Rats prefer high sucrose concentration over lower

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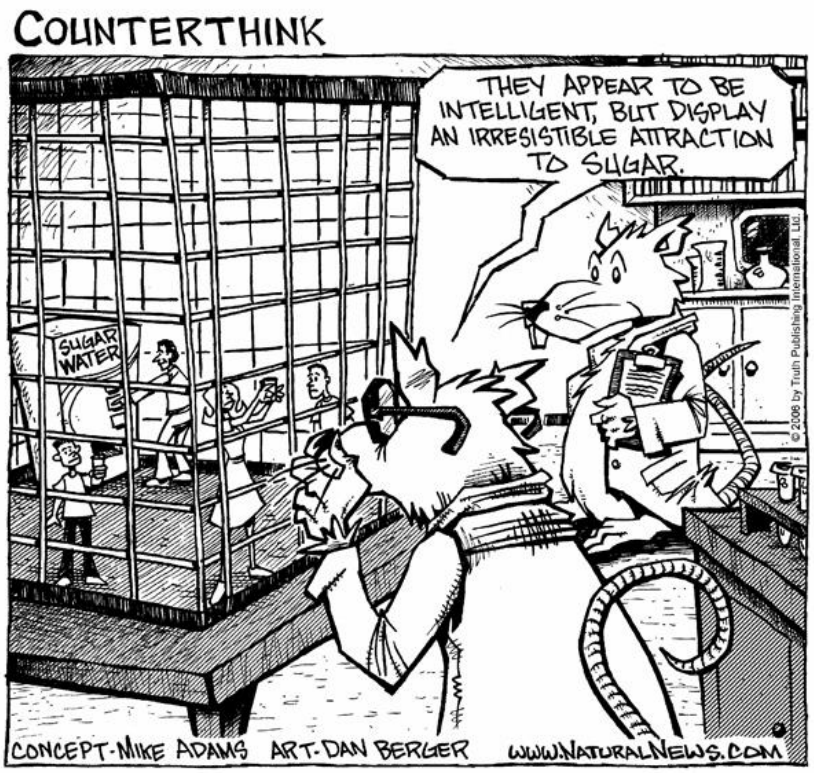

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Grant

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Foreword

The social rodent lab is located at the Heinrich Heine University in Dusseldorf. The director, Marijn Van Wingerden is supported by the Volkswagen Stiftung “Freigeist” fellowship in his project “The neural Basis of Social Valuation – the Confluence of Neuroscience, Psychology and Economics”. In order to uncover the neural basis of social valuation, the lab applies a quantitative, interdisciplinary approach to social decision making in rodents.

Abstract

Neuroscience is a field of science concerned with explaining brain processes and functions. One of these major and essential processes is social cognition. While our proficiency to take part in social interactions is an essential part of the human being, the brain mechanisms involved are barely known.

Mental and social disorders are major focuses of research within neuroscience and constitute an unavoidable problem to solve. However, to develop successful chemical therapies against such disorders, basic brain functioning in the frame of behavioural cognitive functions must be better understood.

The social rodent lab working on experimental psychology is following this path to uncover the basis of social valuation in the brain. In order to do this, the rat, as a social mammal, is the chosen model for understanding neural activation systems. Here we show that our rats can discriminate between higher and lower concentrations of sucrose solutions. In the frame of this work, the experimental setup used was designed, developed and optimized for future studies to identify an indifference point between a sucrose reward and social reward. This endeavour shows promising first steps towards allowing for the testing of molecules that can improve social behaviours.

In the meantime, in vivo electrophysiology will provide the link between neuron activity and observed behaviours.

Keywords

- ✓ Behaviour
- ✓ Sucrose
- ✓ Rats
- ✓ Rodents
- ✓ Preference
- ✓ Discrimination
- ✓ Reward
- ✓ Social behaviours
- ✓ Social disorder
- ✓ Social brain
- ✓ Psychology
- ✓ Neuropsychology
- ✓ Three-chambered task
- ✓ Neurophysiology
- ✓ Electrophysiology

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Introduction

A study suggests that 11.5% of the European population has experienced a mental disorder, counting only data obtained during the year of the survey (Alonso and Lépine 2007). Diseases like autism, depression, schizophrenia, psychotic disorders and the majority of other mental disorders known are one of the greatest challenges of the century to comprehend, mostly because of their complex character.

Mental disorders are frequently due to the gathering of multiple factors including both genetics and environmental (Landrigan 2010), (Jaffee 2007). That is why even today with the modern means we have, it is still problematic to identify the exact causes and mechanisms behind the disorders.

An important aspect of those mental diseases is the social component. But why are social behaviours so essential? Social cognition can be defined as the aptitude to understand other people, their intentions, feelings and thoughts (Adolphs 2009). By sharing information with others, social contact allows us to understand the world and better predict the events coming, to improve our actions. Humans are constantly trying to improve social contact by every means. “It has been argued that our social nature defines what makes us human "(Adolphs 2003).

Mental disorders constitute a social deficit, which has a negative impact on the affected individual. This social deficit can be assimilated as social isolation and human ability to create social connection is so deep that when the isolation feeling appears, it can affect our ability to think clearly. Furthermore, the effects can be as much mental as physical (Cacioppo and Patrick 2008).

