AGGRESSIVE AND SEXUAL MOTIVATION IN MALE HAMSTERS: BEHAVIORAL AND NEURAL PERSPECTIVES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Tal Richards

May 2013

AGGRESSIVE AND SEXUAL MOTIVATION IN MALE HAMSTERS: BEHAVIORAL AND NEURAL PERSPECTIVES

Tal Richards, Ph. D.

Cornell University 2013

The purpose of this work was to test the social behavior network theory, suggesting that different social behaviors share overlapping neural mechanisms. This was done by focusing on aggressive and sexual motivations, which share many features, foremost among which is that both are stimulus-driven. These two motivations were evaluated using the same novel method, where subjects could push a heavy door towards a conspecific stimulus, with increased pushing reflecting higher motivation.

Behaviorally, males tested for aggressive motivation showed increased desire to fight following a winning experience and decreased desire to fight following a losing experience, supporting the validity of the method for quantifying this motivation. Males tested for sexual motivation with diestrous females showed very high levels of pushing, despite these females being aggressive, but similar to the aggression study, motivation was reduced in males who had lost a fight with a female.

Neurally, we first compared aggressive and sexual motivation by looking at the neural activation of males that lost a fight with another male and comparing it to that of males that lost a fight with a diestrous female. This analysis found that although the overall activation levels in all SBN regions were not different between the two conditions, evaluating the correlations with measures from the fights showed very different patterns for the two conditions. Males that lost to a male

exhibited negative correlations between fight measures and the activation in the various measures, while males that lost to a female showed a much weaker pattern involving mostly positive correlations. Exploring the neural mechanism of aggressive motivation in the motivation apparatus revealed a similar pattern involving only negative correlations despite the wide variety of regions investigated, suggesting that this motivation may simply reflect the lack of inhibition of reaction to a provoking stimulus. The pattern corresponding to sexual motivation in the apparatus was once again entirely different from that of aggressive motivation, although similar to the pattern seen in males that lost to a female. These findings cast doubt on some central assertions of the SBN theory.

BIOGRAPHICAL SKETCH

Tali was born in 1986 and graduated from Tel Aviv University in 2004 with a B.Sc. in biology, magna cum laude. She came to Cornell to pursue a Ph.D. in psychology (with a behavioral neuroscience concentration) soon after, where she remained for many many years pursuing the work described here.

This work is dedicated to all the hamsters who sacrificed their life for this project. RIP.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AcbC accumbens nucleus, core
AcbSh accumbens nucleus, shell
AH anterior hypothalamic area
BLA basolateral amygdaloid nucleus, anterior part
BLP basolateral amygdaloid nucleus, posterior part
BSTAI bed nucleus of the stria terminalis, anterointermediate part
BSTAM bed nucleus of the stria terminalis, anteromedial part
BSTAV bed nucleus of the stria terminalis, anteroventral part
BSTPI bed nucleus of the stria terminalis, posterointermediate part
BSTPM bed nucleus of the stria terminalis, posteromedial part
Ce central amygdaloid nucleus
Cg cingulate cortex
DLPAG dorsolateral periaqueductal gray
DMPAG dorsomedial periaqueductal gray
IL infralimbic cortex
LH lateral hypothalamic area
LPAG lateral periaqueductal gray
LPO lateral preoptic area
LSD lateral septal nucleus, dorsal part
LSI lateral septal nucleus, intermediate part
LSV lateral septal nucleus, ventral part
MeAD medial amygdaloid nucleus, anterodorsal part
MeAV medial amygdaloid nucleus, anteroventral part
MePD medial amygdaloid nucleus, posterodorsal part
MePV medial amygdaloid nucleus, posteroventral part
MO medial orbital cortex
MPN medial preoptic nucleus
MPO medial preoptic area
ORB orbital cortex
Pa paraventricular hypothalamic nucleus
PLCo posterolateral cortical amygdaloid nucleus
PMCo posteromedial cortical amygdaloid nucleus
PrL prelimbic cortex
SuM supramammillary nucleus
VMH ventromedial hypothalamic nucleus
VP ventral pallidum

General introduction

Uncovering the way in which neural activity produces behavioral output has been a central objective of the field of neuroscience. While historically that involved attempts to identify specific regions that produce complex behaviors or higher cognitive functions, over the years there has been a gradual shift towards a more domain general view (Finger, 2001). One notable example for this shift within the field of behavioral neuroscience has been the study of social behavior, which while seeking to implicate specific regions in each of a variety of social behaviors (e.g. sexual behavior, aggression, maternal care), instead identified a great deal of overlap in the regions involved in each (Newman, 1999). This led to the social behavior network theory, which suggested that rather than linking each region to just one behavior, several key regions form an integrated network such that different social behaviors are associated with different patterns of activation (Newman, 1999). Yet, despite the general acceptance of the theory and the framework it provided, the way in which these activation patterns generate the complex behavioral patterns comprising mammalian social behaviors was never explored. Since each of the social behaviors typically involve a sequence of multiple simpler behavioral components, we decided to take a first step towards addressing this matter by focusing only on the motivational component of the two primary male social behaviors – sex and aggression. Isolating the motivational component was meant to eliminate the part of the neural activation associated with the performance of either of these behaviors, and instead try to identify the potentially common elements involved in the decision to actively engage with a conspecific, be it male (fight) or female (mate). For that purpose, we designed a new method that measures the hamsters' motivation to engage with a conspecific by measuring the amount of effort they were willing to exert in order to do so, which allowed us to compare the two motivations under

conditions where the behavioral output associated with each was the same. Our hypothesis was that when correlating the level of motivation with the neural activation across the social behavior network and associated regions (see O'Connell & Hofmann, 2011 for expansion), there would be a significant degree of overlap between the regions whose activations contribute to the motivation to fight and those whose activations contribute to the motivation to mate. Such finding would support the idea of a "general social arousal mechanism" in common to both behaviors (Kollack-Walker & Newman, 1995) and would advance our understanding of the way in which differences in the patterns of activation across a network of regions can lead to contextually-different yet comparable behaviors.

