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Example Report

Evaluation of XXX in Neuropathic Pain Model

Introduction

This study evaluated a selective ion channel antagonist, xxx, in a rat model of chronic pain, compared to both a positive control (gabapentin) and a proprietary reference compound (yyy). The model involves unilateral chronic constriction injury of the L5 spinal nerve. The endpoint measures include both a standard behavioral measure of pain threshold and a behavioral measure of the aversive component of pain perception.

Methods

Subjects

A total of 66 male Sprague-Dawley rats were employed in this study. At the time of baseline paw withdrawal testing (3 days post –L5 ligation surgery), five animals were not hyperalgesic and were dropped from additional testing. The remaining 61 animals, weighing 210-300 g at the time of initial drug injection, were randomly assigned to the different treatment groups. Two animals died during the course of the experimental procedure (one animal from the 20 mg/kg xxx group, one animal from the vehicle control group). The data from all remaining 59 animals that were tested during both the acute and the chronic phases of the experimental procedure are reported here. The final sample sizes for each treatment group were: Vehicle, n =10; yyy (10 mg/kg) n=9, xxx1 (0.3 mg/kg) n=9; xxx2 (3 mg/kg) n=12; xxx3 (20 mg/kg) n=10; gabapentin 90 mg/kg n=9. Animals were housed in groups of three and maintained on a 12 h/12 h light/dark cycle with food and water available *ad libitum*.

Surgical Procedure

Animals were anesthetized using halothane anesthesia (3% induction; 2-2.25% maintenance). The rats were placed in a prone position and the left paraspinal muscle was separated from the spinous process at the L4-S2 levels. The L6 transverse process was carefully removed with a small rongeur to identify visually the L4-L5 spinal nerves. The L5 spinal nerve was isolated and tightly ligated with 6-0 silk thread. The muscle layer was sutured and the skin incision closed with wound clips. An antibacterial solution was applied to the surgical site. Post-surgical signs of infection or overt signs of

discomfort were closely monitored. Discreteness of the lesion was indicated by the presence of normal motor function in the adjacent L4 hindpaw area following recovery from anesthesia.

Behavioral Testing

Paw withdrawal testing was performed using the up/down method (Dixon, 1980). Animals were retrieved from the colony room and habituated to a Plexiglas test chamber on top of a mesh screen for 15 min. The size of the chamber for free movement of the animal and the mesh screen allows for application of calibrated von Frey monofilaments to the plantar surface of each hindpaw. The force applied to each paw by the monofilaments ranged from 3 mN to 200 mN. For each trial, the 50% withdrawal threshold for each hindpaw was calculated using the following formula: $[X_{th}]_{log} = [vFr]_{log} + ky$, where [vFr] is the force of the last von Frey used, k = 0.2268 which is the average interval (in log units) between the von Frey monofilaments, and y is a value that depends upon the pattern of withdrawal responses (Dixon, 1980). The difference in paw withdrawal threshold between the left (ligated) and right (intact) hindpaws was calculated, and then averaged across the 3 trials of the test session to yield the final calculation of withdrawal threshold for each animal. Thus, the larger the difference in left minus right hindpaw threshold, the greater is the degree of hyperalgesia. As values approach zero difference following treatment, this indicates attenuation of hyperalgesia.

Place avoidance testing was performed according to the method of LaBuda and Fuchs (2000). Specifically, animals were placed within a 30x30x30 cm Plexiglas chamber positioned on top of a mesh screen. One half of the chamber was painted white (light area) and the other half of the chamber was painted black (dark area). During behavioral testing, animals were allowed unrestricted movement throughout the test chamber for a 30-min test period. Testing started immediately with mechanical stimulation (476 mN von Frey monofilament) applied to the plantar surface of both hindpaws at 150 sec intervals. The mechanical stimulus was applied to the left paw (ligated) while the animal was within the dark area of the chamber, and to the right paw (non-ligated) while the animal was within the light side of the chamber. The number of seconds an animal spent within the light side of the chamber was recorded, and converted to a percentage score for each 5-min period of time.

