) and the resting test subjects. Swimming activity of each rat was monitored via a television camera mounted overhead, which relayed information including latency to find the platform, total distance traveled, time and distance spent in each quadrant etc. to a video tracking system (Actimetrics, Evanston, IL).

2.3.2 Visible platform task

On the day prior to initiation of water maze testing, a visible platform test was performed as a general estimate of visual acuity. In this pre-test, a highly visible (white) cover fitted with a small white flag was attached to a small, square platform submerged beneath the surface of the water (described below), which effectively raised the surface of the platform to approximately 1.0 cm above the surface of the water. Each rat was given one or more trials with a 90 sec time limit to locate the platform visually. This was accomplished by lowering the rat into the water in the NE quadrant and allowing the rat to locate and climb onto the platform, which was located in center of the opposite quadrant of the pool. When the rat was successful (on its own accord, i.e. without experimenter assistance) it was then given a series of 4 additional trials (with a 1.0 min inter-trial interval) and the latency (in sec) to locate the platform was recorded. On each of these trials the platform was moved to the center of a different quadrant and the animal was always placed into the pool from the opposite quadrant until the test was conducted once in all 4 quadrants.

2.3.3 Hidden platform task

For the water maze test itself, the escape platform was submerged. The platform was an invisible (black) 10 cm square, submerged approximately 1.0 cm below the surface of the water and placed in the center of the NE quadrant. Each rat was given 4 trials per day for 4 consecutive days to locate and climb onto the hidden platform. A trial was initiated by placing the rat in the water facing the pool wall in one of the 4 quadrants. The daily order of entry into individual quadrants was pseudo-randomized such that all 4 quadrants were used every day. For each trial, the rat was allowed to swim a maximum of 90 sec, in order to find the platform. When successful the rat was allowed a 30-sec rest period on the platform. If unsuccessful within the allotted time period, the rat was given a score of 90 sec and then physically placed on the platform and also allowed the 30-sec rest period. In either case the rat was given the next trial after an additional 1.5 min rest period (i.e., total inter-trial interval=2.0 min).

2.3.4 Probe trial (transfer test)

On the day following the last trial of the hidden platform task, a single probe trial was conducted, in which the platform was removed from the pool to measure memory for the previous platform location. The animal was placed in the pool and time and distance traveled in each of the 4 quadrants plus the number of crossings over the previous platform location, were recorded.

2.4 Measurement and analysis of performance

All data were collated and entered into Microsoft Excel spreadsheets, and subsequently imported into SigmaStat version 2.03 for statistical analyses. Statistical analyses were conducted without knowledge of group identity. Latencies, swim path lengths, swim speeds, and time spent in the previous target quadrant of the pool (Probe Trial) as well as the number of crossings of the previous platform location were compared across groups using Repeated Measures ANOVA, with post-hoc comparisons made using Fischer's PLSD test at a significance level of $p < 0.05$.

In many cases the data sets did not meet the criteria for either normality or equal variance (or both), and so the data were first either transformed or ranked and then analyzed by ANOVA. All figures show the untransformed data, while statements of statistical significance are based on the transformed or ranked data (when performed). It should be noted that in the analysis of the data from the Visible Platform Test, no data transformation resulted in normality; statistical analysis used least-squares transformed data.

3. Results

3.1 Visible platform test

Fig 1 illustrates a trend toward impairment in locating a highly visible platform in the watermaze pre-test, for all treated groups compared to untreated (Saline-Saline). In the overall ANOVA (2-way repeated measures), this trend produced a significant Group effect, $F_{4, 45} = 3.0$, $p<0.03$. Performance improved over the 4 trials, as indicated by a significant Trial effect, $F_{3, 12} = 5.3$, $p<0.01$. There was no significant Group x Trial interaction. In post-hoc Fischer's tests, the impairment in locating the visible platform was statistically significant in all trials for animals receiving "X" at the lower dose, and in the latter two trials for animals receiving "X" at the higher dose.

