CONFIDENTIAL **FINAL**

ClientXXXX: Evaluation of XXXX in the Foot-shock induced USV model in Rats

STUDY REPORT

Study/Report No.	XXXXXXXXXXX-ZZZZZ for NDI YYYYY for Client
Report issue date	DATE
Date of Experiment	DATE - DATE
Undertaken at	UNIVERSITY XXX under the direction of YYY and under the overall supervision of NeuroDetective International, Inc

STATEMENT AND SIGNATURE

CompoundXXX: Evaluation of CompoundXXX in the Foot-shock induced USV model in Rats

This study was conducted in the CCC Laboratory at XXX University, under the direction of YYY, and under the overall supervision of NeuroDetective International, Inc. All procedures were approved by the Institutional Animal Care and Use Committee at XXX University, and animal care was in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

WRITTEN BY:

YYY Professor, Study Director XXX University	Date

REVIEWED BY:

Forrest A. Haun, Ph.D. Chief Executive Officer NeuroDetective International (NDI), Inc.	Date

QUALITY ASSURANCE:

ZZZ (TITLE), Quality Assurance person at WWW assigned to this study by NDI	Date

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GLOSSARY OF TERMS

D.W	Distilled water
I.P	Intraperitoneal injection
USV	Ultrasonic vocalization

1. SUMMARY

Test Article: Compound XXX
Test System (Species/Strain/Substrain): Wistar Rats (Charles River, Portage, MI, USA, 49024)
Sex: Male

Results of Study:
The effects of a proprietary compound designated COMPOUND XXX were studied using a fear-conditioned ultrasonic vocalization (USV) paradigm. Two primary dependent measures were used: 1) number of USVs emitted, and 2) change in number of USVs emitted compared to baseline. The duration of USVs emitted was also recorded and analyzed and was treated as a secondary dependent measure, because USV duration generally shortens as the absolute number of USVs emitted decreases. A number of statistically significant effects were found from these experiments:

- COMPOUND XXX at the 5.0, 10.0 and 20.0 mg/kg doses significantly reduced the number of USVs emitted compared to vehicle controls.
- All doses of COMPOUND XXX tested (0.1, 1.0, 5.0, 10.0, and 20.0 mg/kg) significantly reduced the number of USVs emitted compared to baseline levels.
- COMPOUND XXX at 5.0, 10.0 and 20.0 mg/kg significantly reduced the duration of USVs compared to vehicle controls.

In Conclusion, these findings strongly suggest that COMPOUND XXX has anxiolytic and/or antidepressant effects.

2. INTRODUCTION

TITLE: Effect of COMPOUND XXX on Foot-shock induced Ultrasonic vocalization (USV) frequency in male Wistar rat.

PURPOSE: The objective of this study is to confirm the anxiolytic and/or antidepressant effects of COMPOUND XXX

TESTING LAB.: CCC Laboratory
XXX University, LOCATION

RESPONSIBILITIES:

Study Director	AAA Professor
Animal facility manager	BBB, B.S. Colony Technician
Archivist	CCC, MA, Title.

BACKGROUND:
COMPOUND XXX was examined after IP administration to evaluate its potential anxiolytic and/or antidepressant effects using the Foot-shock induced Ultrasonic vocalization model, which is one of the best validated conditioned animal models of Anxiety/Depression in rats. In general, benzodiazepine anxiolytics and the new pyrimidinylpiperazine (5-HT1A receptor related) anxiolytics (De Vry et al., 1991, 1992; Traber and Glaser, 1987) suppress ultrasonic vocalization.