Impaired social behaviours are against our own nature and undoubtedly there are reasons to assume that social cognition is an important brain function. According to results provided by the supporters of the social brain hypothesis, the complexity of social behaviour is a major basis of interspecies differences correlating to forebrain size, illustrated in birds and mammals, particularly primates (Dunbar and Shultz 2007).

However, critiques on comparative brain studies have indicated the need for comprehensive, interdisciplinary studies for a full understanding of the issue of cognitive function and behaviour (Healy 2007). The underlying mechanisms are poorly known, due in part to the complexity of the brain (Behrens, Hunt et al. 2009) and it is essential to improve our knowledge of the associated brain mechanisms in order to properly treat mental disorders.

Today it is widely accepted that there are specific brain areas that are dedicated to social interaction. The first areas known to be involved in social cognition were

discovered by inflicting brain lesions in monkey models and by studying the impact of unfortunate brain injuries in the famous Phineas Gage (Kihlstrom 2010). Nowadays, with the appearance of advanced neuroimaging techniques, it has been shown that four areas are the main actors in social behaviour: (1) the posterior superior temporal sulcus (pSTS) and the adjacent temporo-parietal junction (TPJ), (2) the amygdala, (3) the temporal poles, and (4) the medial prefrontal cortex (MPFC) and the adjacent anterior cingulate cortex (ACC).(Frith 2007).

Despite the progress made there is still a long way to go. Between biology and psychology, neuroscience tries to determine underlying mechanisms.

Much of the behaviour of humans and other animals is directed towards seeking out rewards (Steinberg, Keiflin et al. 2013). So to properly appreciate the link between social behaviour and the brain, the role of the brain reward system should be considered, as it plays a key role in reinforcing social behaviour (Sobota, Mihara et al. 2015).

Our brain reacts to rewards, like monetary rewards, by stimulating the release of molecules by our neurons, explaining the feeling of pleasure. Dopamine is known as one of the main molecules involved in this reward system (Routtenberg 1978). Rewards appear to stimulate this system and it has been indicated that there is a "common neural currency" for rewards (Izuma, Saito et al. 2008). As such, social contact can be understood as a social reward. Monetary reward being not considered as a reward for animals, food rewards are commonly used as an appropriate substitute.

In this field of study, ethical implications must be considered. Experiments on humans are a broad controversial topic and, despite guidelines written after WWII, the subject matter is far from closed. After the Milgram-experiment (Milgram 1963) and the Stanford prison experiment, other considerations arise and it has been established nowadays that "No research should be conducted in psychology or medicine which violates the biological or psychological integrity of any human being regardless of the benefits that might, or even would definitely, accrue to the society at large" (Zimbardo 1973).

Animal models are often used as an alternative to human research. At first glance animals seem really different from humans but at a physiological level, they are quite similar. The attribution of related behavioural and physiological processes in humans and animals suggests that animal research can be generalized to humans in some cases (Farrar, Kieres et al. 2003). However, many differences between species remain. Despite our general genetic proximity, some animals are good human-like models for one thing and some for another.

Furthermore, as stated, mental disorders can be caused by a combination of factors and can include a combination of symptoms. Today mental disorders are well classified by their symptoms (DSM-5

2013) and it is impossible to create an animal with the exact same disorder that you wish to study. Often, the goal is to isolate the symptoms of a particular condition to study them individually.

Much of our social behaviour arises from neurobiological and psychological mechanisms shared with other mammalian species (Adolphs 2009) with rodents being chosen today as preferential subjects for psychological studies (van Wingerden and van den Bos 2015).

The advantage of working with rodents is that their brains are very similar to human brains; that provides a good comparison method or model of how the human brain works (van Wingerden and van den Bos 2015). Rats and mice make up 95% of animals used in scientific research and today , rats are the most used for studies in experimental psychology (FBR 2015). Their complex social behaviours and the relative ease of breeding and training them make them a great choice for experiments on behavioural studies (Jacob 1999). It has been shown that rats can be easily trained for multiple tasks, they show intelligence, which means they develop a capacity for logic, understanding, learning, emotional knowledge, problem solving and a wide variety of tasks (Gill, Smith et al. 1989). Furthermore, they can be used to analyse primordial behaviours that can be partly compared to human behaviours (van Wingerden and van den Bos 2015).

This study is a preparation in order to allow further studies using our results. The social rodent lab aims at exposing the brain mechanisms of social valuation using multiple paradigms and social tasks, combined with electrophysiology on rats. These pilot experiments are a part of a broader project initiated in 2014.

In the future, the goal is to obtain an indifference point between an unsocial reward and a social reward in the rat model. For this purpose, a proper reward must first be chosen and calibrated. Sucrose appears a great choice as it has been shown in several studies that it is a proper reward for this model. (Muscat and Willner 1989)

Past studies have already shown that rats can discriminate between different concentrations of sucrose solutions (Muscat and Willner 1989). If discrimination involves similar behavioural processes in human and animals, it would be expected that higher concentrated sucrose solutions would be chosen at a higher rate than the less concentrated solutions. However, a precedent study showed that rats will in some cases not choose the higher concentration of sucrose when it reaches 30% sucrose or more (Farrar, Kieres et al. 2003). For this reason, before using sucrose as an equivalent reward for social contact, it is necessary to know exactly what range of concentrations are preferable to use.

