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## MOUSE ANXIETY MODELS AND AN EXAMPLE OF AN EXPERIMENTAL SETUP USING UNCONDITIONED AVOIDANCE IN AN AUTOMATED SYSTEM - INTELLICAGE

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### **ABSTRACT**

Anxiety disorders are the most common forms of mental illness of the adult population of the U.S.A. Use of animal models for anxiety has made an important contribution to clinical and pharmacological anxiety research as well as basic research of the mechanisms involved. We review the current operant conditioning models that are used in anxiety research as well as paradigms using unconditioned fear responses. We then outline how they could theoretically and in practice be adapted to the settings of a novel automated system, the IntelliCage. The IntelliCage is a computer-controlled environment for socially housed mice that automatically records a number of behavioral parameters for each mouse and in addition allows to individually condition and test mice. IntelliCage allows fast and efficient test procedures for evaluating fear responses with a minimum of handling stress and a maximum of standardization. To confirm suitability of the IntelliCage for the study of anxiety, we present results on a modified Vogel water-lick conflict for mice. After 18 hours of water deprivation, mice were exposed to the following three conditions: punished drinking, punished drinking after injection of Diazepam, and punished drinking after injection of saline. The IntelliCage automatically quantified and recorded behavioral parameters indicative of anxiety for each individual. None of the parameters differed between untreated and saline treated control mice. In contrast, animals treated with Diazepam significantly differed from the two other groups in several aspects indicative of decreased anxiety elicited by the aversive stimulus. The results were robust and remained unchanged after correction for individual differences and drug-induced differences in activity. Our study shows that the IntelliCage can be used to assess anxiety and anxiolytic drug effects in a fast and efficient way.

**KEYWORDS:** Diazepam, Vogel water-lick conflict, anxiolytics, aversive stimulus, automated behavioral observation

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## INTRODUCTION

According to the "Anxiety Disorders Association of America (ADAA)" anxiety disorders are the most common mental illness in the U.S.A. with 13.3% (19.1 million) of the adult population (ages 18-54) being affected. Anxiety disorders cause costs of more than \$42 billion a year, almost one third of the \$148 billion total mental health bill for U.S.A. according to "the Economic Burden of Anxiety Disorders," a study commissioned by the ADAA, based on data gathered by the association and published in the *Journal of Clinical Psychiatry*.

Animal models of anxiety have been used in psychopharmacology mostly in relation to the success or failure of a given model in predicting the clinical anxiolytic potency of pharmacological agents (Green & Hodges, 1991; Ohl, 2003). At the industrial level such models are mainly used in screening procedures and for the discovery and testing of new or modified successful anxiolytics (Green & Hodges, 1991; Sanger et al., 1991; Stephens & Andrews, 1991). The academic use of such models is mainly the construction, modification and validation of theories of anxiety and the mechanisms of anxiolytic drug action (Green & Hodges, 1991; Ohl, 2003).

Animal models of anxiety in psychopharmacology are models of the effect of benzodiazepines (BZ), which mainly function via specific BZ-receptors in the brain. The current hypothesis is that the immediate action of BZs and other anxiolytics is to facilitate GABA neurotransmission, i.e. the enhancement of the GABA-mediated inhibition of other neurotransmitter systems. Behavioral and other effects of BZs would then appear as the consequence of this primary action (Haefely, 1985a; Haefely, 1985b; Clement & Chapouthier, 1998).

Animal models make a fundamental contribution to the area of anxiety research at the clinical, industrial and scientific level. Individual differences in susceptibility to anxiogenic stimuli and variable responses to different types of threats possibly can be modelled in animals (Green & Hodges, 1991; Shekhar et al., 2001). Genetic and pharmacological studies of reactive and non-reactive mouse strains may result in important insights into general neurological mechanisms underlying anxiety (Green & Hodges, 1991; Ohl, 2003). As our experiments have shown, the use of Intellicage (IC), an automated system for behavioral observation and conditioning of socially housed mice, allows effective screening of mouse strains for anxiety levels. In addition, the system allows testing of the same animals for the effect of anxiolytic agents in a very straightforward and cost-effective manner, due to the low amount of required human interference. We provide evidence that IC could be used to investigate anxiety and anxiolytic drug action.

