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Clavulanic acid: A competitive inhibitor of beta-lactamases with novel anxiolytic-like activity and minimal side effects

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ABSTRACT

Clavulanic acid is a member of the beta lactam family of antibiotics with little or no intrinsic antibacterial activity of its own; instead, it is used to enhance the activity of antibiotics by blocking bacterial beta-lactamases. Because clavulanic acid by itself is very safe, orally active and shows good brain penetrance, we sought to determine if it had any potential as a psychotherapeutic. Clavulanic acid was a tested across three mammalian species, hamsters, rats and cotton-top tamarin monkeys in a series of behavioral assays designed to screen for anxiolytic activity. In addition, several studies were done in rodents to compare the behavioral profile of clavulanic acid to the commonly prescribed benzodiazepines, particularly with respect to their unwanted side effects of motor depression, amnesia and neuroendocrine dysregulation.
Our findings show that clavulanic acid is a highly potent anxiolytic in rodents without altering motor activity in the open field test, normal learning and memory in the Morris water maze, or normal stress hormone release. Orally administered clavulanic acid significantly reduces measures of anxiety in male/female pairs of cotton-top tamarins. In addition, male tamarins showed a highly significant increase in sexual arousal as measured by the number of penile erections. The fact clavulanic acid has anxiolytic activity in the tamarin holds the promise that this drug may be an effective therapeutic for the treatment of anxiety disorders in humans.

1. Introduction

The escalating cost of drug development combined with patent expirations leaves the pharmaceutical industry with an aging pipeline and fewer new molecules. One innovative strategy to boost pipeline productivity in CNS therapeutics is to find new indications for off-patent drugs initially developed for peripheral indications. Key to this search is identifying a class of drugs with low toxicity. Since their discovery over 60 years ago, beta lactam antibiotics like penicillins and cephalosporins have become some of the most commonly prescribed drugs in the world with a long standing history of safety. Their potential as CNS therapeutics has been ignored by the pharmaceutical industry, probably because of their low brain penetrance and the liability surrounding bacterial resistance to the overuse of antibiotics. However, this may be changing as it was recently discovered that ceftriazone, one of the few brain-penetrating cephalosporins, can increase expression of brain glutamate transporter, an action that may help protect against neurodegenerative diseases caused by glutamate neurotoxicity (Rothstein et al., 2005).

Clavulanic acid (CLAV) is a member of the beta lactam family of drugs and while it is structurally similar to penicillins and cephalosporins, it has weak antibacterial activity with no therapeutic value as an antibiotic. However, when given in combination with some beta-lactam antibiotics like ticarcillin, CLAV can extend the spectrum and enhance the activity of the antibiotic (Crosby and Gump, 1982; Wüst and Wilkins, 1978). This synergistic activity is possible because CLAV acts as an irreversible competitive inhibitor of bacterial beta-lactamases that naturally degrade and inactive beta-lactam antibiotics (Brown et al., 1976; Reading and Cole, 1977; Payne et al., 1994). Clavulanic acid by itself is orally active and stable. The bioavailability is approximately 64 to 75% (Davies et al., 1985; Bolton et al., 1986). Peak plasma concentrations occur between 45 min to 3 h after ingestion (Bolton et al., 1986) with a plasma half-life of ca. 1 h (Nakagawa et al., 1994). The CSF/plasma ratio is around 0.25, evidence that CLAV readily passes the blood-brain barrier (Nakagawa et al., 1994).

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Given these favorable bioavailability characteristics and the absence of antibacterial activity, we sought to determine if CLAV had any potential as psychotherapeutic. CLAV was a tested across three mammalian species, hamsters, rats and tamarin monkeys in a series of behavioral assays designed to screen for anxiolytic activity. Our findings show that CLAV is a highly potent anxiolytic with a unique profile of activity unlike any of the commonly prescribed drugs used to treat anxiety disorders.

2. Methods

2.1. Hamster model of anxiety

A robust and simple bioassay for screening potential anxiolytics is the golden hamster seed finding test (King et al., 2002). Briefly, hamsters are deprived of food overnight. The following day they are exposed to the additional stress of being taken from their home cage and placed in a novel environment for a few minutes. This manipulation stimulates the release of the stress hormone cortisol. During their absence from the home cage, sunflower seeds are hidden under the bedding in one of the corners. When returned to the home cage, hamsters routinely scramble along the walls for 1–2 min before settling down, locating and eating the seeds. However, animals treated with the known anxiolytics, e.g. chlordiazepoxide, fluoxetine, buspirone, find seeds in less than 10 s.

2.1.1. Animal care

Male Syrian golden hamsters (*Mesocricetus auratus*) (140–150 g) breed at the University of Massachusetts Medical School from stock acquired from Harlan Sprague–Dawley Laboratories (Indianapolis, IN) were housed individually in Plexiglas cages (24 cm × 24 cm × 20 cm), maintained on a reverse light:dark cycle (14 L:10D; lights on at 19:00 h) and provided food and water *ad libitum*. Animals were acclimated to the reverse light: dark cycle for at least two weeks before testing. All behavioral tests were conducted during the dark phase of the circadian cycle. Animals were acquired and cared for in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 85-23, Revised 1985) and adhere to the National Institutes of Health and the American Association for Laboratory Animal Science guidelines. The protocols used in this study and those using rats described below were in compliance with the regulations of the Institutional Animal Care and Use Committee at the University Massachusetts Medical School.

2.1.2. Experimental design

A range of concentrations of CLAV (saline vehicle, 0.1, 1.0, 10, 100, 1000 ng/kg) was tested in six groups of hamsters (4–8/group) following intraperitoneal administration of drug in a volume of ca. 0.2 ml. In a subsequent study, CLAV was tested in doses of 100 pg ($n=6$), 10 ng ($n=10$), 1 μg ($n=13$), 100 μg ($n=16$), and 10 mg/kg ($n=16$) and water vehicle ($n=13$) following oral administration. All tests were conducted during the dark phase of the circadian cycle under dim red illumination. Prior to testing all animals were fasted for 12–14 h. Sixty-ninety minutes after drug administration, animals were taken from their home cage and placed into a holding cage for 2 min. During their absence, six sunflower seeds were buried under the bedding in one corner of their home cage. Animals were placed back into their home cage randomly facing any one of the empty corners and timed for their latency to find the seeds in a 5 min observation period. Latency times were analyzed with a one-way ANOVA followed by Scheffe's post hoc tests.