In this study, we are going to test rats in different conditions, exposing them to a choice between 2 different concentrations of sucrose. The experiment consists of investigating whether our rats can discriminate between different sucrose concentrations and if they discriminate

differently, if the differences between the two sides are not the same.

1. Methods

1.1. Animals subjects and housing

Subjects used were 9 Long-Evans males rats (*Rattus norvegicus*) bred by Charles River Labs, Calco, Italy. The animals were housed in groups of three per cage in translucent standard laboratory cages type IV (E. Becker & Co. GmbH, Castrop-Rauxel, Germany).

Food and water were available *ad libitum* in the home cages. As rats are nocturnal, they were held in a reversed day/night cycle (light off at 0700 pm until 0700 am). This allowed testing during their active phase. The temperature in the colony chamber held constant (20 ± 2 °C) and humidity was controlled (60% ± 5%). Rats were weighed daily during the whole experimental phase and prior to monitor their health (Annexe 1). Rats were 5–6 months old at the beginning of the experiment and weighed between 428.5 and 509.5 g (mean ± SEM=463.11 ± 8.70g). Cages were enriched with plastic tunnels. All the experimental procedures and settings used were approved by the LANUV *(Landesamt für Natur-, Umwelt- und Verbaucherschutz North Rhine-Westphalia,* Germany) and in accordance with the German animal Welfare act.

1.2. Experimental setup/ apparatus

The experiment was conducted in an open field setup (60cmx60cm) Figure 1, subdivided into 3 equivalent chambers.

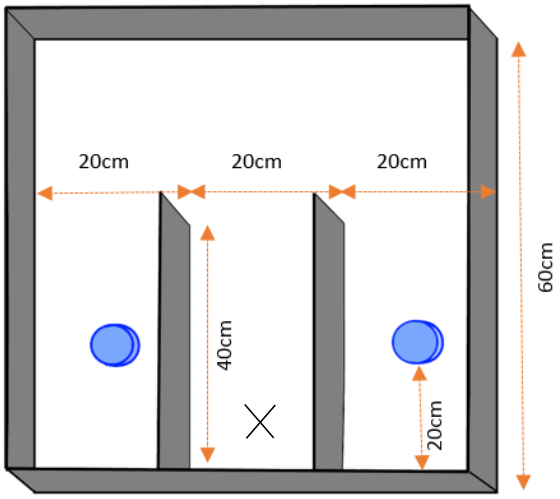

Figure 1. Three Chambers Maze. X *marks the starting point.*

The middle chamber was considered the starting box. Walls were made from PVC and opaque. All the chambers were partly open to allow rats freely moving between chambers. In the outer compartments, 2 petri dishes were symmetrically placed to avoid differences between the chambers.

The experimental room was constantly under red-light and all the screens and materials producing light were covered with red foils to keep the rats in the darkness. 99% of the rat retina consists of rods, which sense only light and dark, and only 1% consists of cones (LaVail 1976). Rats have just two types of cones (called "dichromatic" vision): a short "blue-UV" and the middle "green" cones (Szel 1992), those cones permit them to see a range between Ultraviolet and orange that why red is like darkness for them.

The experiment was recorded by an infrared camera placed above the setting in order to allow subsequent analysis.

1.3. Experimental design

A within subject design was chosen to decrease individual differences and give more power to the statistics.

1.3.1. Habituations days

Before the experiment rats were handled extensively by the experimenter over 2 weeks. In order to minimise stress and to get the animals used to the test setup, they were allowed to habituate for 10 mins each day for 3 consecutive days before the start of the experiment.

Rats were also familiarized with the liquid reward used by exposing them to a 2% sucrose solution in their home cage during the week before the experiment.

1.3.2. Reward

As a reward, four different sucrose solutions were used: 0%, 5%, 10% and 20%. For the solutions, 99.5 % D (+) – Saccharose (*Carl Roth GmbH + Co. KG)* was dissolved in tap water. The solutions were freshly prepared on the morning of the experiment.

1.3.3. Experimentation Schedule

All testing was carried out between 09:30 a.m. and 04:30 p.m. The experiment lasted 12 days, as each rat encountered the 6 conditions twice during the experiment Table 1.

0%/5%	0%/10%	0%/20%	5%/10%	5%/20%	10%/20%

Table 1. The 6 Rewards combinations

To control for a possible side bias, each reward combination was inverted between the first and the second appearance of it. The order of appearance of each sucrose combination during the 12 days was randomized (Annex 2).

1.3.4. Experimentation conduct

The experiment consisted of 2 phases:

First, rats were introduced to the maze for a 10 minute habituation within the setup. During this phase, a petri dish was present on each side of the setup but was left empty. Then, rats were removed and the maze was cleaned with 70% ethanol. A clean petri dish was then filled with 50ml of the solution and put back in place. The time between the two phases was kept constant and lasted 3.5 min.

In a second phase, the rat was put back in the setup where it was again allowed to freely move for a 10 min period, now with the liquid reward present in the petri dishes.

After introducing the animals to the setup, the experimenter left the experimental room to avoid perturbation of the rat's behaviour.

1.4. Video tracking

The movement and behaviour of the rats was analysed from the recorded videos using Ethovision XT5.