3. TEST ARTICLE AND VEHICLE INFORMATION

3.1. Test Article

Name:	COMPOUND XXX
Lot No.:	Lot#. XXXXXX
Sources or supplier:	_____
Physical properties:	_____
Purity:	_____
Storage condition:	Stored in a dry, dark, controlled access area and maintained at -20℃ temperature

3.2. Positive Control

Name:	Buspirone hydrochloride
Lot No.:	Lot# 048K1674
Sources or supplier:	Sigma-Aldrich
Physical properties:	White powder
Purity:	> or = 99%
Storage condition:	Stored in a dry, dark, controlled access area and maintained at 4℃ temperature

3.3. Vehicle for Test Article & Positive Control

Name:	0.9% physiological saline
Formulation of vehicle	made with mixture (D.W: Saline = 99.1: 0.9) The 0.9% physiological saline solution was made dissolving sodium chloride (>98%, ICN Biomedicals, Inc., Lot # 5099E) in purified water

(> 99%) to a concentration of 0.9%

4. METHODS AND EXPERIMENTAL DESIGN

4.1. Test System

Species:	Rat
Strain/Substrain:	Male Wistar
Justification for species selection:	The characteristics of the animal used (age, strain, species) were comparable with those described in the scientific literature (De Vry et al., 1993). The Wistar rat is generally recognized as appropriate for stress-induced ultrasonic vocalization testing and considerable historical data are available.
Source:	Charles River, Portage, MI, USA, 49024
Body weight range:	208 - 286g body weight range at the beginning of the experiment. Subjects are tested repeatedly with at least a 10-day washout period between dosings.

4.2. Animal Husbandry

Method of identification:	Tail markings using permanent marker
Housing:	Animals were delivered to the laboratory at least one week before the experiment, during which time they acclimatized to the laboratory conditions. Animals were housed in groups of 4 in plastic cages (55x35x20 cm).
Quarantine and acclimatization:	The animals were acclimated to laboratory conditions for a minimum of seven days prior to initiation of dosing.
Food:	Pelleted feed (Mazuri Rodent Pellets 5633, PMI International, 505 N 4th Street, Richmond, Indiana, USA, 47374) were provided *ad libitum*. Heavy metals and insecticide residues were certified by the vendor. Contaminants were not observed in animal feed at a level expected to interfere with the integrity of this study.
Water:	Tap water was provided *ad libitum* consistent with rodent housing care protocols at XXX University.
Bedding:	Animal bedding (Beta Chips, NEPCO, 115 Sweet Road, Warrensburg, New York, USA,

	12885) was supplied during the study period. Low contaminant levels were verified by the vendor and were not high enough to interfere with the integrity of this study.
Environmental conditions:	Animal rooms with controlled conditions as follows: Temperature: 20 to 26 ℃ Relative humidity: 40 to 60 % Ventilation: 10 to 20 times/hour Light: 150 to 300 Lux with 12 hour light/dark cycle (Lights on 0700)
Animal ethics and welfare:	Standard operating procedures related to this experiment have been reviewed and approved by the Institutional Animal Care and Use Committee at XXX University and are in compliance with the NIH Guide for the Care and Use of Laboratory Animals (National Academy Press, 1999).

4.3. Administration of the Test Article/Vehicle

Formulation method of the dosing solution:	The suspensions for test and reference articles were prepared on the day of the experiment. Prepared articles were not thereafter subjected to any physical-chemical analyses. The quality of the preparation was verified indirectly from their observed pharmacological effects.
Dose volume:	All articles were administered in a volume of 1ml/kg body weight. The doses of COMPOUND XXX are expressed in mg/kg body weight.

4.4. Subjects

Seventy male Wistar rats, weighing 208 – 286 g at the start of the study were obtained and used in this experiment to test five doses of COMPOUND XXX, plus saline and buspirone. Three doses of COMPOUND XXX were tested first, followed by two additional doses of COMPOUND XXX that were added to the dose response curve at a later date (10 days following washout from the first dosing). At each of the dosing times, the doses of

COMPOUND XXX were tested along with vehicle and the positive control (buspirone).

4.5. Apparatus

Four standard rodent experimental chambers equipped with shock grid floors and an ultrasonic vocalization detector were used for these experiments. All equipment and software are supplied by Med-Associates (St. Albans, Vermont, USA). The frequency range for USVs was set for 20 – 40 kHz for USVs of at least 30 dB. The time resolution for USVs was 30 msec.