2.2. Rat model of anxiety

The elevated plus-maze was developed for screening anxiolytic and anxiogenic drug effects in the rat (Pellow et al., 1985). The method has been validated behaviorally, physiologically, and pharmacologically. The elevated plus-maze consists of two open arms and two enclosed arms. Rats will naturally make fewer entries into the open arms than into the enclosed arms and will spend significantly less time in open arms. Clinically effective anxiolytics, e.g., chlordiazepoxide or diazepam, significantly increase the percentage of time spent in the open arms as well as the number of entries into the open arms. Conversely, anxiogenic compounds like yohimbin or amphetamines reduce open arm entries and time spent in the open arms.

2.2.1. Experimental design

Male, Wistar rats weighing 250–300 g were group housed in a normal 12:12 light:dark cycle with light on at 0800 h and provided food and water *ad libitum*. The plus-maze consisted of two open arms, 50 cm long, 10 cm wide, with walls 40 cm high made of clear Plexiglas. The two enclosed arms had the same dimensions but included a roof. The Plexiglas for the enclosed arms was painted black. Each pair of arms was arranged opposite to each other to form the plus-maze. The maze was elevated to a height of 50 cm. Based on the dose range data from the hamster seed finding test, rats were tested with a single dose of 1.0 μg/kg CLAV. Eighteen animals were tested in the plus-maze 90 min following the intraperitoneal injection of CLAV, or vehicle control in a volume of ca. 0.3 ml. The order of treatments was counter balanced with at least 48 h between injections. At the start of the experiment, the animal was placed at the end of one of the open arms. Over a 5 min observation period, animals were scored for the latency to enter the closed arm, time spent in the closed arm and the number of open arm entries following the first occupation of the closed arm. The study produced tables of repeated measures. The data between treatments were compared with two-way, repeated measures ANOVA followed by Bonferroni post hoc tests.

2.3. Non-human primate model of anxiety

The cotton-top tamarin (*Saguinus oedipus*) is listed as an endangered species. This monkey has a high stress temperament, making it difficult to breed and rear in captivity (Snowdon et al., 1985). Captive tamarins have the highest prevalence of stress-induced colitis and colon cancer of any monkey studied (Clapp et al., 1988). The stress of captivity contributes to the onset of inflammatory bowel disease since this condition is extremely rare in wild populations (Wood et al., 1998; Wood et al., 2000) and remission occurs when captive monkeys are returned to the environmental conditions of the natural habitat (Wood et al., 1995).

2.3.1. Experimental design

Animals were housed and cared for at the Department of Psychology Callithrichid Research Laboratory, University of Wisconsin, Madison. Eight male/female pairs of tamarins with long standing pair bonds were used for these studies. There were no offspring since males were vasectomized. Animals were tested following oral treatments of CLAV and vehicle control. Both members of a pair received the same treatments at the same times. Each animal served as its own control being tested both with CLAV or vehicle one week apart. The treatment schedule was counter-balanced. CLAV was dissolved in water and dispensed onto a small cookie piece in a volume of 100 μl. In a pilot study, three of the eight male/female pairs of tamarins were given 1 μg/kg CLAV over a two day period as described below. There was no observable change in behavior so the dose was increased to 1 mg/kg body weight. A single CLAV cookie piece was given to each member of a pair. Animals were given three CLAV cookie pieces each day for two consecutive days. The 1st cookie piece was given at 8:00 AM prior to the morning feeding, the 2nd at 11:00 AM prior to the noon feeding and the 3rd at 2:00 PM prior to the late afternoon snack. All animals were scored for anti-anxiety activity 60 min after the 3rd treatment on the second day.

This treatment regimen of three doses each day for two consecutive days was chosen to acclimate the animals to the treatment procedure (day 1) and to achieve steady-state blood concentration before testing (day 2). A pharmacokinetic assessment of CLAV was run which estimated an oral dose of 1 mg/kg every 3 h would produce steady-state levels yielding an average plasma concentration of 2.5 μg/ml at the time of testing (See Supplementary data file A). Since the CSF/plasma ratio is 0.25 the estimated concentration in the brain would be around 0.3 μM. Given the rate of clearance of CLAV, treatments from day 1 should not have contributed to blood levels of drug on day 2.

Two observers blind to the treatment independently scored behavioral activity. To elicit anxious/stressful behavior a novel object was placed into the home cage. A different novel object was used for each test session and the object presentation was counter-balanced. Following the presentation of the novel object animals were scored for a duration of 15 min for: 1) latency to approach and latency to touch the object, 2) time spent in contact or proximity with the object, 3) face and head movement (frowns, head shakes and head cocks that reflect tension), 4) scratching (a validated measure of anxiety in macaques), 5) piloerection (autonomic stress response), and 6) visual scanning (nervous vigilance). On the first test session, it was noted that males treated with CLAV showed a high incidence of penile erection; hence the number of erections; mounts and female solicitations were also scored and statistically compared using a Wilcoxon Signed-Rank Test. The research protocol and care of tamarins was approved by the University of Wisconsin, College of Letters and Science Animal Care and Use Committee.

2.4. Comparing clavulanic acid and chlordiazepoxide in the rat model of anxiety

Chlordiazepoxide (Librium®) is a commonly prescribed anxiolytic that has been thoroughly characterized in preclinical studies. The effective anxiolytic dose in the plus-maze is 10–25 mg/kg (Lister, 1987; File and Aranko, 1988; Shumsky and Lucki, 1994). In this range of doses, chlordiazepoxide is a sedative and depresses motor activity complicating the interpretation of any behavioral assay that requires locomotion (McElroy et al., 1985). However, it was discovered animals develop a tolerance to the motor depression with repeated daily administration of chlordiazepoxide for several days (Shumsky and Lucki, 1994). Hence in these studies, rats ($n=6$) were given a single intraperitoneal injection of chlordiazepoxide (10 mg/kg) each day for seven days prior to the start of the experiment. Although CLAV has no effect on motor activity, it was necessary to treat an equal number of rats with daily injections of CLAV (100 ng/kg) to insure a balanced experimental design. In addition, there was a third group of rats ($n=6$) receiving daily injections of saline vehicle.