Chapter 1: behavioral and theoretical factors underlying aggressive motivation

Introduction

Aggressive motivation could be defined simply as the desire to fight. Such a definition may sound trivial, but it is far from it. The fundamental question in the study of aggression has been whether aggressive behavior is ever the goal of the individual, making it a primary motivation, or is it always a means to an end. A straightforward comparison could be drawn to male sexual behavior, where it is clear that the behavior (mating) is the goal of the individual. When theorizing on the causes of aggression, one approach has been Lorenz's hydraulic model, which claims that aggressive energy is spontaneously generated and seeks release (1966). This approach has been quite controversial (for an in-depth analysis see Hinde 1969). Since so far there has been no clear evidence for such an "aggression deprivation" effect without constant exposure to aggression-inducing stimuli (e.g. Kudryavtseva 2004), we will not discuss this approach further. Instead we would like to focus on the opposing approach, advocated by researchers such as Scott (1971) and Marler (1976), who stated that aggression is always triggered by a particular stimulus and in a particular context. The goal of this approach has been to identify the various factors contributing to the occurrence of aggression, factors which for the purpose of this discussion will be roughly divided into contextual factors (particular situations), internal factors, and properties of the stimulus (the target of the aggression)[1].

When identifying contexts that contribute to the occurrence of aggression, the most obvious trigger is being threatened or attacked (Scott & Fredericson 1951; Marler 1976), especially in a situation in which one cannot escape (Archer 1988). However, individuals defending themselves in such situations are not motivated by the desire to fight, but rather by the need to defend

---

[1] There are clearly some overlaps between the categories when it comes to matters such as the behavior of the stimulus, but this makes no difference for the current paper.

themselves, so defensive aggression was suggested to be aversive for the individual, rather than appetitive (Rasa 1976). Another important factor contributing to aggression is territoriality, since animals are often found to be more aggressive when tested in their home cage than in a neutral arena (Scott & Fredericson 1951; Miczek & O'Donnell 1978). However, territorial aggression is another case in which the goal is defense, this time of resources, rather than the performance of aggressive behavior in itself. Competition for a resource is believed to be another major contributor to aggressive behavior, but this type of aggression has been difficult to elicit in rodents, and paradigms that managed to induce it revealed that it is less vigorous than fighting without the presence of a resource, likely because the animals' goal is the resource and it actually draws their attention away from the conspecific (Scott & Fredericson 1951; Lagerspetz 1964). Situations in which an individual is subjected to pain or frustration have been shown to result in aggression towards a nearby conspecific (Hinde 1969), although paradigms using such situations to study aggression in animals tend to be fairly artificial (e.g. no frustration seen in the context of a competition, Scott & Fredericson 1951; Hinde 1969), so they will not be discussed further. Finally, it has been suggested that under some circumstances, the mere proximity to a male conspecific could be sufficient to elicit aggression without any added aversive stimulus such as pain or frustration (Hinde 1969; Marler 1976; Archer 1988). Ultimately, such confrontations function to increase the reproductive success of the males who win them by allowing them more access to mate with females (e.g. Miczek et al., 2001), however securing access to females is unlikely to be the direct cause of the fighting behavior since the presence of a female does not increase inter-male fighting in male rodents but rather is more likely to lower it (Scott, 1966). Here we classify this fighting for "social status" as the only one of the aforementioned contexts reflecting true aggressive motivation, since it is triggered by mere proximity to a male

conspecific with no additional triggers. The choice to fight rather than walk away, despite the fact that there is nothing to be directly gained from fighting, suggests that the aggressive behavior itself is the goal in such cases[2].

When facing a conspecific, the choice between attack and avoidance is dependent not only on the previously mentioned contextual factors, but also on internal factors. We define internal factors as anything having to do with the individual themselves, such as physiological factors and prior experience. Here we will focus only on the latter, specifically on the outcomes of the individual's past agonistic encounters (whether it won or lost). When theorizing about the effects of experience on rodent aggression, it has been suggested that fighting should follow the principles of reinforcement learning (Scott & Fredericson 1951), with winning being rewarding and leading to an increase in attack tendencies, while defeats are punishing and lead to a decrease in aggressiveness and an increase in escape and avoidance. Accordingly, many studies have shown that winning affects behavior in subsequent interactions. Specifically, winning repeated encounters with nonaggressive opponents leads to a decrease in attack latency and an increase in attack behavior in both mice and hamsters (Ginsburg & Alee 1942; Lagerspetz 1964; Brain & Poole 1974; Parmigiani & Brain 1983; Potegal et al. 1993; Martinez et al. 1994; Oyegbile & Marler 2005). Studies examining the effect of being attacked and defeated by a more aggressive individual on behavior in subsequent interactions found the opposite effect – a decrease in aggressiveness and an increase in submissiveness and fear in both mice and hamsters (Ginsburg & Alee 1942; Lagerspetz 1964; Frishknecht et al. 1982; Potegal et al. 1993). Methods that assess the reactions of subjects to a conspecific across a perforated barrier rather than in an agonistic encounter have also been used to address the general effect of winning or losing. The results of

---

[2] Similar to the way sexual behavior is the goal when encountering a female, with the production of offspring being the ultimate consequence of successful mating.

this type of analysis in both mice and hamsters were that winners spent more time next to a male conspecific than losers did (Avgustinovich et al. 1997; Petrulis et al. 2004). One of these studies also compared the winners and the losers to naïve controls (Avgustinovich et al. 1997), finding that while losers spent significantly less time in proximity to the conspecific stimulus than the controls did, winners did not differ significantly from controls.

In addition to contextual and internal factors, the decision to react aggressively to a conspecific is also influenced by properties of the conspecific stimulus itself. One such property that interacts with the effects of experience is familiarity, specifically the familiarity formed through an agonistic interaction. One of the studies that addressed the effects of winning also examined how familiarity with the opponent might interact with those effects (Parmigiani & Brain 1983), finding that dominant mice showed an increase in attack behavior towards novel opponents compared to familiar ones. The effect of familiarity of the losers with their opponents in an agonistic interaction was not specifically evaluated, although losers' tendency to submit to non-aggressive opponents as well as to aggressive ones suggests that the losers generalize the fear of the opponent to all other males (Ginsburg and Alee 1942; Frishknecht et al. 1982; Potegal et al. 1993). Studies that used an investigation method to evaluate the effect of familiarity with the opponent on loser behavior, on the other hand, found that hamsters that were repeatedly defeated spent less time in proximity to a barrier across which was the hamster who beat them compared to a novel hamster (Lai & Johnston 2002; Petrulis et al. 2004). Additionally, winners tested in that manner spent less time in proximity to the hamster they beat compared to a novel hamster (Petrulis et al. 2004), in line with the findings described above.