1.4.1. Detection using contrast

In order to optimize video settings before the start of the experiment, a pilot footage was conducted to determine the optimal contrast between the floor of the setup and the moving rat. The initial floor of the setup was grey and with the camera used, the program that was detecting using contrast difference could not detect the rat optimally. Therefore, the grey floor was

replaced by a plastic white floor, which created a high contrast between the floor and the animal. This pilot test allowed us to use Ethovision XT5 tracking without problems.

1.4.2. Ethovision XT5 functions

Ethovision XT5 allows for creating a template that can be applied to all videos for the analysis. This template permits splitting the arena of your setup into zones where you want to record activities, Figure 2.

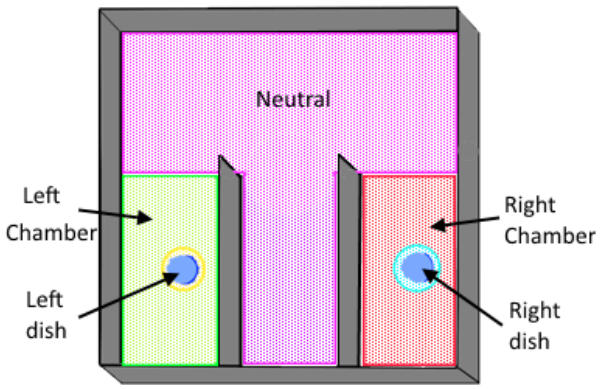

*Figure 2. Areas of interest; five regular zones and 2 Cumulative zones, green and yellow for **Cumulative left zone (CL)** and red and turquoise for **Cumulative right zone (CR).***

We demarcated the left and right chamber areas, the neutral parts of the setup and the petri dishes.

We also created two cumulative zones including Left chamber with Left dish and Right chamber with Right dish.

The program can thus calculate the time spent by the rat in each zone we defined.

1.5. Analysis

The data from the cumulative zones was our main interest. First, the relative duration of the time spent in the zone had been calculated by comparing phase 1 and phase 2 results using this equation:

$$Relative\ duration\ \% = \frac{Test\ Phase\ (s)}{Habituation\ phase\ \ (s)} \times 100$$

Next, we averaged the relative durations for each concentrations in each condition.

Then we calculated a diffrence score for each condition, in order to compare the discrimination between the different conditions using the following equation :

$$Time\ spent\ High\ concentration\ area - Time\ spent\ Low\ concentration\ area$$

Interested in preference between side within each condition a Dependent T-test was performed in which the time spent in the high concentration area was directly compared to the time spent in the low concentration area. As not all samples were normally distributed we performed a logarithmic tranformation.

Holm's sequential bonferroni correction was applied to protect against multiple testing bias, avoiding type I errors.

Finally, a repeated ANOVA using IBM SPSS statistics 20 was performed to compare the individual difference scores in each condition.

The result for the first test for rat 6 was excluded from the data because it appeared as an outlier due to anormal behavior during the test phase. No other major deviation was found from the assumption of the ANOVA.

All tests were performed with α=0.05.

2. Results

The experiment provided us with 216 videos with half of them corresponding to the habituation phase and the other half the test phase. After analysing the videos with Ethovision XT5, we obtained a data sheet gathering all the results with the variables created in the template as shown in Figure 2.

2.1. Result show that rats prefer high over low concentration.

In all the conditions tested, the high concentration side was preferentially chosen by the rats. Figure 3 (p11) displays the results obtained by calculating the average of the relative duration among the areas for each rat. The relative time spent by each rat shows a tendency towards spending more time on the side containing the high sucrose solution, despite a high variance between conditions.

Data show clearly that the high concentration is always preferred by our rats. We transformed our data under the logarithm to respect the assumptions required by the T-test. The result for each pairs of concentrations are significant,

- (1)0%/5% T(8)=-5.938, **p<0.0005** ;
- (2)0%/10% T(8)=-7.516, **p<0.0005** ;
- (3)0%/20% T(8)=-9.126, **p<0.0005** ;
- (4)5%/10% T(8)=-6.999, **p<0.0005** ;
- (5)5%/20% T(8)=-7.021, **p<0.0005** ;
- (6)10%/20% T(8)=-3.898, **p<0.05** ;

All the test were confirmed by a Holm's sequential Bonferroni correction.

The dependent T-test confirmed the observations made our rats preferred high concentration over lower.

Due to the means of the two sides and the direction of the *t*-values, we can conclude that there was statistically significant more time spent in the high side concentration among the population tested.

The mean and the standard deviations for each condition are displays on Table 2.

Conditions	**Mean**	**Std. Deviation**
(1)ln C0% - ln C5%	-1,25816	,63564
(2)ln C0% - ln C10%	-1,20921	,48263
(3)ln C0% - ln C20%	-1,34616	,44252
(4)ln C5% - ln C10%	-1,41075	,60470
(5)ln C5% - ln C20%	-1,15276	,49257
(6)ln C10% - ln C20%	-,78931	,60755

Table 2. Means and Standard deviation for the Dependent T-test between concentrations for all conditions.

On the Figure (p12) each condition were averaged for all the rats and it can be notice that rats spent between 2 and 4 times longer in the high concentration side.

The concentration of sucrose impacted their choice of which side to occupy the most.

2.1. Results show no difference between conditions.

The one way repeated measure ANOVA didn't show a statistically significant effect of the condition for the side preference, *(F(5,40)=1.360 P=0.260).*

On Figure 5 (p12) the display of the difference scores show that the time spent in the high sucrose concentration area

appear to be weakly negatively correlated with the magnitude (0%-5%/0-10%/0-20%). Related to the other condition this doesn't appear to be a general tendency.