2.5. Controlling for learning and memory in the rat anxiety model

The Morris water maze was developed to test spatial memory (Morris, 1984). The pool is divided into quadrants usually designated North, South, East and West. The water in the pool is made opaque with milk powder. Hidden just beneath the surface in one of the quadrants is a platform that serves as an escape route for rats placed into the pool. An animal is placed somewhere in the pool from a variety of different start points and is timed for latency to find the platform, percent time spent in each quadrant, distance traveled and swimming speed. The animals have no visual or spatial cues in the pool and must rely on extra-maze cues, i.e., objects set up outside the pool that can be seen by the swimming animal. Through a series of trials a rat develops "place learning" or knowledge about the position of the platform based upon the extra-maze cues. The platform can be moved to a different quadrant each day combining spatial memory with working memory. This paradigm involves extinction of the prior memory and resolution of a new spatial problem.

2.5.1. Experimental design

The water maze consisted of a black plastic circular pool ca. 150 cm in diameter and 54 cm in height filled to a level of 35 cm with water made opaque with powdered milk. The pool was divided into four quadrants with a platform 10 cm in diameter submerged 2 cm below the surface in the northwest quadrant. The water was maintained at a temperature of 25 °C. Around the pool were several visual cues. Above the pool was a video camera for tracking the movement of the experimental animal. The data collection was completely automated using the software developed by HVS Image (Hampton, UK). Before testing, rats were familiarized with the pool and the platform was placed in the northwest quadrant. Each day for 4 consecutive days, animals were placed into pool at random sites and given two min to find the platform. Following these familiarization trials, animals were tested for *spatial navigation*. Animals were treated 1 h before testing with 1.0 μg/kg CLAV ($n=9$) or vehicle ($n=9$). The first day of testing began with the platform in the expected northwest quadrant. All behavior was videotaped for a 2 min observation period. After testing the animals were dried off and placed back into their home cage. On each subsequent day the platform was moved to a new quadrant and the rat started at different positions. The rat was always placed into the pool facing the sidewall. The start positions relative to the platform were different for each of the four trials; however, the platform was always in the same relative position in each quadrant. It was positioned 20 cm in from the side of the pool and in the left corner from the center facing out.

On the day following the last day (Day 4) of spatial navigation, animals were tested for *cue navigation*. In these tests, the platform was raised above water level. One hr before testing, animals were treated with CLAV or saline vehicle. The same animals treated with CLAV during spatial navigation were treated with CLAV for cue navigation. Animals were run through a series of two min trials with 35 min between trials. At each trial, the platform was moved to a different quadrant. The cue navigation study was identical to the spatial navigation except the platform was visible and the testing was done over five consecutive trials done on a single day. Animals were scored for latency to find the platform, percent time spent in each quadrant, path distance and swim speed for all testing periods.

For both spatial and cue navigation studies, the latency to find the hidden platform, path length, swim rate, and quadrant times between CLAV and vehicle treated animals were compared with a two-way, repeated measures ANOVA followed by Bonferroni post hoc tests.

2.6. Controlling for the neuroendocrine stress response in the hamster model of anxiety

The hamsters' stress response was elicited to test CLAV for effects on the hypothalamic-pituitary-adrenal axis. The simple procedure of placing an adult male hamster into a novel environment for 5 min causes a significant, predictable increase in blood levels of cortisol (Weinberg and Wong, 1986). This novelty test was used to assess the effects of CLAV on stress-induced release of cortisol. Two groups of male hamsters were treated intraperitoneally with either CLAV (1 μg/kg, $n=6$), or saline vehicle ($n=4$). A third group ($n=4$) received no treatment or isolation stress and served as a control for basal levels of cortisol. Sixty minutes after treatment animals were taken from their home cage and placed into a novel cage for 5 min. Immediately afterwards animals were sacrificed by decapitation and trunk blood collected for radioimmunoassay of cortisol. All animals were tested under reverse light:dark conditions 4 h into the dark cycle. Data were compared with a one-way ANOVA followed by Fisher PLSD post hoc tests.

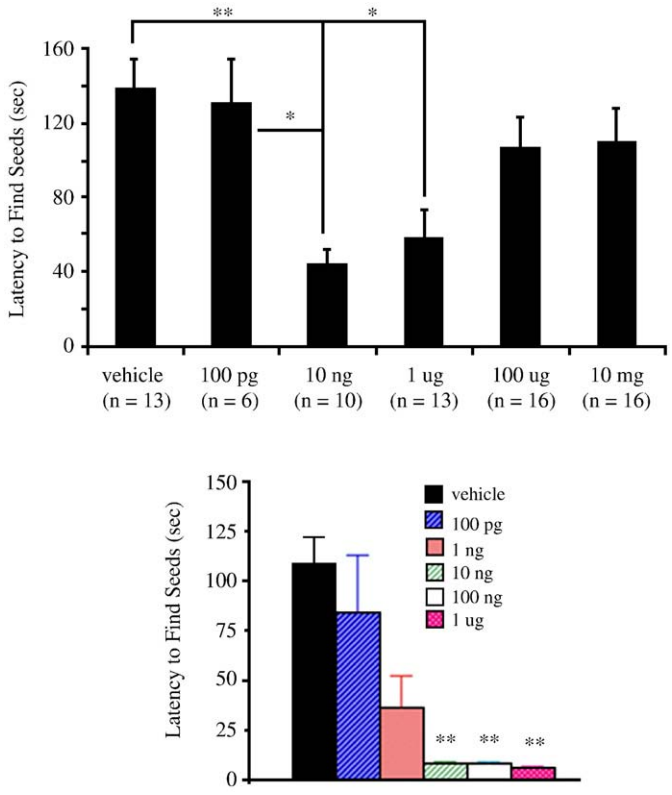

Fig. 1. Hamster seed finding. Shown in the top panel are the mean scores for latency to find seeds following CLAV treatment across a broad range of doses. Note the inverted-U shaped dose response curve. The bottom panel shows a dose-dependent reduction in seed-finding in the low dose range. (*p<.05) (**p<.01).