4.6. Testing Procedures

The procedures used in this USV method are similar to those used by Molewijk et al., 1995. These procedures comprise three separate phases, performed over the course of three consecutive days. In the first phase (Day 1), each rat was placed individually in a USV chamber and exposed to 6 randomly distributed shocks (0.8 mA, 8 sec duration) over the course of a 7 minute session. In the second phase (Day 2), each rat was placed in the same USV chamber for 2 minutes, during which time it received a single shock (0.8 mA, 8 sec duration). The rat was then returned to its home cage. After 30 minutes, each rat was returned again to the same USV chamber for a 10 minute trial period, during which no shocks were delivered. During this 10-minute period (referred to as the "pre-test" session) USVs were recorded; these USVs constituted baseline data for comparisons to responses in the final phase of the procedure (Day 3). Rats that emitted fewer than 80 USVs (each being separated by 30 msec) during the pretest session were excluded from further testing. The animals retained for further testing were ranked according to the number of USVs emitted in the pre-test session and then assigned in a balanced block design to one of the experimental groups (5 doses COMPOUND XXX), to the buspirone group, or to the vehicle group. On Day 3, two trials were given to each animal, one for 2 minutes and the other for 10 minutes, using procedures identical to those on Day 2, except that a drug injection was given immediately after the 2 min trial. Then, during the following 10 minute test session, the number and duration of USVs was recorded. These procedures (Days 1 to 3) were repeated again 10 days later.

5. DATA ANALYSIS

Raw data were entered into calculation sheets previously controlled, checked and protected, using GraphPad Prism 5 (GraphPad Software Inc., USA). All data entered were compared

by two individuals, and in this way verified before data analysis.

Statistical analysis: Results are presented as means followed by a standard error of the mean (SEM). Animals were matched for number of USVs emitted across each condition during the pre-test session and then assigned to each test condition in a balanced-block design. As described in the Subjects section (above), the full dose range of COMPOUND XXX was tested at two separate times (10 days apart). The mean value of the vehicle groups' data was not significantly different at these two testing times; therefore the data for these two groups were combined into a single Vehicle group for analysis purposes. Moreover, as a result of combining data from the two testing times, some animals are represented in the combined dose response curve more than once, with data from different treatment conditions. The combined data were analyzed using a one way between groups analysis of variance. A Dunnett's post hoc analysis was subsequently conducted for assessing statistically significant differences between vehicle control and treatment conditions. Values of $p<0.05$ were considered as statistically significant.

6. ARCHIVES

All raw data, the original Study Protocol and all amendments, the original report, correspondence and all other study-related documentation will be securely maintained for 5 years according to standard archiving procedures in the CCC Laboratory at XXX University.

7. PROTOCOL ADHERENCE

The study was performed in accordance with the NDI study protocol No. XXXXXXXXXXX-ZZZZZ There were no deviations from the study protocol during the study period.

8. RESULTS & CONCLUSION

USVs were assessed during the 10 minute test session on Day 3, after rats had been treated with either buspirone (2.0 mg/kg; N=16), COMPOUND XXX (0.1 [N=7], 1.0 [N=7], 5.0 [N=7], 10.0 [N=14], and 20.0 mg/kg [N=9]) or vehicle control (N=18). The number of

USVs, percent change in USVs from baseline, and duration of USVs emitted for each of these treatment groups and vehicle is shown in the figures below.

8.1. Analysis 1: Mean(±SEM) number of calls

Assessed 30-min post-treatment (Day 3)

USV Counts: Overall, there was a statistically significant difference in number of USVs between groups, $F\ (6, 71) = 4.61$, $p < 0.001$. Subsequent Dunnett's post-hoc tests showed significantly fewer USV's emitted by buspirone and by COMPOUND XXX at 5.0, 10.0 and 20.0 mg/kg, compared to vehicle control.

8.2. Analysis 2: Mean(±SEM) percent change in USV level (% of Baseline)

% Change = Day3/ Day2 * 100

Change in USV Counts (% of baseline): USV counts on Day 3 (post-treatment) were compared to baseline levels of USV counts on Day 2, using a percentage change measure. Overall, there was a statistically significant difference for change in USV counts between groups, $F\ (6, 71) = 9.14$, $p < 0.0001$. Subsequent Dunnett's post-hoc tests showed statistically significant reductions in USVs for buspirone and all COMPOUND XXX doses compared to vehicle controls.