3. Discussion

The results displayed clearly showed that the population of rats tested can discriminate between high and low sucrose concentrations of a liquid reward as previously shown in(Farrar, Kieres et al. 2003). In the other hand the ANOVA shows that the variance between the time spent in the high concentration and the low concentration does not shows difference between conditions.

For rats it doesn't matter if there is 5%, 10%, 15% or 20% difference between the two sides. It was expected that a correlation might exist between gap size of solution concentrations in each condition and the time spent on the preferred side. In accordance with (Muscat and Willner 1989) and (Farrar, Kieres et al. 2003) it was expected that rats would show a higher preference correlated with the augmentation of the sucrose concentration. However, our results showed that it was not the case, our rats doesn't displayed difference in their preference between conditions.

This may be explained by the design of our experiment which was not the same as (Farrar, Kieres et al. 2003), here we used a maze and we gave a choice to the rats then we calculate the time spent in zone and not the amount of intake. (Muscat and Willner 1989) used a design more close to our but the rats used were not adults and weighing 290-350 g, as they didn't complete their growth theirs taste preference were maybe not fully developed.

However it was also anticipated to expose a switching point where the concentration on the high side is too high, leading to a devaluation of the reward because the solution would be to sweet for the rats. Results from (Farrar, Kieres et al. 2003) indicated that the rats valued the 30% sucrose concentration less than both the 3% and 10% sucrose concentrations. We didn't used concentrations until 30% but the result from (Muscat and Willner 1989) show this switching point below 10%. Related to that we guessed that the switching point could appear around 10% or 20%. If we look at our results between 0%/5%, 0%/10% and 0%/20%, the preference decreases respectively. However, when considering all conditions, this data is not representative and, moreover, not statistically significant.

The data collected did not show all the expected results, which may be explained by the apparatus and the paradigm used.
It must also be taken into account that the strains used are not the same. We used the Long-Evans strain and (Farrar, Kieres et al. 2003) used Sprague–Dawley rats; a preview study shows that, rats of the Long-Evans strain appear to have a greater appetite for 5% glucose solution than do those of the Sprague-Dawley strain (Freggly 1992) that can be also the case for the other concentrations, explaining the difference in our results.

Now we know that the rats used in this study can discriminate between two sucrose concentrations. Ensuing, it will be possible to calibrate the reward system until identifying an indifference point between a sucrose reward and social reward. Such a system, once established, may open fields of research in testing molecules of interest that can change the tendency of the rats behaviours.

Many labs are initiating studies to improve the understanding of brain's mechanisms. With a better knowledge of the exact functions and positions of the neurotransmitters that are involved in the regulation of social behaviours and their valuation, we hope to develop some specific techniques to manipulates, stimulating or inhibiting those areas.

In vivo electrophysiological recording already showed that it can be used to map how intensively specifically-targeted neurons are firing in real time (Steinberg, Keiflin et al. 2013). Controlling as much as possible the conditions where the rats are exposed and knowing the brain area involved, it is here possible to uncover how the brain processes primordial behaviours as social interaction.

Furthermore, as a screening platform for molecules of interest in the treatment of behavioural deficits, this system can be used to show how chemicals are acting at a neural level, related to the behavioural level. Oxytocin is already known as a molecule widely involved in social activity (Sobota, Mihara et al. 2015) and the possibility to calibrate its dosage to behavioural response to improve social behaviour is a perspective of great interest. Several studies could also be initiated with cross-species observations to uncover the factor driving the evolution of social cognitive functions (Hernandez-Lallement, van Wingerden et al. 2014).

Conclusion

To conclude, rats as models offer a powerful means to investigate the evolution and neural substrates of social behaviour (Hernandez-Lallement, van Wingerden et al. 2014). We argue here that rats can discriminate between two solutions of sucrose, however, the results were not sufficient to corroborate with all expectations. The strain and the apparatus used may appear as the principal causes to explain these deficits. However, this study was a pilot study, whose purpose was not only to test how this population of rats discriminates sucrose solutions, but also to establish and calibrate the entire apparatus of the system. The three chambered maze was designed entirely for this purpose and had not yet been established in the social rodent lab. In order to run more consequent experiments in this maze, initial troubleshooting to solve eventual problems that could arise had to been completed first.

Following the path opened by Hernandez-Lallement, van Wingerden et al. 2014, further studies will expose precisely how neurotransmitters that control our social behaviours work, depending on the situation rats are exposed to. Applying recent techniques such as

electrophysiology recording and psychopharmacological manipulations we could try to uncover their mechanisms and modes of action. We hope to create a more precise map of the corresponding brain areas. This research will be part of the greater efforts to tackle the challenge of developing specific and successful treatment options for behavioural and mental disorders.