2.7. Controlling for motor activity in the rat model of anxiety

Male, Wistar rats weighing 250–300 g were group housed in a normal 12:12 light:dark cycle with light on at 0800 h and provided food and water *ad libitum*. Eighteen animals were tested for general motor activity in an "open field." Following the IP injection of 1.0 μg/kg CLAV, or vehicle control in a volume of ca. 0.3 ml, animals were placed into a large clean Plexiglas cage (48×32×40 cm) devoid of bedding. This open field was delineated into equal quadrants by tape on the underside of the cage. Animals were scored for motor activity by counting the number of quadrants traversed in 1 min. The order of treatments was counter

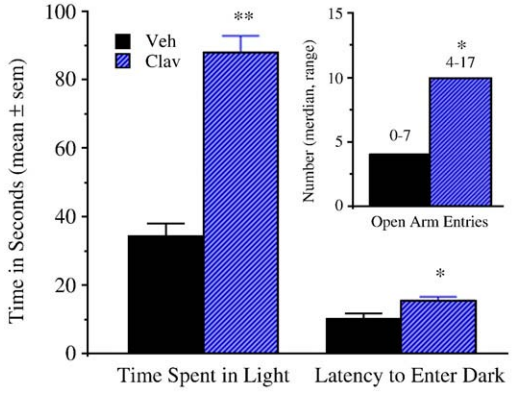

Fig. 2. Rat elevated plus maze. Shown are the mean scores for time spent in the open arm (light) and latency to enter the enclosed arm (dark) of the elevated plus maze following the intraperitoneal administration of CLAV (1 μg/kg) or saline vehicle (Veh). The insert shows median and range for the number of open arm entries for both treatments. (*p<.05) (**p<.01).

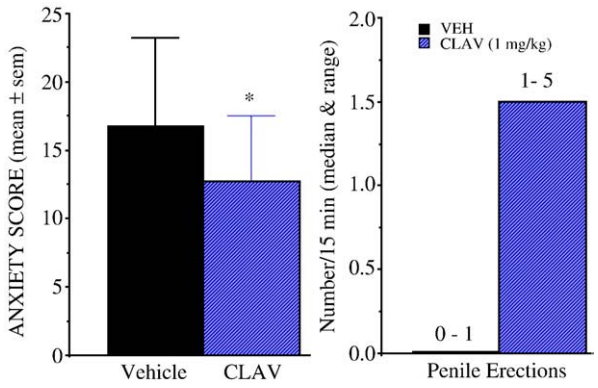

Fig. 3. Tamarin anxiety test. Shown in the left graph are the mean composite anxiety scores recorded during a 15 m in observation period from tamarin monkeys following treatment with oral CLAV (1 mg/kg) or vehicle. The right graph show the median and range for the number of penile erections observed during the same observation period. (*p<.05).

balanced with at least 48 h between injections. The data between treatments were compared with two-way, repeated measures ANOVA.

3. Results

3.1. Effects on hamster model of anxiety

Oral CLAV across a broad dose range caused a inverted-U shaped dose response in the latency to find seeds ($F_{(5,68)}=3.65$, p<0.01) (Fig. 1). When compared to vehicle, the 100 pg, 100 μg, and 10 mg doses showed no significant difference. Doses of 10 ng and 1 μg were significantly different from vehicle (**p<0.01) and the 100 pg dose (*p<0.05). CLAV dose of 10 ng showed a p<0.05 difference compared to 100 pg. When tested specifically in the low dose range CLAV at 10 ng and above significantly (p<0.01) reduced latency times to less than 8.0 s as compared to saline vehicle with a mean latency of 104 s. These results show a nice logarithmic dose response curve for the test. Does up to 1 ng/kg were not significantly different from vehicle control.

3.2. Effects on rat model of anxiety

There was a significant difference between treatments for latency to enter the dark ($F_{(1,18)}=8.53$; p<0.01). When treated with CLAV (p<0.05) animals stayed in the starting open light position longer than when treated with vehicle (Fig. 2). The time spent in the open arm was highly significant between treatments ($F_{(1,18)}=144$; p<0.0001). The time spent in the open arm was significantly increased

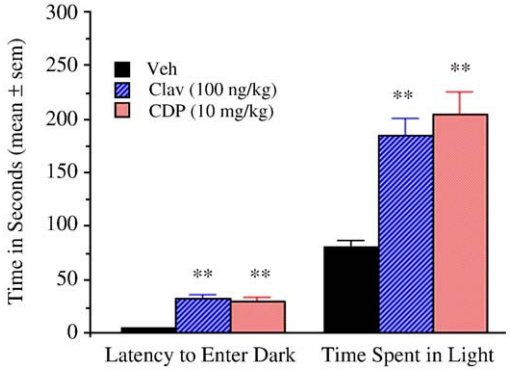

Fig. 4. Clavulanic acid versus chlordiazepoxide. Shown are the mean scores for time spent in the open arm (light) and latency to enter the enclosed arm (dark) of the elevated plus maze following the intraperitoneal administration of CLAV, chlordiazepoxide (CDP) or saline vehicle (Veh). (**p<.01).

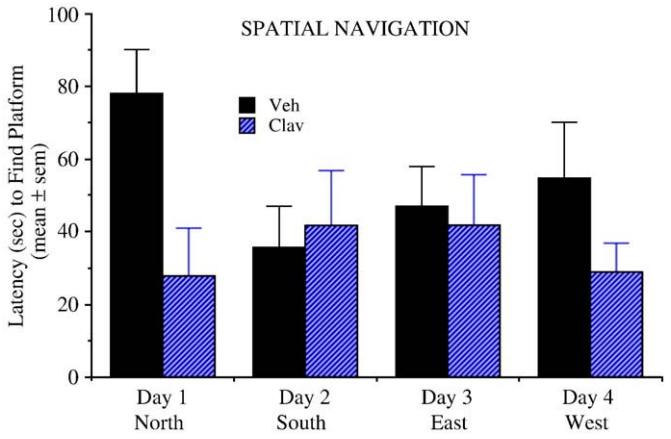

Fig. 5. Spatial navigation in the water maze. Show are the mean latencies to find the platform following CLAV and vehicle (Veh) treatments over four consecutive days. North, South, East and West refers to the location of the quadrant in which the platform was submerged on each particular day. There were no significant differences between treatments.

for CLAV ($p<0.01$) as compared to vehicle. The open arm entries were also significantly different between treatments ($F_{(1,18)}=44.0$ $p<0.0001$) with CLAV ($p<0.01$) showing increased movement into the lighted open arms as compared to vehicle.