## **MODELS OF ANXIETY**

Punishment-based conflict procedures have been employed for over four decades in the identification and characterization of anxiolytic agents (Millan & Brocco, 2003). The technical specifications of IntelliCage, the validity and reproducibility of the conditioning models as well as the popularity of the different operant models, the Vogel-water-lick or the Geller-Seifter conflict and variants of them seem to be easily applicable to testing mice.

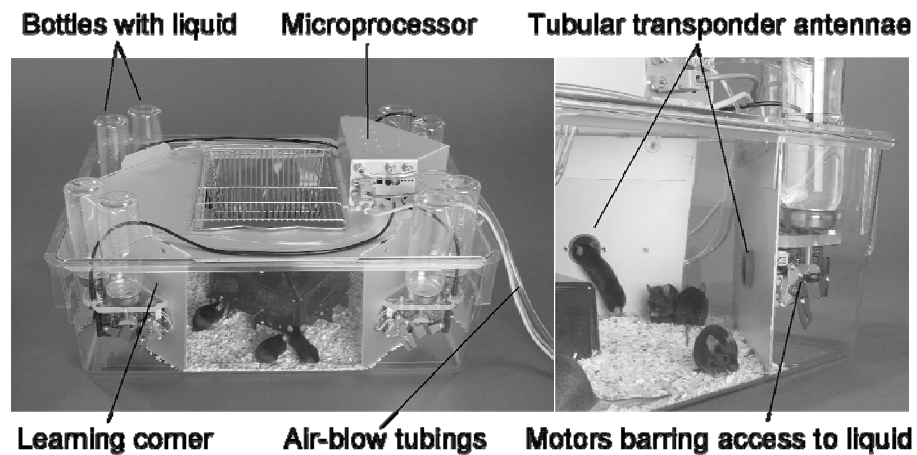
### **Geller-Seifter conflict**

In the original procedure (Geller & Seifter, 1960), rats were trained in operant chambers to operate a lever to obtain food. Presentation of an auditory cue signaled a change in the reinforcement contingencies. Further responding resulted in both an increased availability of food, and foot-shock. In other words, this procedure involves a multiple schedule of reinforcement. In the first segment of the schedule (signaled by an auditory or visual cue), response is reinforced at irregular intervals. In the second segment (the conflict component), every response is simultaneously reinforced (signaled by a different signal); and punished (by the delivery of a brief, inescapable electro-shock). The suppression of the response in the conflict component can be specifically attenuated through the administration of anxiolytics; their potency in the experiment being proportional to their clinical potency (reviewed in Green & Hodges, 1991; Millan & Brocco, 2003). However, the response to the simple food-rewarded component without punishment is not enhanced by the anxiolytics. The disadvantages of this classical procedure include a long period of training (one to several weeks) until the animals reach a stable base-line response to the conflict component as well as the necessity for long-term food restriction. Once the subjects have learned the tasks in the Geller-Seifter paradigm response rates in all operant components remain relatively stable over long periods (Green & Hodges, 1991). This makes the Geller-Seifter conflict a suitable test for repeated drug-testing in order to demonstrate reliable and repeatable responses to anxiolytics over time in individual subjects.

### **Vogel water-lick conflict**

The Vogel water-lick conflict is a modification of the Geller-Seifter conflict paradigm that was established to eliminate the long periods of training. It is widely used for anxiolytic research. In this modified procedure mice (or rats) first learn to drink from a water-spout in an operant chamber. Then, after a period of unpunished licking, response (=licking) is punished with a footshock. Consequently, licking is suppressed in control animals. Anxiolytics lessen this suppressed behavior, while non-specific effects on drinking per se can be assessed via non-punished or ad-libitum drinking (Green & Hodges, 1991; Rodgers et al., 1997; Vanover et al., 1999; Millan & Brocco, 2003; Ohl, 2003; Witkin et al.,

2004). The response rates, despite of punishment, are enhanced by active anxiolytics such as chlordiazepoxide and barbiturates, but not amphetamine (Stephens & Andrews, 1991). The major drawback of using the Vogel water-lick conflict is the lack of a systematic analysis of drug-effects on non-conflict behavior (Green & Hodges, 1991). Later versions of the water-lick paradigm aimed at improving replicability by pre-selection of subjects that lick water but are sensitive to shock induced suppression (Petersen & Lassen, 1981; Petersen & Jensen, 1984).