To evaluate aggressive motivation separately from fighting competence, researchers have developed methods to test rodents' desire to fight in non-agonistic situations. One type of test

that has been used to evaluate aggressive motivation is the amount of time that mice with winning experience spent near a perforated partition (across which was their opponent) right before a fight, since the extent of this "partition behavior" was found to positively correlate with attack duration during the subsequent fight (Kudryavtseva et al. 2000). However, since the amount of time spent reacting to the mouse on the other side does not correspond either to an active choice to fight or to performing any action bringing about the occurrence of a fight, this does not provide a direct assessment of aggressive motivation according to our operational definition. Furthermore, despite the interpretation that the mice were showing "intent to overcome the partition to bite the loser" (Kudryavtseva et al. 2000, p. 389), no behaviors reflecting that intent (such as attempts to climb over the partition or undermine it) were quantified. Paradigms that did use quantifiable measures of rodents' active desire to engage in an aggressive interaction and willingness to work for it have utilized operant tasks, based on the common notion that a behavior is motivated if it can function as a reinforcer (Teitelbaum 1966). The subjects used in experiments of this type are typically male mice who initially go through repeated aggressive encounters with submissive opponents, meant to establish stable and rapid attack behavior and eliminate the fear of a counter-attack by the opponent (Potegal 1979). Subsequently those subjects go through operant training reinforced with brief attack opportunities on non-aggressive victims. One kind of operant action male mice have been shown to perform in order to fight is running towards a goal box where a stimulus mouse was kept. Such paradigms include simple runway running, where mice exhibit faster runway running when approaching a stimulus rather than an empty goal box (Legrand 1970); T-maze learning, where mice learned to turn towards the stimulus side of the T-maze significantly more often than chance (Tellegen et al. 1969; Tellegen & Horn 1972; Kelsey & Cassidy 1976); and crossing an

electrified grid, which mice did faster when approaching a male conspecific rather than an empty goal box (Lagerspetz 1964). The results of these studies all suggest that attack opportunities were reinforcing. A significant priming effect was found in all these studies, meaning that subjects performed significantly better following a brief attack opportunity (Tellegen et al. 1969; Legrand 1970; Lagerspetz 1964; Tellegen & Horn 1972; Kelsey & Cassidy 1976). However, when examining only the trials that were not preceded by an attack opportunity, subjects were found to perform at chance level in two of the three T-maze studies (Tellegen et al. 1969; Tellegen & Horn 1972). In addition, crossing an electrified grid without a pre-fight was not significantly faster towards a submissive opponent than towards an empty goal box (Lagerspetz 1964). Both of these findings suggest that mice require prior aggressive arousal in order to be motivated to seek out an opponent to attack. Another type of operant task is one in which the subjects remain in their home cage and are trained to perform an action on a device inserted into their cage, reinforced by brief introductions of a submissive conspecific into their home cage. Using such tasks, male mice have been shown to press a bar significantly more frequently when reinforced by attack opportunities than when given no reinforcement (Connor 1974). Mice also learned to nose-poke significantly more frequently into a hole that was reinforced by attack opportunities than in a hole that was not reinforced (Fish et al. 2002). Interestingly, neither of these tasks required any aggressive priming for the mice to perform, unlike the T-maze and grid-crossing methods, perhaps because mice tend to be more aggressive when tested in their home cages (Scott & Fredericson 1951; Miczek & O'Donnell 1978).

When evaluating all of these operant methods on the contexts in which animals are expected to be aggressively motivated, it is clear that none of these methods involves the proximity-induced, stimulus-reactive aspects of natural aggression. Moreover, none of them can tell us anything

about whether aggression is a primary motivation of untrained animals or whether it could be elicited by proximity alone even in naïve animals, without any additional aversive stimulus such as pain (e.g. Scott 1971).

In this study we introduce a novel paradigm for quantifying aggressive motivation. Unlike the operant methods described above, our method allows the subjects to investigate the stimulus animals non-aggressively through a perforated flap door, allowing them to interact that way if that is all they are interested in. However, if they are interested in fighting then they can push the flap door to get through to the stimulus animal, a task which was rendered highly effortful by attaching weights to the bottom of the door. The fact that the subject is constantly exposed to the stimulus while exerting the effort allows us to directly evaluate aggressive motivation in a reactive manner, since the only reason the subjects would push the door would be in order to contact and fight with the stimulus animal. To incorporate the directionality aspect of the decision to act aggressively, the apparatus also included an empty goal box identical to the one holding the stimulus animal, with an identically weighed door. Subjects were not trained to enter either goal box, and were only tested once.

Unlike other aggressive motivation studies, we used the golden hamster (*Mesocricetus auratus*) as our animal model. Hamsters are solitary animals (Nowack & Paradiso 1983) and they make ideal candidates for the study of primary aggressive motivation because a pair of naïve hamsters placed together in a neutral arena usually end up fighting each other vigorously within minutes (unpublished observations), which is an exemplar case of proximity-induced aggression. Thus, we chose to look at the important question of whether animals with no fighting experience would show unconditioned aggressive motivation, without ever having experienced the consequences of a fight, in a situation in which the only trigger for aggression was the presence of a

9

conspecific. Moreover, we tested whether winning a single fight would be enough to increase aggressive motivation in order to see if the increase in attack behavior found following an animal's first win (Brain & Poole 1974; Oyegbile & Marler 2005) was due only to increased fighting competence. We also tested whether a single loss would be enough to suppress aggressive motivation without involving any pronounced fear conditioning. Lastly, we tested whether a single fight is enough for the subjects to remember their opponent by comparing winners and losers tested with novel stimuli and ones tested with their fight opponent.

Materials and Methods

Overall Design

Five behavioral groups were used to study the effects of fighting experience on aggression. Each group had 12 subjects. One group consisted of males with no fight experience and four groups were of males that went through a single fight. Of these four groups, two groups were of winners and two groups were of losers. From each of those pairs of groups, subjects from one group were tested with unfamiliar male stimuli and subjects from the other group were tested with their familiar opponents. We will refer to our 5 groups as: naïve subjects tested with a novel male (NNM), winners tested with a novel male (WNM), winners tested with their familiar male opponent (WFM), losers tested with a novel male (LNM) and losers tested with their familiar male opponent (LFM).

The experiment included three phases: training, aggressive encounters, and the motivation test; a full experimental cycle took 3 days. On the first two days, all subjects learned how to get through a flap door in the training apparatus (the training phase did not involve a conspecific stimulus). Later on the second day males that were assigned to a fight condition went through an aggressive

encounter. On the third day all subjects were tested for aggressive motivation in our motivation apparatus, encountering a conspecific behind a flap door for the first (and only) time.

Animals

The study used male golden hamsters 3-4 months old from our laboratory colony (derived from Charles River stock). They were weaned at one month old and housed individually in solid-bottom polycarbonate cages (45x25x15 cm) containing sani-chip bedding with food and water ad libitum and maintained on a reversed 14:10-h light/dark cycle. Subjects had no aggressive experience prior to this experiment (discounting juvenile play-fights with their siblings prior to weaning).