3.3. Effects on non-human primate model of anxiety

There was no significant difference between treatments in latency to approach or latency to touch the novel object. Neither was there any difference in time spent in proximity to the object. However, using the combined measures of anxious/nervous behaviors (3, 4, 5 and 6) to establish a composite anxiety score, we found there were significantly fewer of these behaviors following treatment with CLAV (12.7 + 4.8) than with vehicle (16.8 = 6.4) Wilcoxon $t=28$, $n=15$ $p<0.05$) (Fig. 3). There were no sex differences in drug response.

The unexpected result was the increase in erections by males. All eight males showed at least one erection in 15 min when treated with CLAV whereas only three of these eight showed erections with vehicle (Fig. 3). The mean was 2.25 erections in 15 min with CLAV and 0.37 with vehicle. Cotton-top tamarins in captivity show an average base rate of 1.5 erections per hour during their active diurnal period, a rate comparable to VEHICLE treatment (Snowdon and Ziegler, 2007). Animals treated with CLAV show a rate of 9.0 erections per hour. These results were significant by a Wilcoxon test ($t=0$, $n=7$, $p<0.02$). Two of the eight females showed solicitation behaviors with CLAV treatment whereas no females showed solicitation with vehicle. There was no significant difference in mounting behavior between CLAV and vehicle treatments.

3.4. Effects of clavulanic acid and chlordiazepoxide in the rat model of anxiety

There was a significant difference between treatments ($F_{(2,15)}=21.45$, $p<0.001$) for the latency to enter the dark. The latency to enter the dark enclosed arms was significantly greater for animals treated with CLAV and CDP ($p<0.01$) as compared to vehicle control (Fig. 4). There was also a significant difference between treatments ($F_{(2,15)}=17.14$, $p<0.001$) for the time spent in the light. The time spent exposed to light in the open arms was also significantly greater for the CLAV and CDP ($p<0.01$) treated animals as compared to vehicle (Fig. 4). There was no significant difference between treatments for open arm entries (data not shown).

3.5. Effects on learning and memory in the rat model of anxiety

There was no main effect for drug treatment ($F_{(1,\ 16)}=4.17$, $p<0.057$), days of testing ($F_{(3,48)}=0.51$, $p>0.5$) or interaction between factors ($F_{(3,48)}=1.92$, $p>0.1$) (Fig. 5) for latency to find the platform. However, animals treated with CLAV showed shorter latencies to find the platform on Days 1 and 4 with a trend towards significance.

The strategy for finding the platform was similar for both treatments (Fig. 6) as judged by the percentage of time the animals spent in each quadrant. For any quadrant on any day there was no significant difference between treatments. There was a significant difference between days for percentage of time spent in any particular quadrant (e.g., CLAV, North Quadrant, $F_{(3,32)}=38.81$, $p<.0001$). Animals spent a significant portion of their time in certain quadrants on certain days. For example, on Day 1 both CLAV and vehicle animals spent most of their time in the North quadrant as compared to the other quadrants ($p<.01$). This was to be expected since they had knowledge of the location of the platform in this quadrant from the familiarization procedure.

Although the strategy for finding the platform as measured by percentage of time spent in each quadrant was similar between CLAV

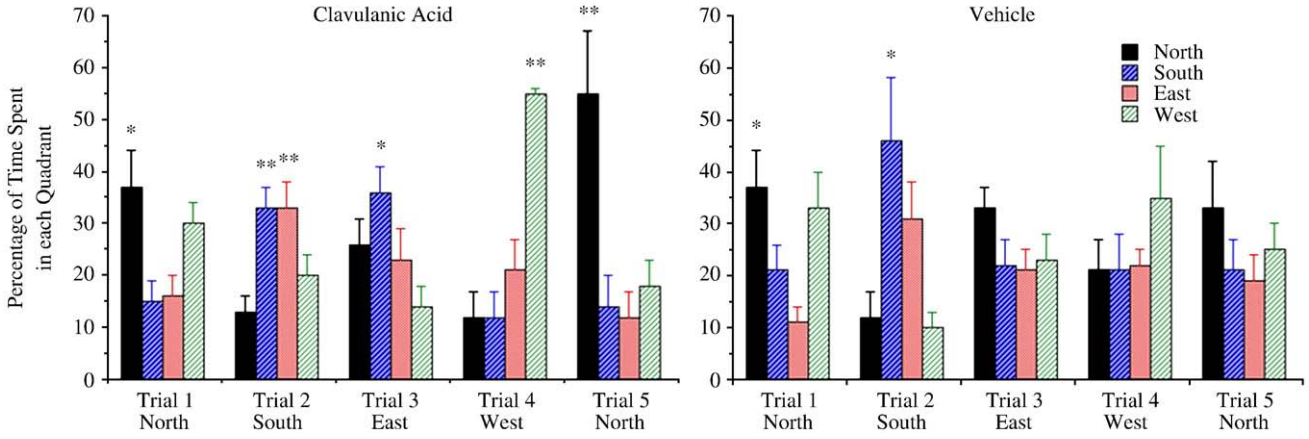

Fig. 6. Strategy to finding the hidden platform with spatial navigation. Shown is the mean percentage of time rats spent searching each quadrant for the hidden platform for each of four consecutive testing days following clavulanic acid or vehicle treatment. On each particular day the platform was moved to a new quadrant as indicated by the North, South, East and West locations. So for example, on Day 1 the platform was hidden in the North quadrant and rats treated with CLAV or vehicle spent significantly more time swimming in that quadrant as compared to the others. (**$p<.01$).