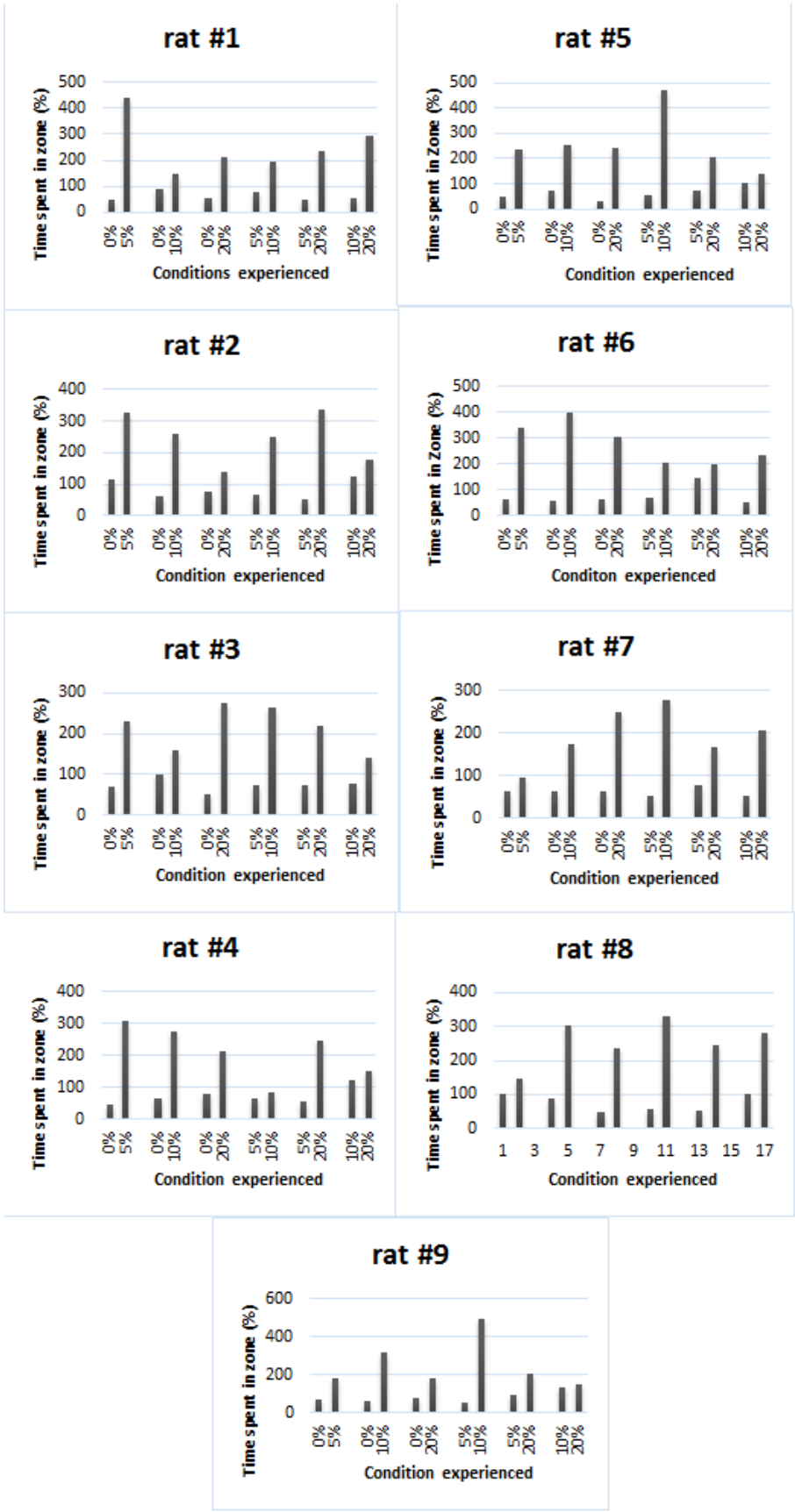

Figure 3. Relative duration of time spent in zones. Display of each condition for all rats