General Procedure

On day one, animals were surgically prepared with ligation of the L5 spinal nerve according to the procedure described above and allowed a 3-day post-surgical recovery period. On day 4, baseline paw withdrawal was determined using the procedure described above. Animals with less than 50% decrease in withdrawal threshold for the ligated paw relative to the non-ligated paw were excluded from further testing. Immediately following the testing, animals were weighted, administered one of six coded solutions (9:00 AM), and then 45-min later, retested for mechanical paw withdrawal thresholds and place avoidance. On days 5-7, animals were weighed daily followed by administration of one of six coded solutions (9:00 AM). On day 7, the animals were re-tested for paw withdrawal thresholds 45-min following drug

administration and then tested for place avoidance using the procedure described above.

Drug Dosing and Preparation

Animals received a single dose of a coded drug solution once per day for 4 days (days 4-7) at 9:00 AM. For each drug administration, animals were lightly anesthetized with halothane and then administered the drug. Vehicle (1% glacial acetic acid) and xxx (0.3, 3, and 20 mg/kg) were administered orally, yyy (10mg/kg) was administered i.p., and gabapentin (90 mg/kg) was administered s.c. xxx and yyy were stored and prepared fresh daily immediately prior to drug administration according to the directions provided by Company. In brief, a 20 mg/ml stock solution of xxx was prepared and a serial dilution from the stock solution was performed to create the additional concentrations of 3 mg/ml and 0.3 mg/ml. A 10 mg/ml solution of yyy and a 90 mg/ml solution of gabapentin were also formulated each day. "Blinding" during the experimental precedure was ensured by: (1) having one person in charge of creating and coding the drug solutions, (2) having another person in charge of daily drug administration, and (3) having additional personnel in charge of behavioral testing. The person not directly involved in behavioral testing was also in charge of randomization of animals to drug treatment, daily entry of data, breaking of drug code, and other daily project related duties.

Statistical Analysis

The difference between right and left hindpaw withdrawal threshold was calculated for each animal (from 3 trials in each session…see above) and an average obtained for the group. Statistical analysis was performed on the individual animals' data using a repeated measures ANOVA with time as the repeated measure and group (6 levels) as the between subjects measure, followed by post-hoc comparisons (protected t test) of group differences. For the place avoidance test, the percentage of time during the last 15 min of the 30-min test period that each animal remained within the light side of the chamber was calculated. The acute and chronic data were each analyzed separately using a one-way ANOVA with Group (6 levels) as the between subjects measure. Change in body weight was analyzed using a repeated measures ANOVA with time (2 points) as the repeated measure and group (6 levels) as the between subjects measure. Significance was set at $P < 0.05$.

Results

Mechanical Paw Withdrawl Threshold

The mean paw withdrawal thresholds were calculated separately for both the right and left hindpaws across the three trials, and then the left/right difference in withdrawl threshold was calculated and averaged across subjects for each drug compound. A greater negative number indicates hyperalgesia, with a difference of 0 reflecting no difference in withdrawal threshold between the right and left hindpaw. The overall ANOVA revealed a significant main effect for group ($F_{5, 53} = 9{,}19$, $p < 0.001$)

and time ($F_{2, 106} = 28.42$, $p < 0.001$) and a significant group x time interaction ($F_{10, 106} = 3.84$, $p < 0.001$). Post-hoc analysis of the interaction was performed to test the effect of the different test compounds following single administration (acute) or multiple administration (chronic).

For the acute data, there was no change in paw withdrawal threshold following drug administration compared to pre-drug baseline levels in the groups that received vehicle, yyy 10 mg/kg, and xxx1 (0.3 mg/kg). However a significant attenuation of mechanical hyperalgesia was seen in animals that were administered xxx2 (3 mg/kg), xxx3 (20 mg/kg), and Gabapentin 90 mg/kg.