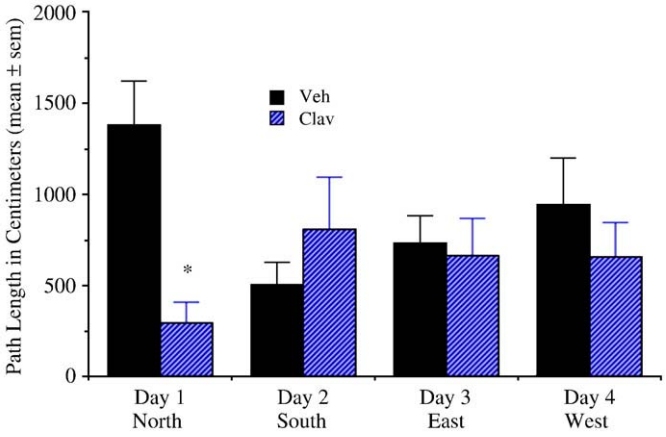

Fig. 7. Distance swam during spatial navigation. Shown is the mean path length swam while searching for the hidden platform for each of four consecutive testing days following CLAV or vehicle (Veh) treatment. North, South, East and West refers to the location of the quadrant in which the platform was submerged on each particular day. (*p<.05).

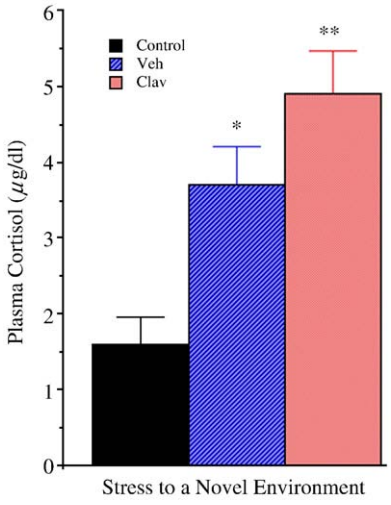

Fig. 9. Neuroendocrine stress response. Shown are the mean plasma cortisol levels in hamsters observed under baseline non stressed conditions (Control) and following introduction to a novel environment with and without CLAV treatment. Vertical lines denote SEM. (*p<.05) (**p<.01).

and vehicle there was a small but obvious difference. Animals treated with CLAV spent more time in the correct quadrant than animals treated with vehicle. This difference is particularly true on Day 2 when the CLAV animals spent over 50% (p<0.01) of their time in the correct (South) quadrant. The vehicle animals spent less than 40% of their time in the correct quadrant, a time not significantly different from the other quadrants. By Day 4 both CLAV and vehicle spent most of their time in the correct quadrant (West). This strategy on Day 4 shows good spatial, working and procedural memory for both treatments.

There was a significant main effect for treatment ($F_{(1,16)}$ = 8.40, p>0.01) on the path length to find the platform. On Day 1 CLAV treated animals (p<0.05) traveled a much shorter distance during the search for the platform than vehicle animals (Fig. 7). There was no significant difference between CLAV and vehicle on swim rate (data not shown).

During cue navigation there was no main effect for treatments ($F_{(1,16}$ = 0.553, p>0.1), trials ($F_{(4,64)}$ = 0.9745, p>0.1) or interaction between factors ($F_{(4,64)}$ = 0.7433, p>0.5) for latency to find the platform (data not shown).

Like spatial navigation, the strategy for finding the platform was very similar for both treatments (Fig. 8) as judged by the percentage of time the animals spent in each quadrant. For any quadrant on any trial there was no significant difference between treatments (e.g., Trial 1, North, $F_{(1,16)}$ = 0.099, p>0.5). There was a significant difference for percentage of time spent in any particular quadrant for either treatment for most of the trials, most notably for CLAV.

The distance traveled to find the platform was not significantly different between CLAV and vehicle animals ($F_{(1,16)}$ = 0.23 p>0.5) (data not shown). While there was no significant main effect for treatment on swim rate ($F_{(1,16)}$ = 0.926, p>0.1), there was a significant trials effect ($F_{(4,64)}$ = 7.87, p<0.001) and interaction between factors ($F_{(4,64)}$ = 2.56, p<0.05). Both treatments, but particularly CLAV showed reduced swim rates by Trial 4 (p<0.01) and Trial 5 (p<0.05) (data not shown).

3.6. Effects on the neuroendocrine stress response in the hamster model of anxiety

There was a significant difference in the stress release of cortisol between treatments ($F_{(2,11)}$ = 10.03 p<0.01). Vehicle (p<0.05) and CLAV (p<0.01) showed more than twice the blood level of cortisol as compared to the untreated, non-stressed control (Fig. 9).

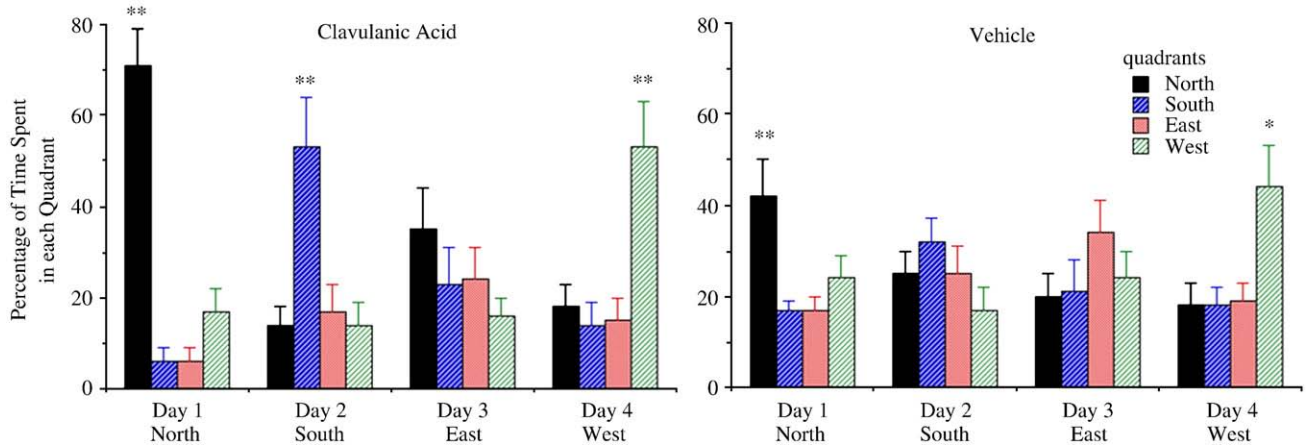

Fig. 8. Strategy to finding the hidden platform with cue navigation. Shown are the mean percentages of time rats spent searching for the platform over five consecutive, two min trials separated by 35 min intervals. Unlike spatial navigation where the platform was submerged, in these studies the platform was above the water and visible to the rat. At each trial, the platform was moved to a different quadrant whose location is indicated by North, South East and West. (*p<.05) (**p<.01).