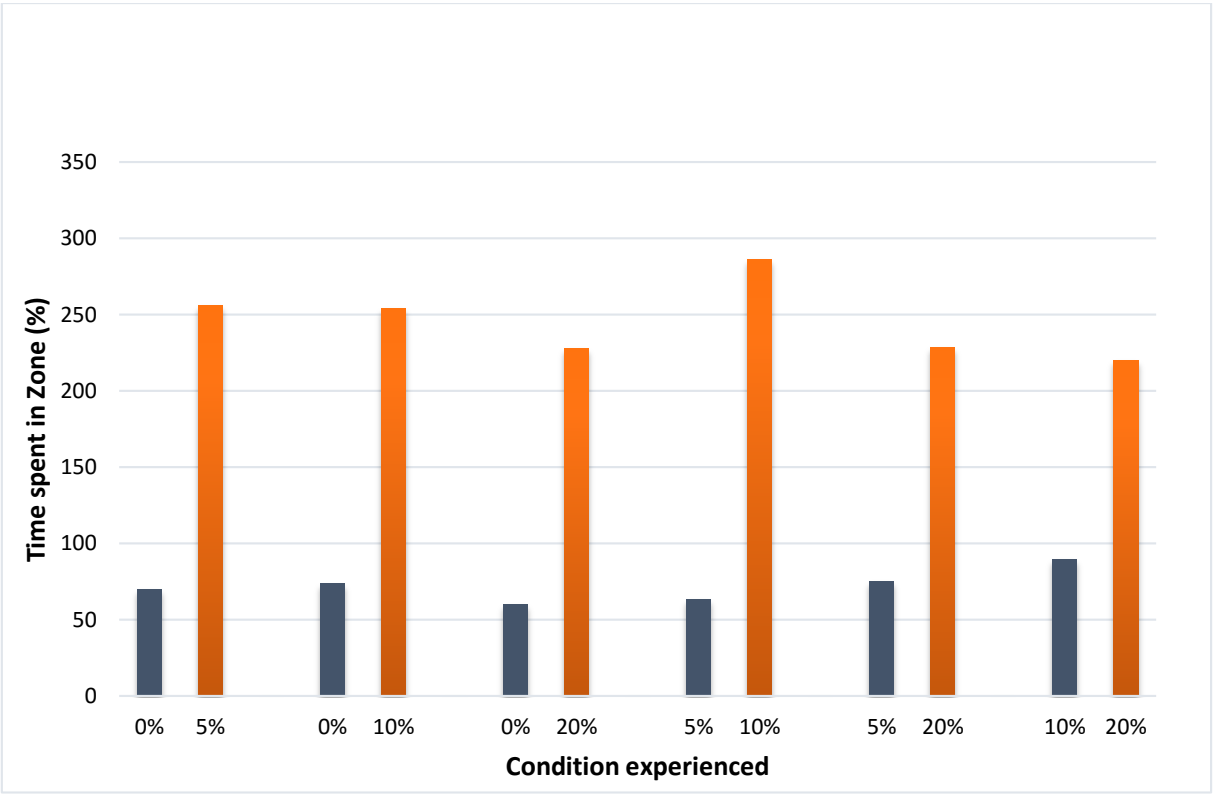

Figure 4. Time spent in zones depending on the condition experienced for the sample N=9. Each pair of concentrations represents a condition. In each condition, the blue bar represents the time spent on the low concentration side and the orange bar on the high concentration side.

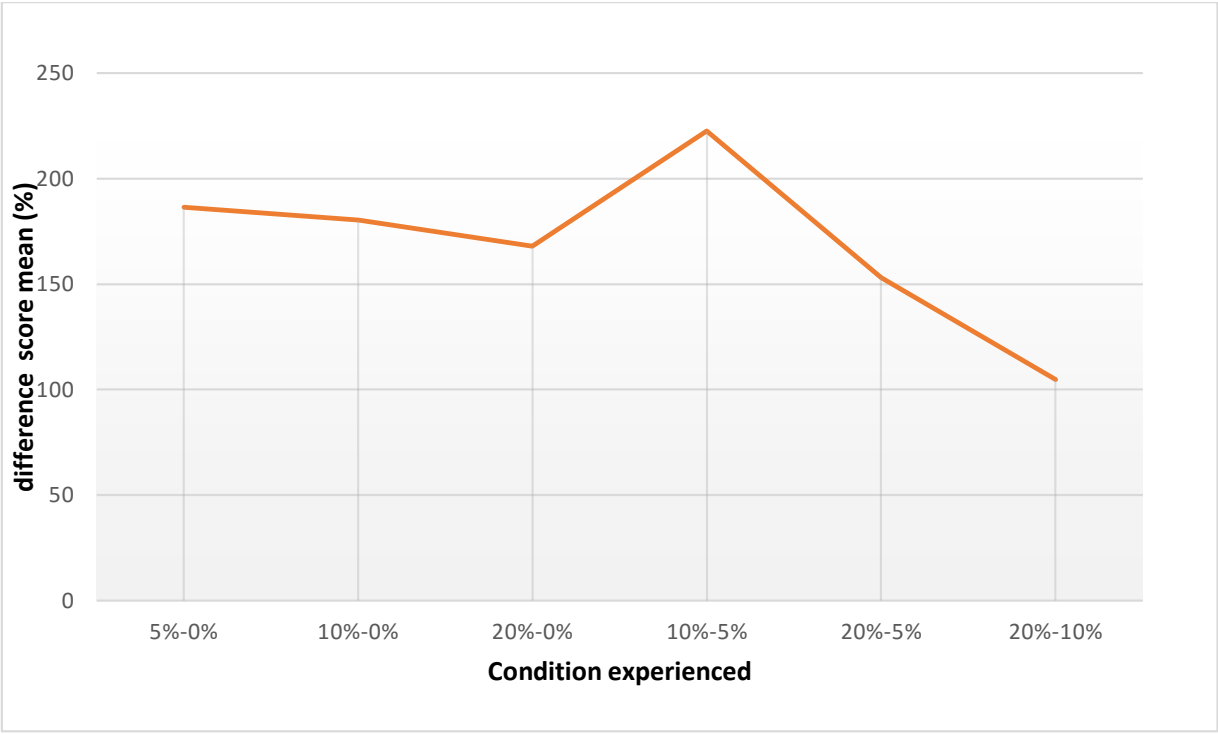

Figure 5. Difference score for each condition experienced. Value of different score in percentage

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Annex 1

Contributor	**Original idea**	**Bibliography**	**Protocol implementation**	**Experiment/ Data gathering**	**Statistical analysis**	**Report**
Principal	Marijn Van Wingerden	Quentin Devaux	Mireille Van Berkel	Quentin Devaux	Quentin Devaux	Quentin Devaux
Secondary	Mireille Van Berkel	Mireille Van Berkel	Quentin Devaux		Mareike Heller and Mireille Van Berkel	