**Figure 1.** IntelliCage system housing mice undergoing automatically permanent monitoring and training for various learning tasks

### The Intellicage

The IntelliCage™ (NewBehavior AG, Hardturmstrasse 76, CH-8005, Zürich, Switzerland) is a fully automated system for the monitoring and testing of socially housed mice. It consists of a large standard rat cage, containing four learning corners (Figure 1). All animals are identified by implanted transponders when approaching and entering a test corner. Each corner contains elements that can be used for operant conditioning. Mice obtain liquid reward, triggered by nose-pokes from either one or both water bottles contained in each corner (total of eight per cage). Tools for conditioning are light diodes in several colors, air-puff punishment and sensors such as licko-meters and infrared proximity sensors. Depending on the complexity of the learning schedules, which are selectable on a controlling computer, each IntelliCage (IC) can handle the learning behavior of up to 16 mice simultaneously (number determined by Swiss animal welfare regulations based on the area of the cage). A system can consist of a maximum of

eight cages per controlling computer allowing testing of 128 mice simultaneously. Because the cage runs experiments and records the data for individual mice, test and control animals can be housed in the same cage and thus under highly standardized conditions. Social housing and the reduced amount of handling decrease many of the side effects known from more conventional procedures. In fact, the IC is perceived as an enriched environment by the mice and stereotypies are not observed. Data collection by the computer also removes observer bias and makes inter-laboratory comparisons possible.

#### *Models of anxiety in IntelliCage*

The IC provides a well-adjusted experimental environment for establishing models of anxiety for mice. All aspects of anxiety research: screening for drug effects, screening of mutant breeds, as well as assessing effects of anxiolytic and to some extent also anxiogenic drugs, can be done in it. One of the manifold advantages IC offers is the standardized home-cage testing of socially housed mice. Testing in a social environment as opposed to the more common single testing influences the outcome of anxiety tests in *Murinae* (Haller et al., 2000; Manzanique et al., 2002; Moragrega et al., 2003). The Vogel water-lick conflict could be directly implemented in IC. The IC also provides the opportunity to combine the advantages of the Geller-Seifter conflict with the Vogel water-lick conflict. It is theoretically possible to test individuals repeatedly in the same environment with different conditioning and test procedures. Thus one of the major drawbacks of the Vogel water-lick paradigm, the lack of information about non-conflicted behavior could be eliminated. New variations of the Vogel water-lick conflict as mentioned above could also be implemented. Mice can be pre-selected with a minimum of handling in the same home-cages. Finally, it would be possible to combine in IC mice models of anxiety with other tests, e.g. for spatial, short- or long-term memory, locomotor activity, and also models of addiction. This allows assessing the effects of anxiolytics not only on the suppression of anxiety on various other parameters.

In the following, we outline hypothetical procedures using IC to perform screening for drug effects and the evaluation of the anxiolytic potency of drugs. These procedures are based upon the theoretical and practical aspects on the Geller-Seifter and Vogel water-lick conflicts and their advancements. In addition, we present results from an experiment, which was conducted in IC in order to evaluate the suitability of IntelliCage for testing anxiolytic drug action with a very simple adaptation of the Vogel water-lick conflict.