8.3. Analysis 3: USV Durations

USV Duration: Durations of USVs were also calculated for vehicle controls and the treatment groups. Overall, there was a statistically significant difference in USV durations between groups, $F\ (6, 71) = 7.67$, $P<0.0001$. A Dunnett's post hoc test showed a statistically significant reduction in USV duration, compared to vehicle control, for COMPOUND XXX at 5.0, 10.0 and 20.0 mg/kg, as well as for buspirone.

8.4. Conclusion

The findings in this study strongly suggest that COMPOUND XXX has anxiolytic and/or antidepressant effects.

9. REFERENCES

Molewijk HE, van der Poel AM, Mos J, van der Heyden JA, Oliver B (1995) Conditioned ultrasonic distress vocalizations in adult male rats as a behavioral paradigm for screening anti-panic drugs. Psychopharmacology (Berl) 117: 32-40

De Vry, J., T. Glaser, T. Schuurman, R. Schreiber and J. Traber (1991) 5-HT1A receptors in anxiety, in: New Concepts in Anxiety, eds. M. Briley and S.E. File (Macmillan, London) p. 94

De Vry, J., R. Schreiber, T. Glaser and J. Traber (1992) Behavioral pharmacology of 5-HT1A agonist: animal models of anxiety and depression, in: Serotonin1A receptors in Depression and Anxiety, eds. S.M. Stahl, M. Gastpar, J.M. Keppel Hesselink and J. Traber (Raven Press, New York) p.55

De Vry J, Benz U, Schreiber R, Traber J (1993) Shock-induced ultrasonic vocalization in young adult rats: a model for testing putative anti-anxiety drugs. European Journal of Pharmacology 249: 331-339

Traber J., and Glaser T. (1987) 5-HT1A receptor-related anxiolytics. Trends Pharmacol. Sci. 8:432–437.

10. TABLES

Table 1 : Mean (±SEM) number of USV calls

Treatment Articles	Dose, mg/kg, ip	N	Number of USV calls	p (vs. Vehicle)
Vehicle	0	18	305.4 ±35.2	-
COMPOUND XXX	0.1	7	235 ± 79.1	NS
	1	7	236.1 ±376.3	NS
	5	7	119.6 ±177.0	<0.05
	10	14	128.6 ±234.5	<0.01
	20	9	107.2 ±047.7	<0.01
Buspirone	2	16	70.9 ±018.9	<0.001

NS = Not Significant

Table 2 : Mean (±SEM) percent change in USV level (% of baseline)
% change = Day3/Day2 *100

Treatment Articles	Dose, mg/kg, ip	N	% of baseline	P (vs. Vehicle)
Vehicle	0	18	83.6 ± 7.2	-
COMPOUND XXX	0.1	7	45.2 ± 10.6	<0.05
	1	7	33.5 ± 8.0	<0.01
	5	7	25.7 ± 15.2	<0.001
	10	14	24.5 ± 5.9	<0.001
	20	9	35.9 ± 17.2	<0.01
Buspirone	2	16	16.0 ± 3.4	<0.001