3.2 Hidden platform test

As expected in this task, there was a significant improvement in performance over testing days, as indicated by a significant Day effect in both the latency ($F_{3, 12} = 77.4$, $p<0.001$) and swim distance measures ($F_{3, 12} = 65.5$, $p<0.001$). Also as expected, scopolamine impaired learning, as indicated by the overall Group effect ($F_{4, 45} = 4.8$, $p<0.01$ in the latency measure, $F_{4, 45} = 4.4$, $p<0.01$ in the swim distance measure). As illustrated in Figure 2, post-hoc Fischer's tests indicated that on test days 1 and 2 animals administered scopolamine generally performed less efficiently than saline controls whether or not donepezil or the "X" compounds were administered. On days 3 and 4, however, performance of animals administered donepezil or "X" at 40 mg/kg (in combination with scopolamine) did not differ (statistically) from saline controls indicating at least a partial reversal of the scopolamine effect later in learning. This was true for both endpoint measures, latency to find the platform and swim path length.

3.3 Swim speeds

There were no treatment effects on swim speed ($F_{4, 45} =2.0$, $p=0.10$). All groups tended to swim more rapidly on earlier than later days (overall Day effect, $F_{3, 12} = 26.4$, $p<0.001$), with no significant Treatment x Day interaction ($F_{135, 199} = 1.5$, $p=0.10$).

3.4 Probe trial

Figure 3 illustrates the performance of the different groups in the probe trial. There were no significant effects of drug treatment on the animals' memory for the prior platform location, as measured by either percentage of time spent in the quadrant of the previous platform location ($F_{4, 45}$ = 0.7, p=0.60), or the mean number of crossings over the previous platform area ($F_{4, 45}$ = 0.9, p=0.50).

4. Summary and Conclusions

1. Donepezil (0.75 mg/kg) and "X" (40 mg/kg) appeared to at least partially reverse the effects of scopolamine in the latter 2 days of hidden platform testing. "X" (40 mg) treated animals also tended to learn the spatial location of the hidden platform faster and more efficiently (shorter swim path length) than donepezil treated animals (trials#6 – 9).

2. There were no treatment effects observed in the probe trials, as indicated by percent time spent in the previous target quadrant and the number of crossings over the previous platform area. This is most likely due to the high (near maximum) level of learning the hidden platform task, as noted above.

3. The dose of scopolamine used in this study with Long Evans rats led to some impairment in the animals' ability to locate a highly visible platform, possibly indicative of adverse effects on visual acuity. This effect was exacerbated by "X".

4. Notwithstanding the possible effect on visual acuity, all scopolamine groups did learn the hidden platform task and performed quite efficiently by the fourth day of testing.

5. References

Stewart, C.A. and Morris, R.G.M. (1993) The water maze. In: A. Sahgal (ed.), **Behavioural Neuroscience, Vol. 1, A Practical Approach**. Oxford (UK), Oxford University Press, pp. 107-122.

Sutherland, R.J., Whishaw, I.Q. and Kolb, B. (1988) Contributions of cingulate cortex to two forms of spatial learning and memory. **Journal of Neuroscience**, 8: 1863-1872.

Terry, A.V., Jr., M. Gattu, M., Buccafusco, J.J., J.W. Sowell, J.W., and Kosh, J.W. (1999) Ranitidine analog JWS-USC-75IX enhances memory-related task performance in rats. **Drug Development Research**, 47 (2): 97-106.

6. Tables and Figures

Table 1: Testing Protocol

Group	July 25 through August 7 (Oral Gavage)
1	Vehicle
2	Vehicle
3	Donepezil 0.75 mg/kg
4	3.0 mg/kg
5	40 mg/kg

	August 3 through August 8	
	30 minutes before testing (IP dosing)	15 Minutes Before Testing (Oral Gavage)
1	Vehicle	Vehicle
2	Scopolamine 0.1 mg/kg	Vehicle
3	Scopolamine 0.1 mg/kg	Donepezil 0.75 mg/kg
4	Scopolamine 0.1 mg/kg	3.0 mg/kg
5	Scopolamine 0.1 mg/kg	40 mg/kg