#### *Hypothetical test procedures*

The Vogel water-lick paradigm has already been applied to the use with mice and is validated for several strains with different anxiolytic drugs (Umezu, 1999; van Gaalen & Steckler, 2000; Millan & Brocco, 2003; Gordon & Hen, 2004;

Mathiasen & Mirza, 2005). The essence of the Vogel water-lick paradigm has been adapted for testing of mice with few changes from the rat experiments. The punishment procedure in the mouse studies was a mild electric shock (0.1 mA for 0.3 s and 0.15 mA for 10 ms) every 20<sup>th</sup> lick. The animals were water deprived for 24 resp. 48 hours (Umezu, 1999; van Gaalen & Steckler, 2000). In the drug effect experiment the animals were accustomed to the test-chambers for 40 min and tested with electric shocks a week later immediately after anxiolytic injection (Umezu, 1999). In the comparative analysis of different strains, mice were tested right after they had shown a minimum number of 20 licks at the water spout (van Gaalen & Steckler, 2000).

According to Millan and Brocco (2003) only the following variables need to be defined:

- Access to water in home cage / test cage (deprivation)
- Test day
- Session duration
- Shock frequency
- Shock intensity

The only modification in the IC would concern the manner of punishment (air-puff instead of electric shock).

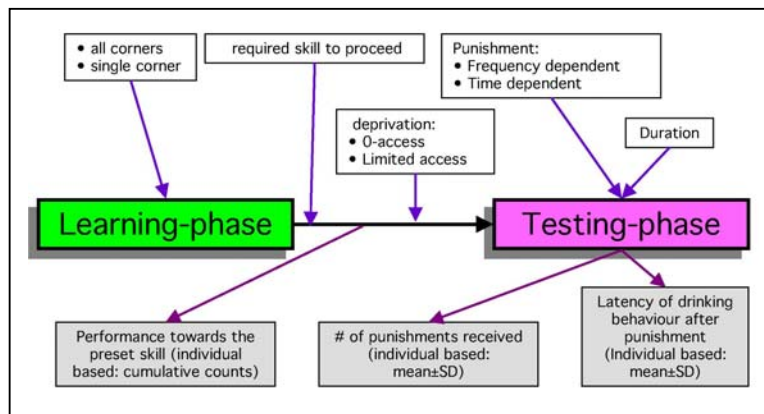
### **Setup I**

A simple setup following the classical Vogel water-lick design: Mice are introduced into IntelliCage and learn to drink water in all available places. Then, following the literature, they are completely water deprived, for a minimum of 24 hours. Deprivation could also include a period of ad-libitum access to water. Such non-zero deprivation schedules include limited access to water e.g. for 20-240 minutes per day. Usually, these non-zero deprivation schedules start at the second day of the experiment after one day of no water access. As a variation and in order to speed up the experimental procedure one could restrict access to water for each mouse to a single corner. After the mice have shown the minimum number of 20 water-licks, i.e. the task has been learned, they can be tested for levels of anxiety by introducing the conflict component. The punishment is an air-puff in IC. Two modes of punishment should be available: frequency dependent and time dependent. Mice are punished either every 1/N<sup>th</sup> lick (5£N£20) or alternatively they receive punishment during N seconds (2£N£5) out of M seconds (N<M). The session duration is of minor importance in IC and can be chosen to be considerably longer than that in the literature (usually between 3 and 15 min) since data acquisition is done automatically and the animals are tested in their home cages. However, it is important to ensure that the total amount of water the animals receive is sufficient (access to water as described above should be included in the deprivation schedule for longer testing).

The statistical output of this simple paradigm would include the number of punishments received and the latency of water-licking after punishment (Fig. 2). The experimental procedure theoretically allows use of a matched-pair test environment (eg. matched t-test). In other words: drug effect could be tested by comparing the number of punishments received by each individual before and after injection of potentially anxiolytic agents. However, to ascertain that mice do not get accustomed to the punishment and to control for the potential effect of handling a control group should be included as well. We suggest that in every experiment all mice receive the same treatment, but only part of them receive anxiolytic drugs while the others receive a sham injection, such as saline solution (vehicle control). This provides the opportunity to disentangle potential habituation effects to the punishment from the effects of the drug in, for example, a repeated measures or a mixed model test. Furthermore, it is possible to use the IC to investigate side effects of anxiolytic drugs on locomotor activity and thirst without punishment. In the final setup the IntelliCage will test mice in four different situations:

- Mice are tested for habituation effects without drugs (vehicle injection).
- Mice are tested for effects of sham injection on locomotor activity and thirst (no punishment I)
- Mice are tested for effects of drugs on locomotor activity and thirst (no punishment II).
- Treatment.