Table 1. People involved in the internship

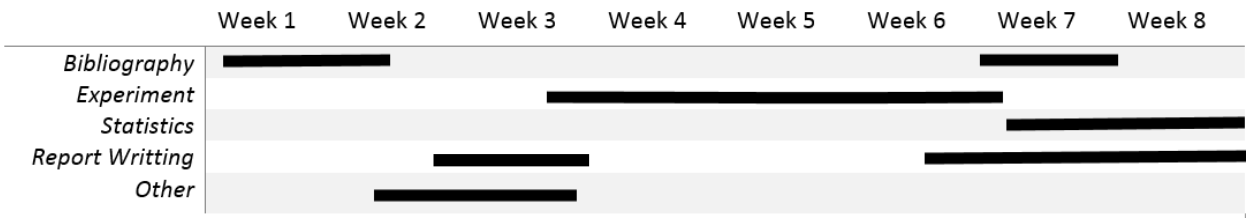

Table 2. Schedule

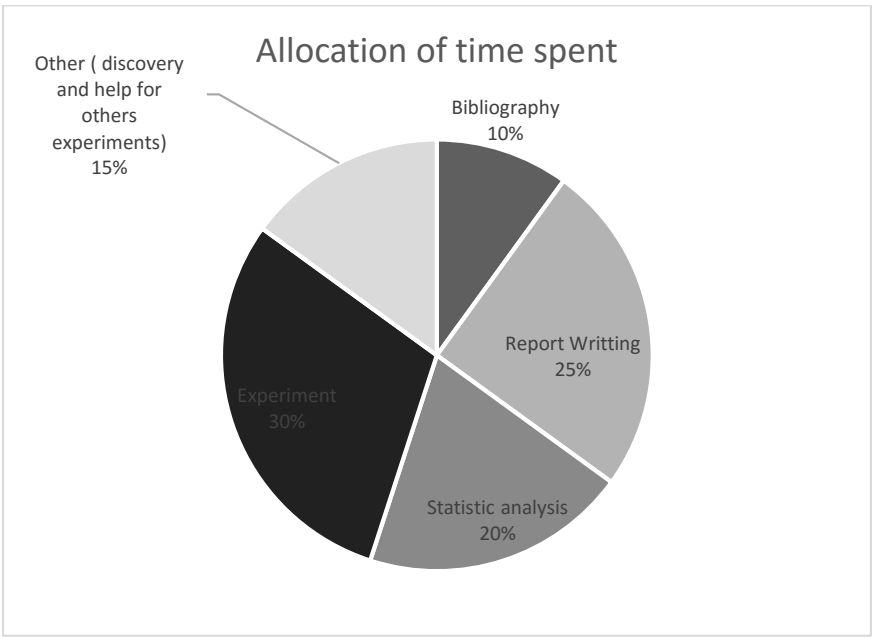

Figure 1. Pie plot representing the time spend for the different phase in %.

Annex 2

Date	Rat	Dose left	Dose right
6/4	1	10%	0%
	2	0%	10%
	3	10%	0%
	4	5%	10%
	5	20%	10%
	6	5%	10%
	7	5%	0%
	8	0%	5%
	9	20%	5%
7/4	1	5%	10%
	2	20%	10%
	3	5%	20%
	4	5%	0%
	5	0%	10%
	6	20%	10%
	7	5%	20%
	8	10%	5%
	9	0%	20%
8/4	1	20%	10%
	2	0%	20%
	3	5%	0%
	4	0%	10%
	5	20%	5%
	6	0%	5%
	7	10%	0%
	8	0%	20%
	9	5%	0%
11/4	1	0%	20%
	2	20%	5%
	3	10%	20%
	4	20%	0%
	5	0%	5%
	6	20%	5%
	7	0%	20%
	8	20%	5%
	9	10%	20%
12/4	1	5%	0%
	2	5%	10%
	3	10%	5%
	4	5%	20%
	5	10%	5%

	6	0%	20%
	7	20%	10%
	8	10%	20%
	9	10%	0%
3/4	1	5%	20%
	2	5%	0%
	3	0%	20%
	4	20%	10%
	5	0%	20%
	6	10%	0%
	7	5%	10%
	8	10%	0%
	9	5%	10%
14/4	1	0%	10%
	2	10%	0%
	3	0%	10%
	4	10%	5%
	5	10%	20%
	6	10%	5%
	7	0%	5%
	8	5%	0%
	9	5%	20%
15/4	1	10%	5%
	2	10%	20%
	3	20%	5%
	4	0%	5%
	5	10%	0%
	6	10%	20%
	7	20%	5%
	8	5%	10%
	9	20%	0%
18/4	1	10%	20%
	2	20%	0%
	3	0%	5%
	4	10%	0%
	5	5%	20%
	6	5%	0%
	7	0%	10%
	8	20%	0%
	9	0%	5%
19/4	1	20%	0%
	2	5%	20%
	3	20%	10%
	4	0%	20%
	5	5%	0%
	6	5%	20%

	7	20%	0%
	8	5%	20%
	9	20%	10%
20/4	1	0%	5%
	2	10%	5%
	3	5%	10%
	4	20%	5%
	5	5%	10%
	6	20%	0%
	7	10%	20%
	8	20%	10%
	9	0%	10%
21/4	1	20%	5%
	2	0%	5%
	3	20%	0%
	4	10%	20%
	5	20%	0%
	6	0%	10%
	7	10%	5%
	8	0%	10%
	9	10%	5%