For the chronic data, there was no change in paw withdrawal thresholds following vehicle and xxx1 (0.3 mg/kg). However there was a significant attenuation of mechanical hyperalgesia in animals that were administered yyy 10 mg/kg, xxx2 (3 mg/kg), xxx3 (20 mg/kg), and Gabapentin 90 mg/kg.

Place Avoidance

The analysis of relative time spent within the light side of the chamber following a single administration of the test compounds revealed no significant difference among the groups ($p > 0.05$). However, there is a strong trend indicating that animals receiving xxx3 (20 mg/kg) and Gabapentin 90 mg/kg were spending less time in the light side of the chamber compared to all other groups, none of which differed very much from each other. This tendency was expected since a decrease in paw withdrawal should also be reflected in a decrease in avoidance behavior to mechanical stimulation of the ligated paw. The lack of statistical significance likely reflects a lack of sufficient sample size to detect group differences with the obtained effect size and variability.

Body Weight

The analysis of body weigh at the time of initial versus final administration of the drug compounds revealed a significant main effect for group ($F_{5, 53} = 2.70$, $p < 0.05$), but no significant main effect for time ($p > 0.85$) or significant group x time interaction ($p > 0.10$). The main effect for group reflects the greater body weight of the vehicle group compared to all other groups. This was a chance outcome and not anticipated since animals were randomly assigned to groups with no knowledge of body weight. Of importance for the present study are the findings of a lack of main effect for time and the lack of the significant group X time interaction. This outcome suggests that drug treatment did not cause a significant alteration in body weight.

Summary

1. As indicated from the data in Figure 1, there is a significant anti-hyperalgesic effect of yyy 10 mg/kg, xxx2 (3 mg/kg), xxx3 (20 mg/kg), and Gabapentin 90 mg/kg. L5-induced mechanical hyperalgesia remained stable and was not influenced by vehicle or the lowest dose (0.3 mg/kg) of xxx. The lack of acute anti-hyperalgesic effect of xxx at the lowest dose might suggest that (1) there is a delayed mechanism of action, (2) chronic administration is required to produce anti-hyperalgesic effects, and/or (3) the dose was insufficient to find an acute effect.
2. The results from the place avoidance test reveal the general trend that the test compounds and the particular doses of these compounds that decrease mechanical hyperalgesia also reverse the avoidance behavior related to mechanical stimulation of the ligated paw.
3. The lack of effect on body weight would suggest that animals receiving drug compounds were eating and drinking in a normal fashion, which might reflect a lack of significant gastric upset by the test compounds.

Additional Information and Interpretation of Side-Effects

In general, the animals in all groups appeared to be in good heath throughout testing, although one animal in the xxx3 (20 mg/kg) group and one animal in the vehicle group died. At no time were animals stopped from receiving drug dosing due to negative side effects of the drug treatment. With regard to xxx, the most common behavioral side effect was sneezing. This occurred most frequently in the 20 mg/kg group (40%), with half that incidence in the lower dose groups (22-24%). Vehicle treated animals had a sneezing incidence of 20%. Another side effect observed was labored breathing, but with equal frequency in all xxx groups and vehicle control group (approximately 10%). Additionally, diarrhea was observed in one animal that received xxx3 (20 mg/kg). The only side effect for Gabapentin was a single incidence of hyperactivity. There were no side effects indicated for the animals that received yyy 10 mg/kg. Overall, compared to vehicle control animals, xxx and yyy appear to be relatively free from negative side effects as evaluated using informal observations of ongoing behavioral repertoire, with the possible exception of heightened sneezing at the highest dose of xxx.

References

1. Dixon, W.J. Efficient analysis of experimental observations. (1980) **Annual Review of Pharmacology and Toxicology**, 20: 441-462.
2. LaBuda, C.J. and Fuchs, P.N. A behavioral test paradigm to measure aversion quality of inflammatory and neuropathic pain in rats. (2000) **Experimental Neurology**, 163: 490-494.

Tables and Figures

Figure 1: Mean (SEM) mechanical paw withdrawal theshold difference between the ligated and non-ligated control paw for animals that received different drug compounds.