Fighting opponents were other subjects from the study, typically from the same experimental cycle, and roughly matched for size. Male stimuli in the apparatus were typically subjects that already completed their experimental cycle, but males of roughly the same age that did not participate in this study were occasionally used.

All methods were approved by the Cornell University Institutional Animal Care and Use Committee.

Apparatus

<u>Training</u>

The training apparatus consisted of a main compartment (30x33x18 cm), a start box (10x13x18 cm) that was attached to the middle of one side of the main compartment, and a goal box (14x18x18 cm) attached in the middle on the opposite side from the start box (see image 1a). The start box and the main compartment were separated by a guillotine door. The walls, floors, and the guillotine door were made of 0.7 cm thick opaque Plexiglas. Additionally, the apparatus had a removable transparent Plexiglas top (used to prevent the hamsters from escaping). The wall

11

between the main compartment and the goal box had a 7cm diameter round opening in it, 1 cm above the floor. The opening was covered with a transparent Plexiglas flap door (13x9x0.3cm) with round perforations that opened towards the inside of the goal box (see image 2a). The door's resistance could be increased by attaching two identical weights to the bottom of the door using Velcro, one on each bottom corner (see image 2a). We used 3 levels of resistance with each of the two weights being 15.5g for level 1, 31g for level 2, and 46.5g for level 3.

Aggressive encounters

All aggressive encounters took place in neutral clean cages with clean bedding, identical to the hamsters' home cages (45x25x15 cm). There was no top on the cage during the encounters so that the loser could escape by jumping out of the cage.

Testing

The testing apparatus had a start box and a main compartment identical to the training apparatus but instead of having a single goal box on the side opposite from the start box it had 2 identical goal boxes opposite each other on the far end of the walls to the left and right of the start box (see image 1b). Both goal boxes had flap doors identical to the flap door of the training apparatus, except that the highest level of resistance was permanently attached to them – 46.5g on each of the bottom corners (see image 2b). Additionally, each flap door was linked to a PASCO PASPORT Rotary Motion Sensor (PS-2120) through the hinge (see image 2), allowing us to determine the angular position of the doors throughout the tests (to record the amount of pushing performed). The sensors were connected to a laptop using PASCO USB links in order to record that information.

Procedure

All parts of the experiment took place under dim illumination, with training and motivation tests
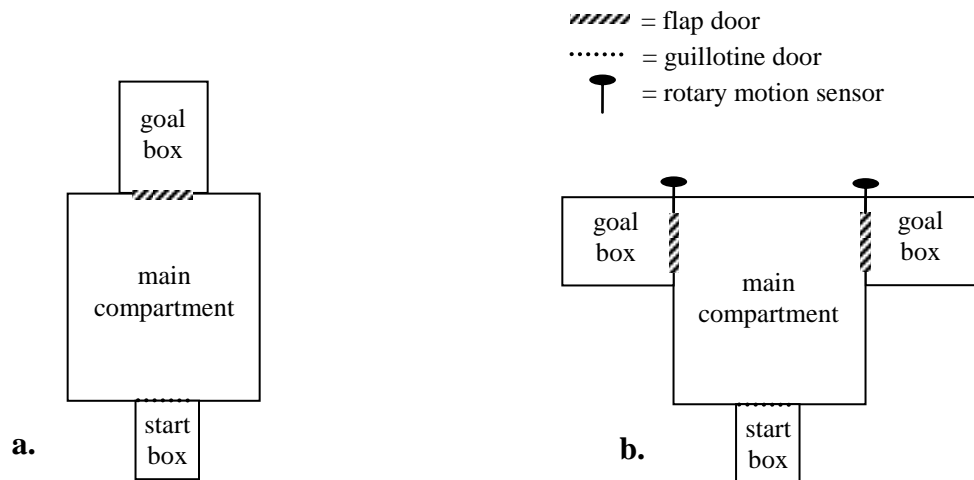
Image 1: layouts of the training apparatus (a) and the testing apparatus (b).
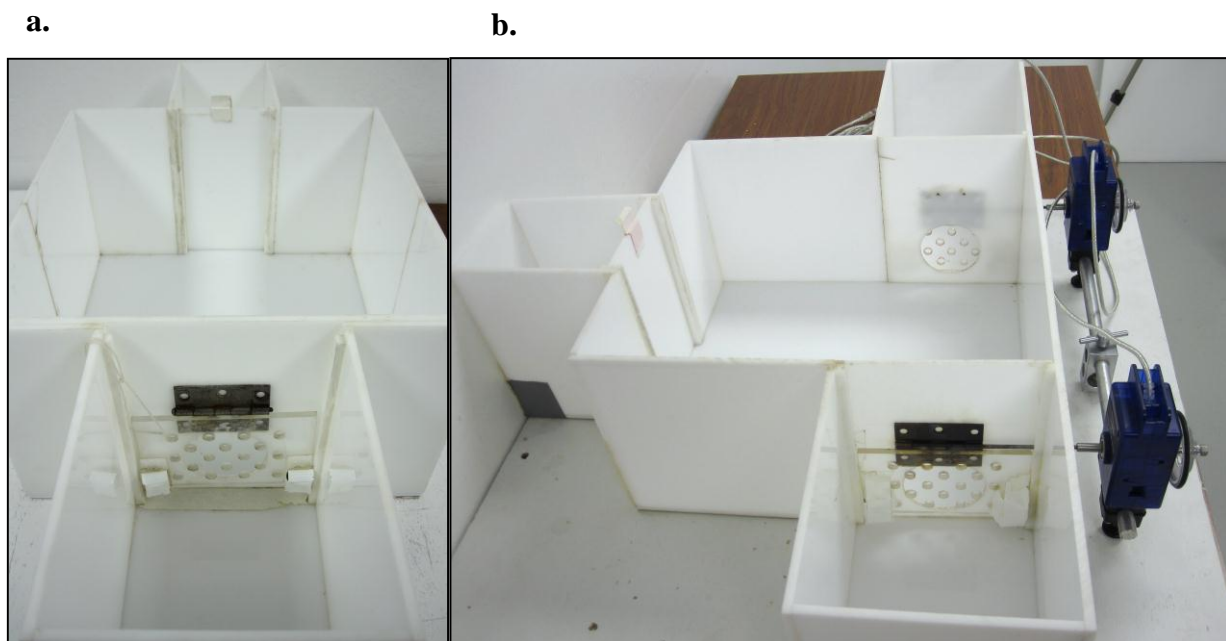

Image 2: training apparatus with level 2 weights (a) and testing apparatus with the sensors (b)

run within the first 3 hours of the dark phase. Fights took place 4-6 hours after the beginning of the dark phase (roughly 2-3 hours after the end of training).