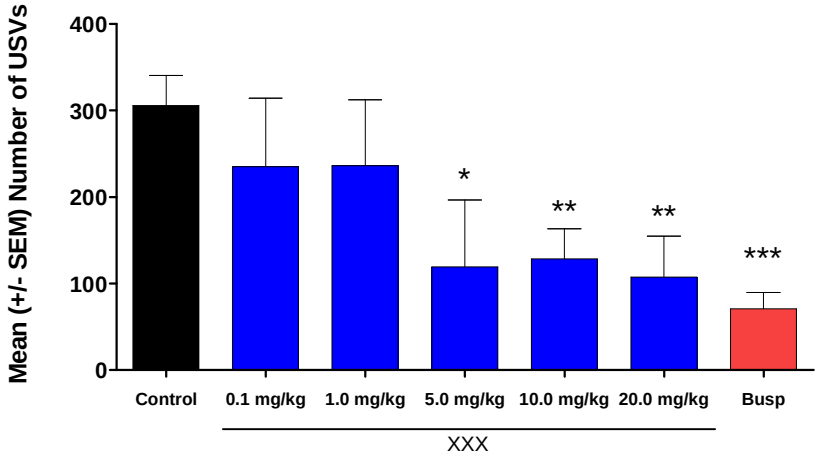
Table 3 : Mean (±SEM) USV Durations

Treatment Articles	Dose, mg/kg, ip	N	Mean Duration (seconds)	P (vs. Vehicle)
Vehicle	0	18	0.59 ± 0.072	-
COMPOUND XXX	0.1	7	0.44 ± 0.10	-
	1	7	0.51 ± 0.099	-
	5	7	0.086 ± 0.010	<0.001
	10	14	0.29 ± 0.093	<0.01
	20	9	0.077 ± 0.0071	<0.001
Buspirone	2	16	0.18 ± 0.046	<0.001

11. FIGURES

Figure 1. Analysis: Mean(±SEM) number of calls

Assessed 30-min post-treatment (Day 3)



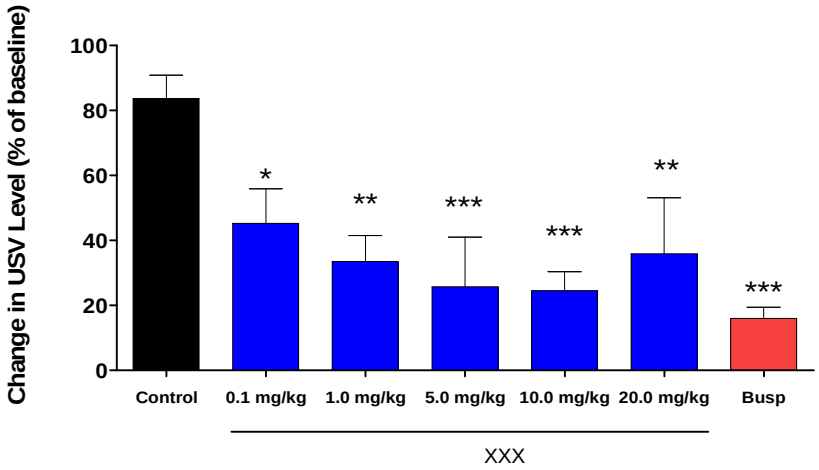
* at least p<0.05 in comparison to control

** at least p<0.01in comparison to control

*** at least p<0.001 in comparison to control

Figure 2. Analysis: Mean(±SEM) percent change in USV level (% of Baseline)

% Change = Day3/ Day2 * 100

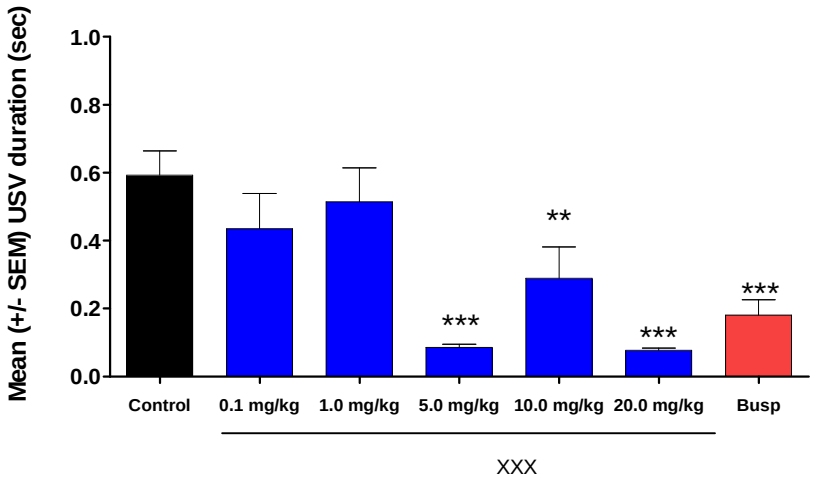


* at least p<0.05 in comparison to control

** at least p<0.01in comparison to control

*** at least p<0.001 in comparison to control

Figure 3. Analysis: Mean(±SEM) USV duration



* at least p<0.05 in comparison to control

** at least p<0.01in comparison to control

*** at least p<0.001 in comparison to control