Good information on procedure may be found in Vanover et al., (1999); Millan and Brocco (2003); Witkin et al., (2004).

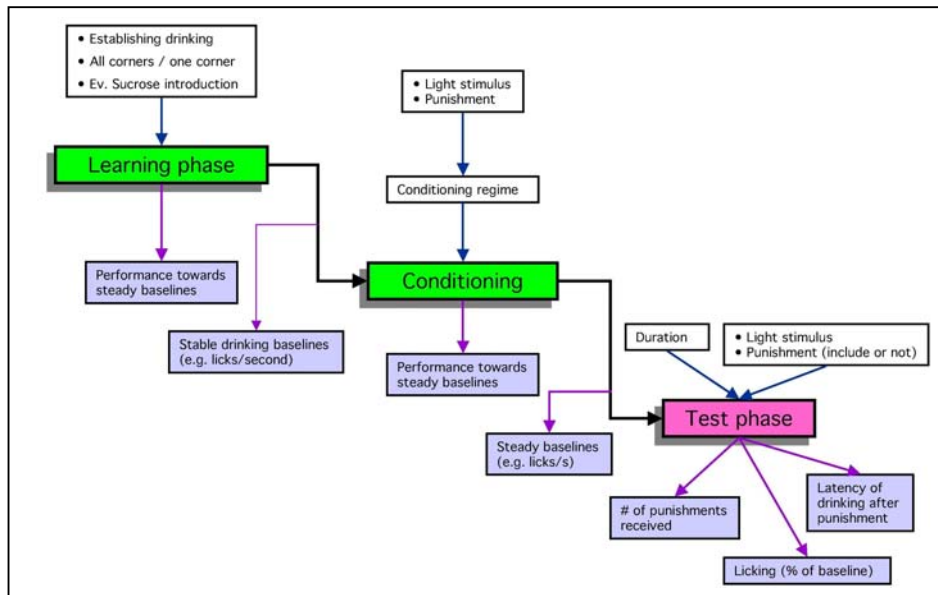


**Figure 2.** Schematic diagram of the different phases in the Vogel water-lick paradigm adapted to IntelliCage. Open boxes contain variables that can be set by users, the grey boxes contain the output deliverables. Data used for quantification of locomotor activity, in addition to the indicated outputs, is the mean number of visits ( $\pm$ SD) of individuals to all corners (even if mice were trained to drink in only one).

Alternatively, it is possible to adapt the procedure as suggested by Witkin et al., (2004) and include a conditioning step in the learning phase, which determines the level at which animals proceed to the testing phase. This would allow assessment of base levels of licking behavior in the unpunished (learning) phase.

### Setup II

Alternative models for testing anxiety in a fear conditioning setup were presented by several authors (Kilts et al., 1981; Petersen & Lassen, 1981; Vanover et al., 1999). In these models, the Vogel water-lick paradigm was combined with the Geller-Seifter model in order to increase statistical sensitivity and reduce the number of animals required and later to allow the use of non-water deprived subjects. The resulting conditioned suppressed drinking procedure, can be adapted



**Figure 3.** Schematic diagram of a conditioned drinking suppression mode in the IntelliCage. White boxes contain variables that should be set by IC users, the grey boxes contain the output deliverables. For locomotor activity quantification it is necessary to record, in addition to the indicated outputs, the mean number of visits ( $\pm$ SD) of an individual in the corners where they were trained to drink.

to IC as well. In the conventional setup, mice are conditioned to associate a stimulus (generally an auditory stimulus) with an aversive unconditioned stimulus, usually a mild foot shock. Subsequently the reactions of the animals to the stimuli

presented during the unconditioned stimulus exposure are investigated. This procedure provides the additional opportunity to investigate extinction of a conditioned stimulus (Marsicano et al., 2002).