<u>Training</u>

The first part of the experiment consisted of two days of training, during which the subjects learned to get through the flap door over several steps with increasing levels of difficulty:

Day 1:

a. The first step was a 2-minute habituation to the training apparatus with the flap door completely open, allowing the subject to explore the entire apparatus.

b. For the second step the door was open at a 30° angle (held with a string), requiring the hamster to crawl under it in order to get into the goal box.

c. For the third step the door was closed without any weights attached to it.

d. For the last step on the first day the door was closed and had the lowest level of weights (level 1) attached to it.

Day 2:

e. The second day of training started with a repetition of the last step from day 1, i.e. level 1.

f. For the next step, the door's resistance was increased to level 2.

g. For the final two steps of training, the door's resistance was increased to the maximum, i.e. level 3. The subjects were required to go through the door at this resistance level twice in order to ensure that they learn that it is doable even though it was quite challenging for some of them.

Each training step (a-g) began with the subject in the start box for about 20 seconds, then the guillotine door was opened to allow it to go into the main compartment (once the subject entered the main compartment the guillotine door was closed behind it). All training steps except the habituation ended once the subject got from the main compartment into the goal box, where there

14

was a sunflower seed as a reward. Once the subject ate the sunflower seed it was transferred from the goal box back into the start box to begin the next step, or put back in its cage if it was the last step for that day. While the subject was in the start box the door's state was modified to the next step and a new sunflower seed was placed in the goal box. The apparatus was cleaned with 50% ethanol between subjects (but not between steps for each subject).

The training was designed to be the minimum necessary for all subjects to be able to push the high-resistance door during the motivation test. Although there was considerable variance between subjects on the amount of time it took them to complete each of the training days, almost none of them required more than 20 minutes on either of the days, and the total duration of training on each day was typically around 10-15 minutes for each subject.

Aggressive encounters

On the second day of the experiment, about two hours after the end of training, pairs of subjects that were assigned to the fight condition went through a single encounter. Both males were placed in a clean cage and allowed to interact with each other freely. There was no time constraint. The trial lasted until the result of the fight was determined by one of the hamsters fleeing by jumping out of the cage – that subject was then classified as the "loser", and the other animal was the "winner". Since such fleeing is only attained as a result a "rolling fight", which is the distinctive pattern of hamster vigorous fighting, involving the pair wrapped around each other while biting and rolling (see Floody & Pfaff 1977), pairs that did not engage in such a fight were not included in this study[3].

Testing

On the third day of the experiment, the subjects' motivation to fight was examined in the testing

---

[3] Over 80% of the pairs did meet the rolling fight criterion; the number of subjects in each group did not include subjects that were excluded from the study.

apparatus. First each subject was habituated to the testing apparatus by being allowed to explore it for 5 minutes. During this time the apparatus was empty and the doors leading to both goal boxes were propped open to allow free investigation. An hour later the motivation test took place. During the test, the flap doors were closed and a male stimulus was placed in one of the goal boxes (chosen pseudo-randomly across tests). The stimulus was tethered so that it could be pulled away from the door when the subject was pushing the door (it was allowed to move freely the rest of the time). This was done to prevent the stimulus from physically blocking the motion of the door while still allowing it to get close enough to the door for the subject to investigate it while the subject was not pushing. Tests lasted for a maximum of 3 minutes, although they ended earlier if the subject got into one of the goal boxes before then (the trial ended as soon as the subject's entire body was in a goal box). Another criterion for ending a test trial was if 60 seconds passed from the first time either of the flap doors crossed the threshold of a 0.5 radian angle (the approximate angle allowing the hamster to crawl under the door). This criterion was necessary in order to avoid frustration or "giving up" by subjects that were less competent at getting through the doors, especially since the presence of the stimulus made it even more challenging to get into the stimulus goal box (due to the small size of the goal box, the presence of an active hamster in it obstructed the subjects' path through the door even without physically blocking the motion of the door).

The testing apparatus was cleaned with 50% ethanol solution before each habituation and each test.

Data collection

For the last two steps of training (on day 2, with the door maximally weighed), the amount of time it took each subject to get into the goal box was measured, starting from the moment it was

out of the start box. The shorter of these two latencies for each subject was used to represent the subject's ability to get through the door (longer durations reflecting poorer ability).

All fights and all motivation tests were recorded using a Sony DCR-TRV900 digital video camera. Additionally, for the motivation tests the angular positions of the flap doors were recorded continuously via the rotary motion sensors onto a laptop running DataStudio Lite software, at a rate of 100Hz.

Fights were scored for the latency to initiate vigorous aggression (a rolling fight), the total duration of vigorous aggression (rolling fights and following chases), and the total duration of the encounter (from first contact until the loser jumped out of the cage).

The tests in the motivation apparatus were first evaluated on the subjects' choice between the stimulus goal box and the empty goal box, based on whether the subject tried to get into either of them ("trying to get in" was defined as pushing the door towards that goal box more than 0.5 radians at least once, regardless of whether the attempt was successful). Tests were scored from the videos for the amount of time subjects spent investigating each door (with investigation defined as having their nose directed at the door within a distance of 1cm). The data files containing the continuous angular positions of the doors throughout the test trials were exported from DataStudio Lite into Microsoft Office Excel for analysis. Two measures for "amount of pushing" were derived for each subject – the total amount of time spent pushing (with pushing defined as the door being at an angle greater than 0.05 radians) and the mean angle of the doors while they were pushed. Each of these measures was computed for both the door leading to the stimulus animal and the door leading to the empty goal box. Ultimately, 3 quantitative measures were derived for each of the flap doors (for each subject) – the percentage of the test the subject spent investigating it without actually pushing (reflecting general interest), the percentage of the

17

test the subject spent pushing it (reflecting persistence), and the mean angle while it was being pushed (reflecting vigor).

Results

Where applicable, mean values are presented as M±SE. All statistical tests were performed using a significance threshold of $P < 0.05$.

Pushing ability

First we evaluated the subjects' ability to get through a weighted door, using the latencies measured on the final two steps of the training (with the door weighted at level 3). Taking the shorter of these two latencies for each subject, we found that subjects varied enormously on their ability to perform the door-pushing task, with the shorter latencies ranging from 11 sec to 412 sec (M=71±9.5). Since the distribution of these latencies was severely skewed, they were normalized using a log transformation (yielding M=1.68±0.05); these transformed values were then used to test whether the 5 groups differed on their pushing ability. A one-way ANOVA did not detect a significant difference between the groups, $F_{4,55}=1.215$, $P=0.32$, suggesting that any difference in pushing found between the groups during the motivation test was unlikely to be the result of an underlying difference in pushing ability.