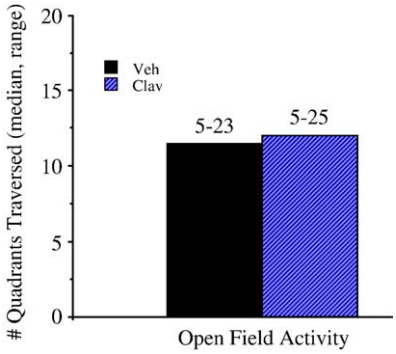

Fig. 10. Motor activity in an open field. Shown are the median scores for the number of quadrants traversed during a 1 min observation period.

3.7. Effects on motor activity in the rat model of anxiety

There was no significant difference between CLAV and vehicle in open field activity (Fig. 10).

4. Discussion

The studies presented here were designed to evaluate CLAV as a potential therapeutic agent for the treatment of anxiety. This assessment used three widely different behavioral assays to establish a drug profile of anti-anxiety that translated from rodents to monkeys. In addition, several studies were done to compare the behavioral profile of CLAV to the commonly prescribed benzodiazepines, particularly with respect to the unwanted side effects of motor depression, amnesia and neuroendocrine dysregulation.

The hamster seed finding assay is a highly sensitive animal model for rapidly screening drugs for anxiolytic activity (King et al., 2002). The model has empirical validity (McKinney, 1989) i.e., anxiolytics like chlordiazepoxide, fluoxetine, and buspirone dramatically reduce seed finding, whereas drugs like desipramine, yohimbin, and clozapine are ineffective. When tested across a broad dose range, CLAV displayed an inverted-U shaped dose response. Interestingly, this hamster model is highly sensitive to these traditional anxiolytics, showing efficacy to doses of 1 μg/kg or less (King et al., 2002). As a robust, high throughput screen, seed finding served to establish a dose range around which to test CLAV in other behavioral assays. Subsequent tests in the elevated plus-maze, the established "gold standard" model of anxiety, showed the acute administration of CLAV in a dose of 1 μg/kg to be effective in increasing the number of open arm entries and time spent in the open arm. This behavioral profile of reduced anxiety served to extend the drug profiling found in seed finding to a second assay system and another species of rodent. When compared in the elevated plus maze to the conventional benzodiazepine anxiolytic chlordiazepoxide, CLAV given over several days at a dose of 100 ng/kg was similar in activity to chlordiazepoxide given over the same period but at 1 mg/kg. These results attest to the potency of CLAV as compared to a commonly prescribed anxiolytic and, in addition, show no indication of tolerance to CLAV over a seven day dosing regimen. While the two CLAV studies testing rats for anxiety in the elevated plus maze differ in design, e.g. acute vs chronic administration and 1 μg/kg vs 100 ng/kg dosing, a comparison of the results is worth noting. The low dose, seven day administration protocol showed greater anxiolytic activity than the higher acute dose based upon time spent in the open arm. Does this reflect an inverted-U shaped dose response shown in the seed finding assay or greater efficacy with chronic treatment? Further studies are needed to address these questions. Also, as noted in the Methods it was not necessary for CLAV treated animals to develop tolerance to the motor inhibiting effects that accompany acute exposure to benzodiazepines. Indeed, animals exposed to CLAV show no depression in motor activity. Although it should be noted, the study testing CLAV for changes in motor activity in an open field used only a single anxiolytic dose, so it is uncertain whether higher doses may impair motor activity.

When comparing CLAV to benzodiazepines it was necessary to screen for the undesirable side effect of amnesia (Shumsky and Lucki, 1994). It is known that diazepam (Valium®) selectively impairs short-term memory and attention while sparing long-term memory (Liebowitz et al., 1987; Kumar et al., 1987). Clavulanic acid treated rats did not show any loss in learning and memory when tested for spatial and cue navigation in the Morris water maze. Indeed, on distance traveled to the hidden platform and percentage of time spent in the correct quadrant for both spatial and cue navigation, CLAV treated animals showed better performance than vehicle.

Another unwanted side effect of benzodiazepines is the suppression of the normal circadian pattern and stress-mediated release of the hormone cortisol (Gram and Christensen, 1986; Petraglia et al., 1986; Hommer et al., 1986). Could the ability of CLAV to reduce anxiety in stressful situations, i.e. food deprivation and novel environment in the seed finding assay, exposure to light and a novel environment in the elevated plus-maze, be caused by the suppression of the natural stress response? The data show CLAV had no ostensible effect on the release of cortisol in response to the mild stress of exposure to a novel environment. At first glance one might think it would be advantageous to suppress the stress response. Indeed, hypercortisolism has been implicated in the pathophysiology of depression (Sachar et al., 1973). Chronic psychosocial stress leading to dysfunctional, hyperactive adrenal glands can be life threatening. However, a responsive hypothalamic-pituitary-adrenal axis is critical for normal physiology and behavior.

The data show CLAV given in a dose of 10 ng/kg body weight has maximal efficacy the hamster seed finding test. The adult male hamsters used in these studies weighed around 125 g. Hence, these animals were given about 1.25 ng of CLAV. CLAV has a volume of distribution approximating the extracellular fluid volume (Davies et al., 1985). The extracellular water content of lean body mass in a rodent is approximately 25% (Wilde, 1945). The concentration of 1.25 ng of CLAV in 31.25 ml of water is 0.04 ng/ml or about 200 pM (formula weight of the potassium salt of CLAV is ca. 240). Since the CSF/plasma ratio is 0.25 the estimated concentration in the brain would be around 50 pM. While there are anxiolytics that show inverted-U dose response curves with maximal efficacy in the 1–10 μg/kg range (e.g. Coop et al., 1991) there are none that we know of in the low nanogram per kilogram range. This dose efficacy may be due, in part, to species differences in drug sensitivity. For example, the male offspring of Sprague–Dawley rats exposed *in utero* to ethinylestradiol, a potent estrogenic drug, show changes in reproductive physiology at a threshold dose of 50 μg/kg (Sawaki et al., 2003). However, CF-1 mice exposed *in utero* to 2.0 ng/kg of ethinylestradiol show significant changes in reproductive physiology (Thayer et al., 2001) demonstrating that the mouse is 25,000 times more sensitive to this drug in this assay system. To this point, hamsters have a sensitivity to commonly prescribed anxiolytics that appears to be 100 fold greater than other rodents as determined in the seed finding assay (King et al., 2002). It is also possible that the high sensitivity of CLAV may be the result of a drug/enzyme interaction. The original studies on CLAV's inhibition of β-lactamases from Gram-negative bacteria reported efficacy in the double digit picomolar range (Reading and Farmer, 1981).