Figure 1

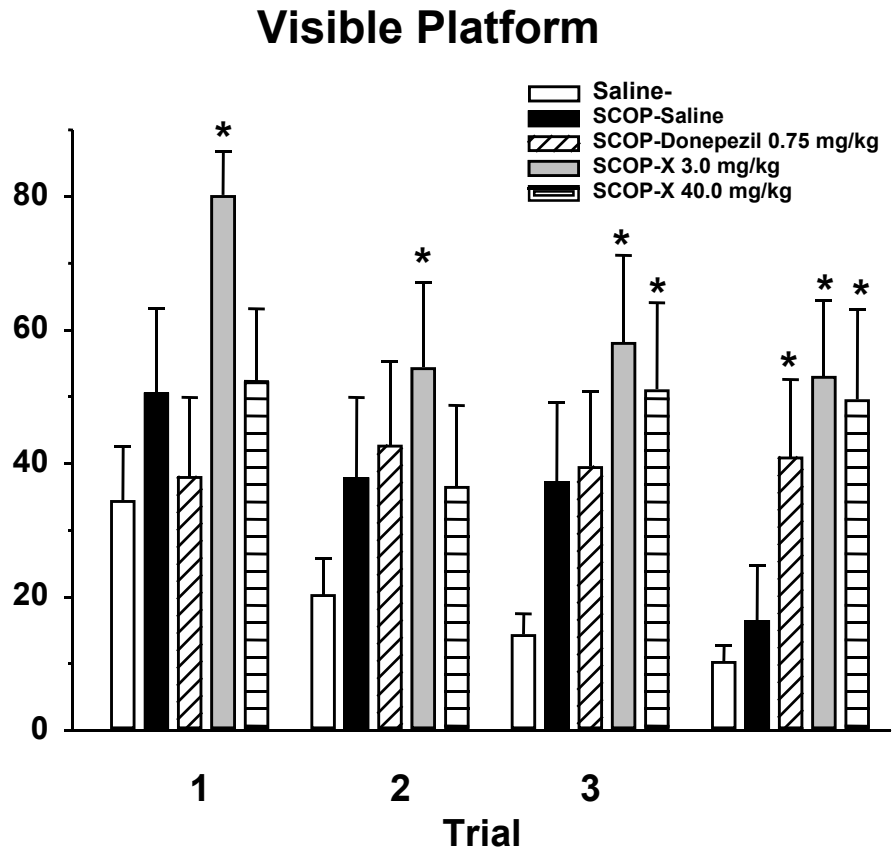

Fig 1. Mean latency in seconds (± s.e.m.) to locate a highly visible platform by the various groups in the watermaze pre-test, over 4 trials. * = $p < 0.05$ vs. Saline-Saline by post-hoc Fischer's LSD test following overall significant two-way repeated measures ANOVA. $N = 10$ rats per group.