Qualitative analysis

As a secondary comparison of subjects' ability to get through the doors, we examined the number of subjects that successfully got into either goal box during the motivation test, comparing it to the number of subjects that pushed hard (angle over 0.5 radian at least once) towards either goal box but did not manage to get into either of them. The number of subjects from each of the 5 groups belonging to either of these categories (got in or pushed hard) is shown

in table 1.1. Binomial tests comparing these counts for each of the 5 groups found no significant difference for any of the groups ($P>0.7$ for all), showing that each group included about as many subjects that were able to enter a goal box as ones that had difficulties doing so. Correspondingly, Fisher's exact test found no significant difference between the groups on that measure ($P=0.9$).

Table 1.1

Group	Got in	Pushed hard
NNM	6	6
WNM	5	7
WFM	7	5
LNM	4	6
LFM	6	5

Number of subjects that got into either goal box ("got in") or tried to get into either goal box but didn't manage to ("pushed hard"), out of 12 subjects in each group. NNM – naïve subjects tested with a novel male; WNM – winners tested with a novel male; WFM – winners tested with their familiar male opponent; LNM – losers tested with a novel male; LFM – losers tested with their familiar male opponent

Next, we compared the number of subjects that tried getting into each of the goal boxes (stimulus or empty) for each of the 5 groups (see table 1.2). Of the 60 subjects, only 3 did not push hard toward either goal box, all of them losers. Moreover, only 2 subjects, one naïve and one winner, pushed hard towards both goal boxes – both of these subjects first pushed hard towards the stimulus, then ended up getting into the empty goal box within a minute from the first hard push (after 53s and 32s, respectively). Therefore, our design yielded a clear choice for either stimulus or empty goal box for 55 of the 60 subjects (92%). We used simple binomial tests to compare the number of subjects that tried to get into the stimulus goal box to the number of subjects that tried to get into the empty goal box for each of the 5 groups. These tests found a significant difference for both winners groups ($P<0.05$ for both) but not for any of the other groups ($P=0.27$ for the naïve subjects, $P>0.5$ for both losers groups). Fisher's exact test found a borderline-significant difference between the 5 groups on the distribution between stimulus, empty and neither, $P=0.05$.

Table 1.2

Group	Stimulus	Empty	Neither
NNM	9	4	0
WNM	11	2	0
WFM	10	2	0
LNM	5	5	2
LFM	4	7	1

Number of subjects that were interested in getting into the stimulus goal box, empty goal box, or neither of them, out of 12 subjects in each group. NNM – naïve subjects tested with a novel male; WNM – winners tested with a novel male; WFM – winners tested with their familiar male opponent; LNM – losers tested with a novel male; LFM – losers tested with their familiar male opponent

Quantitative analysis

Following the qualitative evaluation of direction of motivation, we used a more detailed investigation of the amount of pushing by the subjects from the various groups. To illustrate what the raw pushing data looks like, plots of the angle of each of the two doors as a function of time from two subjects can be seen in figure 1.1. The first plot is from a subject demonstrating a lot of pushing towards the stimulus but none towards the empty goal box (figure 1.1a), while the second is of a subject demonstrating very little pushing towards the stimulus and some pushing towards the empty goal box (figure 1.1b). In order to analyze the amount of pushing across subjects we condensed the detailed pushing data into two numbers –the percentage of the trial spent pushing (with the door's angle over 0.05 radians), and the mean angle of the door while it was being pushed. For general reference, the subject from figure 1.1a spent 0.42 of the trial pushing towards the stimulus goal box with a mean angle of 0.36 radians, with 0 pushing towards the empty goal box, while the subject from figure 1.1b spent 0.1 of the trial pushing towards the stimulus goal box with a mean angle of 0.22 radians, and 0.1 of the trial pushing towards the empty goal box with a mean angle of 0.27 radians. We did not extract any additional information from the raw pushing data for this study.

For the first part of the quantitative analysis, we used paired t-tests to analyze the preferences of subjects from each of the 5 groups for one goal box over the other, focusing on the investigation
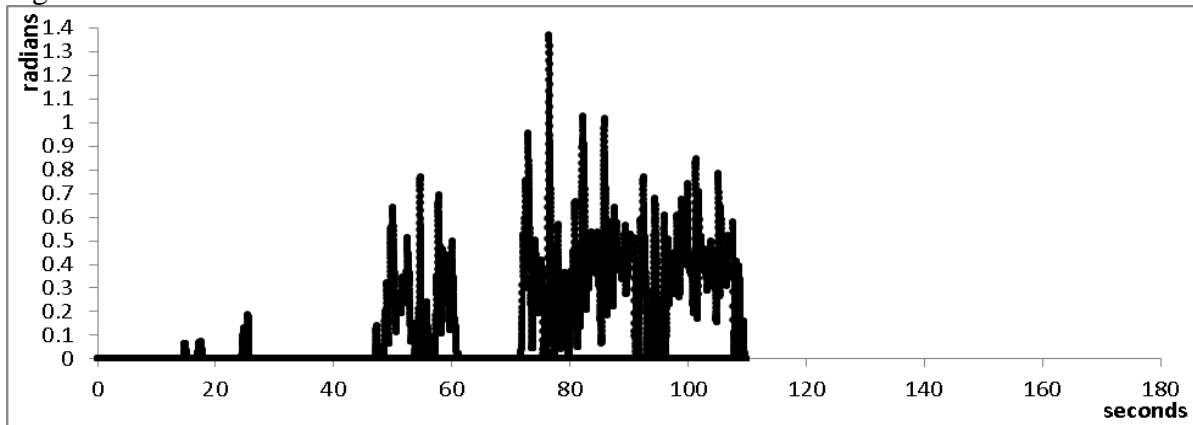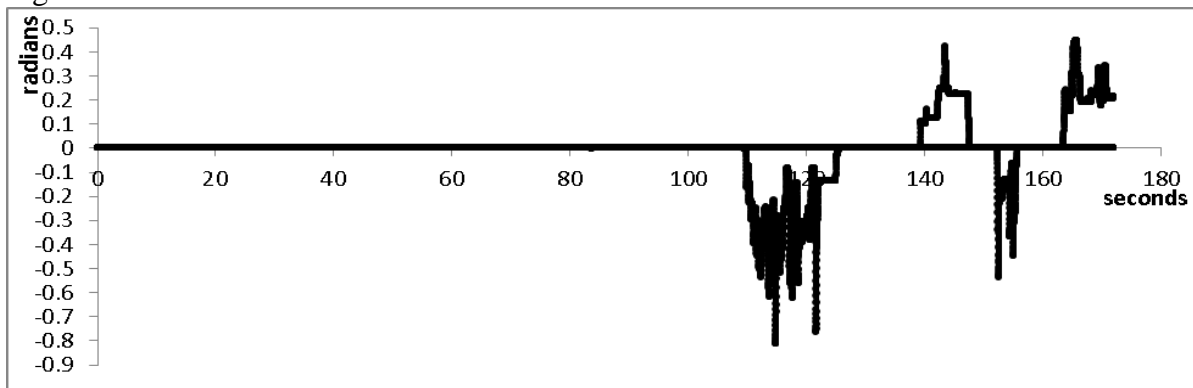
Figure 1.1a