Sparing the stress response together with the absence of motor depression and cognitive impairment makes CLAV unique compared to the benzodiazepine anxiolytics and suggests a highly specific, novel mechanism of action independent of GABA neurotransmission. To test this hypothesis CLAV was screened in binding assays testing for sixty-three different signaling pathways and mediators that included the classical neurotransmitters, ion channels, hormones, brain/gut peptides, prostaglandins, and enzymes (Novascreen Hanover Maryland) (see Supplementary data file B). CLAV showed no significant binding

to any target. These data are particularly notable because they exclude many of the signaling pathways that would have been expected to contribute to anti-anxiety behavior. There are several chemical categories of medications on the market today used to treat anxiety disorders. These categories include beta blockers, monoamine oxidase inhibitors, tricylcic antidepressants, benzodiazepines and selective serotonin reuptake inhibitors (SSRIs). Each of these chemical categories affects a specific signaling pathway. CLAV shows no appreciable binding to the critical receptors and transporters in any of these signaling pathways.

While CLAV does not appear to bind to any of the well known signaling receptors or transporters, it must be altering brain chemistry in some way to achieve anxiolytic activity. In pilot studies we tested this hypothesis by measuring neurotransmitter levels in the area of the nucleus accumbens with microdialysis following CLAV treatment (see Supplementary data file C). The accumbens is part of the limbic forebrain best known for its association with the pathophysiology of schizophrenia and drug addiction but also thought to be involved in sensitization to early life trauma leading to the anxiety disorder PTSD or post traumatic stress disorder (Charney and Bremner, 1999). These preliminary microdialysis studies suggest CLAV increases serotonin and dopamine neurotransmission in the nucleus accumbens. Recent advances in the treatment of anxiety disorders have focused on the activation of the serotonin neurotransmission (Feighner, 1999). Given the work in this area it is not surprising that CLAV's putative release of serotonin is accompanied by robust anti-anxiety behavior in animal models. Interestingly, the enhanced monoaminergic neurotransmission with CLAV treatment may explain, in part, the increased sexual arousal in the stress-prone cotton-top tamarin as discussed below.

Critical to these studies was the ability to move the behavioral profiling identified in rodents to non-human primates. Clavulanic acid given orally at a dose of 1 mg/kg reduced measures of anxiety in male/female pairs of cotton-top tamarins. The time course of action was very rapid and appeared in less than two days of treatments. It is possible the effect could have been observed in 60 min after a single dose of CLAV, as is the case in rodent studies. Nonetheless, the time-course noted in these studies is far better than SSRIs, e.g., fluoxetine and sertraline that are becoming more prevalent in the treatment of anxiety disorders. SSRIs take several days to weeks before achieving clinical efficacy. In addition, the SSRIs suppress libido contributing to sexual dysfunction (Rothschild, 2000).

CLAV not only reduced anxiety in cotton-top tamarins, but it also increased sexual arousal as indicated by the increased rate of penile erections. Tamarins are cooperative breeders where fathers play an important at role in infant care and where there is a close pair bond between mates. Characteristic of these pair bonded animals is a high rate of non-conceptive sex that occurs throughout the female's ovarian cycle as well as in pregnancy. This non-conceptive sex has been hypothesized to be a key behavior for pair bond maintenance (Snowdon and Ziegler, 2007). The mechanism for this CLAV-induced biological effect is unknown. CLAV could have a direct psychogenic effect on libido or the enhanced sexual arousal could be secondary to a reduction in stress. The latter hypothesis is more plausible since stress is one of the major factors contributing to sexual dysfunction (Smith, 1988). Post-traumatic stress disorder has a particularly high incidence of sexual dysfunction (Kotler et al., 2000). Studies in rats show that long-term psychological stress impairs sexual behavior, a result associated with a decrease in monoamine activity in the brain (Sato et al., 1996). Enhancing monoamine activity restores normal sexual behavior following chronic stress. Perhaps CLAV is increasing sexual arousal in tamarins by increasing monoamine activity in the brain. A potential therapeutic effect of CLAV may be in promoting behaviors that strengthen pair bonds.

Given the fact that only a single dose of CLAV was tested in the tamarin study, it is not certain whether doses lower than 1 mg/kg would have been effective. Given the inverted-U shaped dose response curve in the hamster seed finding assay, one could argue that lower doses may have been more effective. While we have attributed the increase in penile erections to the anxiolytic properties of CLAV, it is also possible that the enhanced sexual behavior is independent of low dose CLAV and may reflect a different physiological mechanism. To date there are no published reports of cotton-top tamarins being treated with anxiolytics. In contrast, the closely related New World monkeys, common marmoset (*Callithrix jacchus*) and the black tufted-ear marmosets (*Callithrix penicillata*) have been studied in social conflict paradigms and the Marmoset Predator Confrontation Test (MPCT) as experimental procedures to elicit fear/anxiety-related behaviors. Treatments with 1–3 mg/kg doses Diazepam reduce signs of fear and anxiety in these models and in these species (Cilia and Piper, 1997; Barros et al., 2001).

Using CLAV to reduce anxiety and enhance sexual arousal in the cotton-top tamarin, a species whose existence is jeopardized by its high stress temperament, is very significant. This drug may have a role in animal husbandry to help in the breeding and rearing of endangered species held in captivity. More importantly, the fact CLAV has anxiolytic activity in the tamarin holds the promise CLAV may be an effective therapeutic for the treatment of anxiety disorders in humans and even stress-related gastrointestinal disease.

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