Figure 2

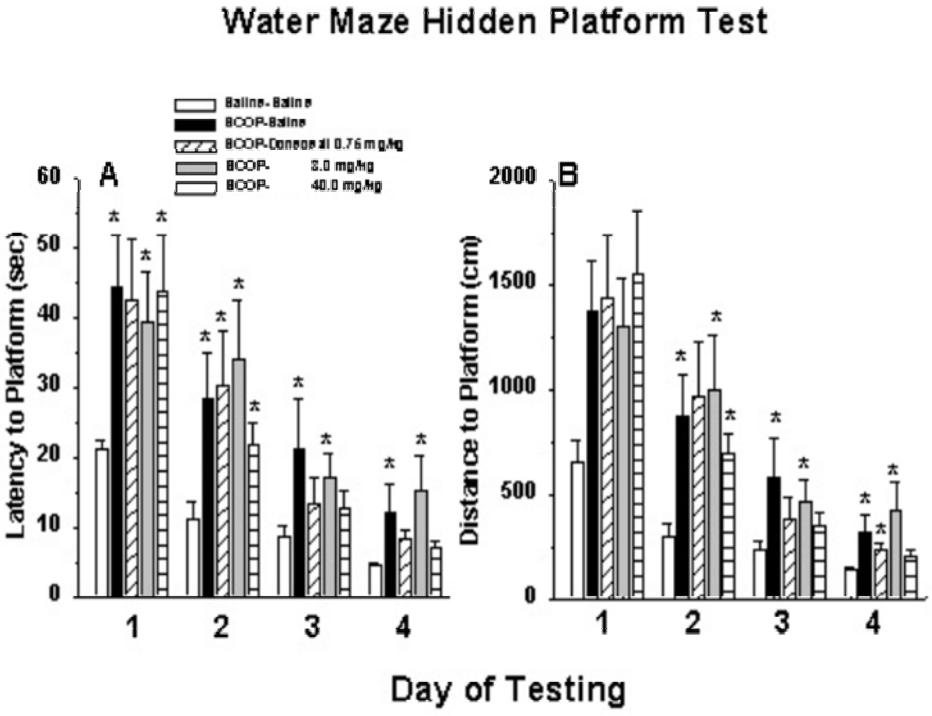

Fig 2. (**A)** Mean latency in seconds (± s.e.m.) and (**B)** distance traveled in cm (± s.e.m.) to locate a hidden platform by the various groups in the watermaze learning task over 4 trials. * = $p < 0.05$ vs. Saline-Saline by post-hoc Fischer's LSD test following significant overall two-way repeated measures ANOVA. N=10 rats per group).

Figure 3

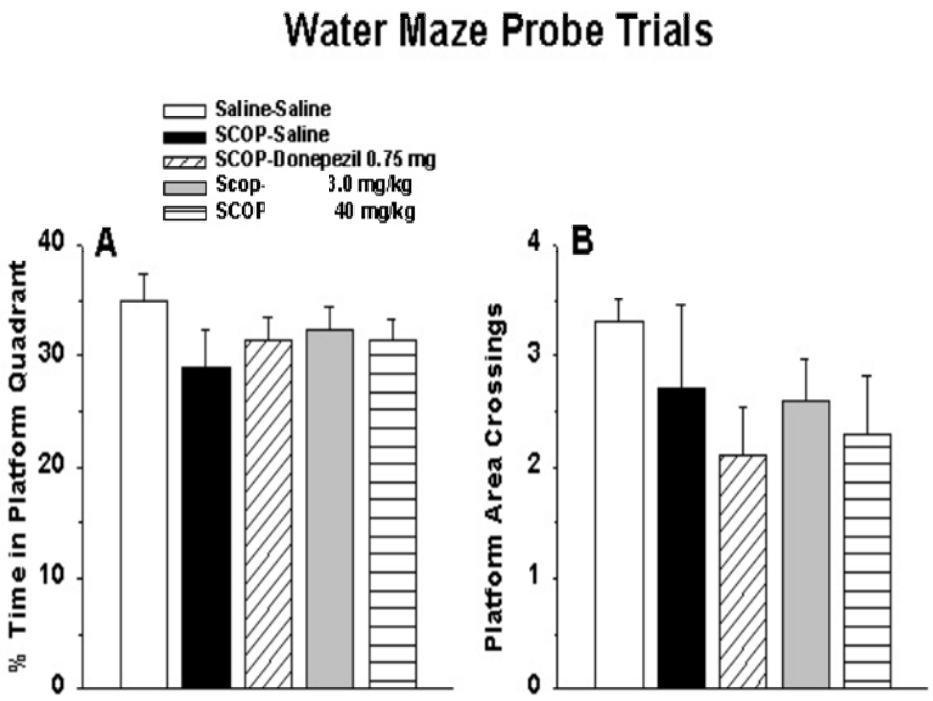

Fig 4. Performance of probe trials by the various groups. Each bar represents the mean (± s.e.m.) of **(A)** percentage of time spent in the quadrant that previously contained the hidden platform; and **(B)** the mean number of crossings over the area (10 sq. cm) of the previous location of the hidden platform. *N*=10 rats per group.