Figure 1.1b

Figure 1.1: angles of both doors as a function of time, for two subjects. Positive direction – door towards stimulus; negative direction – door towards the empty. In both cases the trial was cut 60 seconds after the first time a door reached an angle of 0.5 radian.

21

measure and each of the two pushing measures separately.

First, the analysis of the investigation measure found that subjects from all 5 groups spent a significantly larger portion of the trial investigating the stimulus goal box than the empty goal box, $t_{11}>6$ and $P<0.0001$ for each of the 5 groups (see figure 1.2a). A two-way within-subject ANOVA found no significant interaction between group and goal box (stimulus or empty), $F_{4,55}=0.53$, $P=0.71$, suggesting that subjects from all 5 groups showed the same degree of preference for investigating the stimulus over the empty goal box.

Next, the paired t-test analysis performed on the pushing percentage measure found that only subjects without losing experience (i.e. winners or naïve) demonstrated a consistent tendency to push more towards the stimulus goal box than towards the empty goal box (see figure 1.2b). For group NNM this preference was moderate, with $t_{11}=2.66$, $P=0.02$, while for the two winners groups the preference was stronger, with $t_{11}=8.48$ for group WNM and $t_{11}=4.78$ for group WFM, $P<0.001$ for both. Subjects from the losers groups, however, showed no consistent preference for pushing towards the stimulus rather than towards the empty goal box, with $t_{11}=1.76$ for group LNM and $t_{11}=1.3$ for group LFM, $P>0.1$ for both. A two-way within-subject ANOVA confirmed that there was a significant interaction between group and goal box, $F_{4,55}=5.08$, $P=0.0015$, confirming that the strength of preference for the stimulus over the empty goal box on this pushing measure was significantly different across the 5 groups.

Lastly, the paired t-test analysis on the door-angle measure found that only subjects with winning experience consistently pushed harder towards the stimulus than towards the empty goal box (see figure 1.2c). The strength of preference of the two winners groups was similar, with $t_{11}=4.09$, $P=0.002$ for group WNM and $t_{11}=4.57$, $P=0.001$ for group WFM. The naïve subjects did not show a significant preference on this measure, $t_{11}=1.25$, $P=0.24$. The two losers groups did not

Figure 1.2a: the percentage of the motivation trial spent investigating each door.
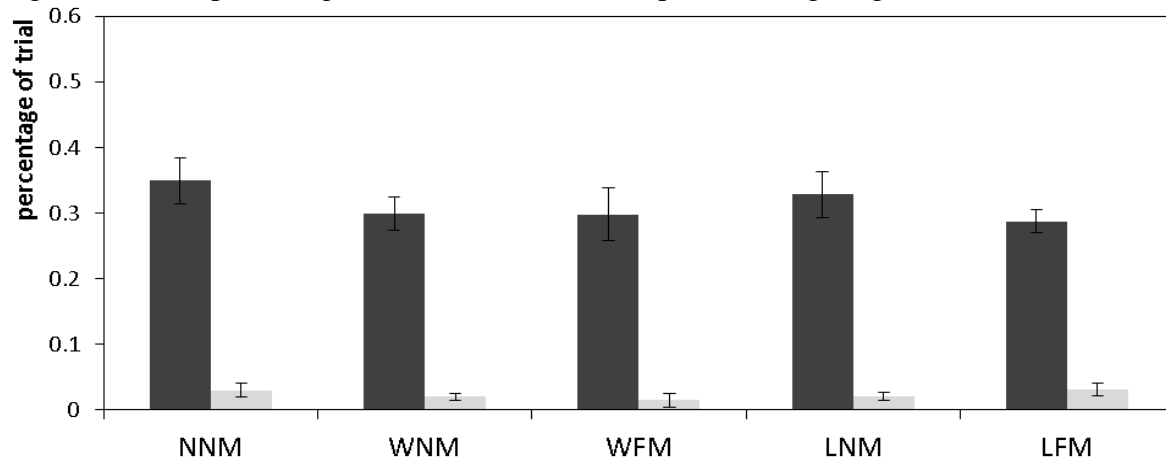

Figure 1.2b: the percentage of the motivation trial spent pushing each door.
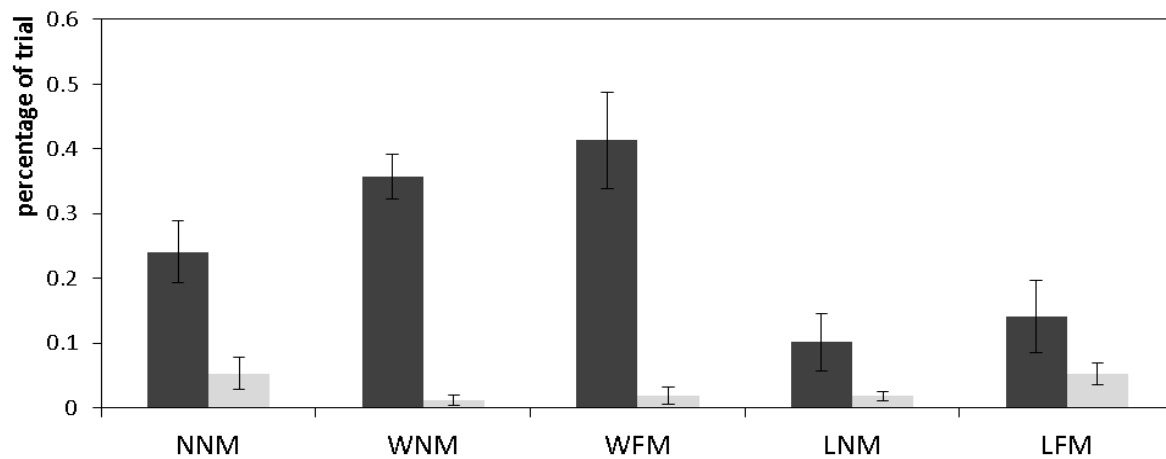