In IC, conditioned drinking suppression could be achieved following the experimental setup of Witkin et. al., (2004) after adapting it to drinking. Potentially, it could be extended with the procedure suggested by Vanover et al., (1999) in order to avoid water deprivation. We will not recapitulate the behavioral procedure given by Witkin and colleagues (2004), since the paper does give very detailed training instructions. Vanover et. al., (1999) introduced punishment for drinking a sucrose solution in order to avoid water deprivation. It would be possible to present sucrose solution in one of the two bottles in each corner and adapt the experimental procedure to a punished sucrose drinking suppression. Finally, the statistical tests and graphical outputs would be simple and straightforward: mainly cumulative records of behavior in the learning and conditioning phase. The output of the experimental phase would include the calculation and graphical display of means and standard deviations separated by the groups of interest (i.e. treated and untreated animals). Additionally, the determination of base levels of predefined behaviors should be considered.

## **AN APPLICATION: A SIMPLE EXPERIMENT IN INTELICAGE ASSESSING ANXIOLYTIC DRUG ACTION**

### **Methods**

We used a simple schedule to test for behavioral differences in mice treated with Diazepam (an agent reducing anxiety in mice and humans). After a deprivation period of 18 hours, 12 mice (C57/B16 females, 7 weeks old) were given access to water in one corner each of the IC. Upon entering the corner, the doors would open, giving the mice ad libitum access to the bottles. The mice were trained to this schedule for two days. Upon completion of these two days, the first time mice drank after deprivation, they received an air puff after one second of drinking. The ensuing visits of the mouse during the remaining time allocated to drinking were not punished (one punishment per 24 hours). On the day following the punished drinking, half of the mice (group A) received an injection of Diazepam intraperitoneally (5mg/kg body weight) approximately half an hour prior to the end of the deprivation period. The control group (group B) received a saline injection. They were then tested with the single punishment procedure as on the previous day. After this first test for drug effects, the animals continued in the punished drinking regime, without additional drug injection for another two days (three days in total after drug injection). On the fourth day (day 6 of the experiment) group B received a Diazepam injection, while group A was injected

with saline solution. The procedure was then repeated as previously (days 6-8). Following this procedure we obtained three balanced sessions of drug injection.

- Punished drinking without injection (PN);
- Punished drinking with Diazepam (PD);
- Punished drinking with saline (PS).

We were interested in various behavioral parameters that would allow to distinguish between less anxious animals that had received Diazepam (PD) and the control treatments (PN and PS). We concentrated on the following variables:

- Latency: time difference between the first punished visit and the next visit. This stands for the amount of time an animal needed to recover from a punishment.
- Number of visits: number of visits to all corners by each animal during the four hours with access to water regardless drinking occurred or not. This measure represents an equivalent of activity.
- Duration of visits: total amount of time animals spent in the corner, where they had access to water during the four hours allocated to drinking.
- Number of licks: total number of licks during the period animals had access to water.
- Duration of licks: the amount of time animals spent drinking.
- Duration of nose pokes: since the animals were not subjected to a conditioning paradigm in terms of nose pokes, this variable measures the total time animals tried to get access to the bottles by nose poking (both cases: successful and unsuccessful).

We calculated a single value for each animal representing the variables above (for  $N > 2$  the mean) during either the punished drinking (PN), the punished drinking with Diazepam (PD) or the punished drinking with saline (PS) period.

### **Results**

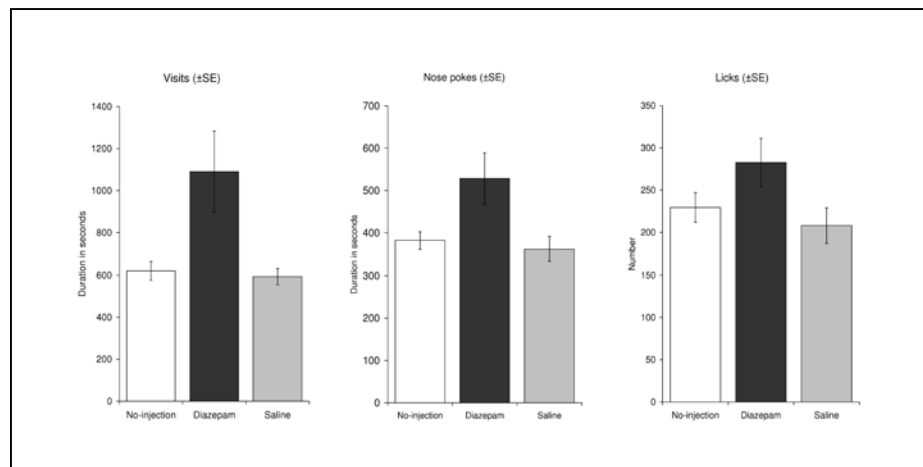
MANOVA (multivariate analysis of variance) was used to test for overall differences between treatments. In the model we used a type3 sum of squares model using treatment and the individuals as explanatory variables, defining treatment as the test hypothesis. The MANOVA showed a significant treatment effect on our variables (Wilk's Lambda=0.3  $F_{12,34}=2.3$   $p=0.03$ ).

We then used univariate tests (ANOVA) to explore the differences for each variable separately. With the exception of latency, all variables differed between treatments (Table 1). Latency did not differ between treatments and the contrasts between treatments revealed no detectable differences, either (e.g. PD vs. PS estimate=-69.7±375  $t=-0.19$   $p=0.9$ ). However, for all other variables there were strong differences between treatments (Table 1).

**Table 1.** Univariate tests for all single variables in the experiment. The statistics test for between treatment differences.

	<b>df</b>	<b>SS3</b>	<b>F</b>	<b>p</b>
Latency	2	1469821.5	0.87	0.43
Number of visits	2	29.76	3.25	0.06
Duration of visits	2	1875163.4	6.70	0.005
Nose pokes	2	195718.5	11.51	0.0004
Number of licks	2	35298.62	6.55	0.006
Duration of licks	2	20265.5	5.27	0.013
Error	22			

Visit duration and the number of visits were not correlated (N=12,  $r=0.1$ ,  $p=0.6$ ). The reducing effect of Diazepam on activity is well known. In fact, the analysis showed that Diazepam-treated animals had lower activity levels, i.e. number of visits (DPD vs. DPS estimate= $-2.0 \pm 0.9$   $t=-2.3$   $p=0.02$ ). Consequently, we used number of corner visits as a surrogate measure of activity and also inserted it as a covariate in the statistical models to correct for activity differences (Figure 4).



**Figure 4.** Differences in several behavioral parameters indicative of anxiety in Diazepam-treated animals using a simple Vogel water-lick paradigm in IntelliCage.

However, the outcome of the statistical tests using number of visits as a covariate (and thereby correcting for drug effects on activity), did not change the

overall pattern of behavioral differences (figure 4). Apart from latency to enter the corner, which was not correlated with any of the other variables, all variables were highly correlated and presumably represented measures of the same behavior (results not shown for duration of licks).

For the sake of simplicity we have not included the statistics for the inter-treatment contrasts while correcting for activity. In summary, the differences between the DPD and DPS treated animals were, with the exception of latency, almost always significant or showed a strong trend (PD vs PS: number of licks  $p=0.02$ ; duration of licks  $p=0.06$ ; nose poke duration  $p=0.002$ ; duration visits  $p=0.006$ ). There was no significant difference between PS and PN in any of the variables ( $p>0.6$ ).

In conclusion, animals treated with Diazepam, were significantly less anxious than animals in the two control groups. The results were robust and remained unchanged after correction for individual differences and drug-induced differences in activity. Our study shows that the IntelliCage can be used to assess anxiety and anxiolytic drug effects in a fast and efficient way, since most of the experiment's time (8 days for 12 mice) was done by computer and test cage, while human handling was only required for the injections.

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