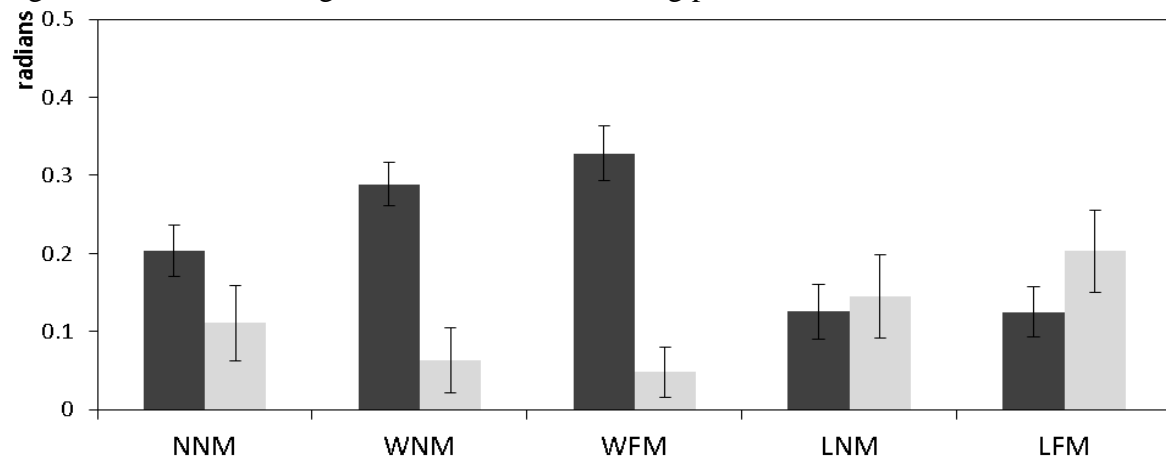Figure 1.2c: the mean angle of each door while being pushed.

Figure 1.2: dark grey bars – stimulus goal box; light grey bars – empty goal box; NNM – naïve subjects tested with a novel male; WNM – winners tested with a novel male; WFM – winners tested with their familiar male opponent; LNM – losers tested with a novel male; LFM – losers tested with their familiar male opponent.

show a significant preference either, although for them the tendency was actually to push harder towards the empty goal box than towards the stimulus goal box, with t_{11}=-0.24, P=0.81 for group LNM and t_{11}=-1, P=0.34 for group LFM. Not surprisingly, a two-way within-subject ANOVA found a significant interaction between group and goal box on this pushing measure as well, $F_{4,55}$=4.82, P=0.002.

For the remainder of the quantitative analysis we focus exclusively on aggressive motivation by investigating the effects of winning, losing and familiarity on the behavior of the subjects only towards the stimulus goal box (disregarding the empty goal box). For this analysis we used both the investigation measure and the two pushing measures. For each of these 3 measures we first used a one-way ANOVA to compare the 5 groups; if they differed significantly on that measure then this was followed by a general linear regression (GLR) analysis. We used a GLR model with indicator variables for winning, losing and familiarity, with the naïve controls as our baseline, in order to be able to tease apart the effects of those 3 factors across the 5 groups. We also included an interaction term, between winning/losing and familiarity, to see if familiarity with the stimulus had a different effect depending on whether the stimulus was a familiar winner or a familiar loser.

First, for the investigation measure the one-way ANOVA found no significant difference between the 5 groups, $F_{4,55}$=0.65, P=0.63 (see figure 1.2a), consistent with the previous part of the quantitative analysis. This measure was not analyzed further.

Next, the one-way ANOVA showed that the 5 groups did differ significantly on the pushing percentage measure, $F_{4,55}$=6.36, P=0.0003 (see figure 1.2b). The GLR model with the interaction term was accordingly significant, $F_{4,55}$=6.36, P=0.0003. However, since the interaction was not significant, t=0.17, P=0.87 (suggesting that there was no significant difference in the effect of

24

familiarity on this pushing measure between the winners and the losers), this term was removed from the model. The final model, "pushing ~ win + lose + familiar", was highly significant, $F_{3,56}=8.52$, $P<0.0001$. The overall effect of familiarity was not significant, $t=0.91$, $P=0.37$, but it was kept in the model in order to be controlled for. So, while controlling for the effect of familiarity, winning a fight was associated with an increase of 0.12 in the percentage of the trial spent pushing towards the stimulus, an effect which was marginally significant ($t=1.72$, $P=0.09$). Losing a fight, on the other hand, was associated with a decrease of 0.14 on that measure, an effect that did reach significance ($t=2.05$, $P=0.04$).

Finally, for the door-angle measure the one-way ANOVA found a highly significant difference between the groups, $F_{4,55}=8.2$, $P=0.00003$ (see figure 1.2c). The GLR with the interaction term yielded the same level of significance, with an adjusted R-squared of 0.33. Since the interaction term was once again non-significant ($t=0.62$, $P=0.54$), it was removed from this model as well. The final model was the same as for the other pushing measure, "pushing ~ win + lose + familiar", yielding a high significance level ($F_{3,56}=10.92$, $P<0.00001$). As in the analysis of the other pushing measure, familiarity did not have significant effect ($t=0.6$, $P=0.55$), and it was kept in the model so that it is controlled for. While controlling for familiarity, winning experience was associated with a 0.096 radian increase in the mean angle of the door while pushing, an effect which was significant ($t=2.24$, $P=0.03$). Losing experience was associated with an effect of similar magnitude but in the opposite direction once again, a 0.088 radian decrease in the mean door angle ($t=2.06$, $P=0.04$).

Correlational analysis

To further test the construct validity of our two pushing measures as representations of aggressive motivation, we looked at the correlations between them and the measures of

aggressiveness from the fight which winners and losers underwent on the previous day. We investigated the correlations separately for the winners and for the losers. Since the previous analyses did not detect a significant effect of familiarity, in the current analysis we combined the WNM and WFM groups into a single "winners" group and the LNM and LFM into a single "losers" group. As to the measures taken from the fights, we used the total durations of the encounters in addition to 3 specific aggressiveness measures – the latency to start a rolling fight, the total duration of the vigorous fighting (including rolling and chasing), and a fight intensity measure defined as the proportion of time spent in vigorous fight from the moment rolling was initiated (this measure was statistically independent from the duration of vigorous fighting, $r=0.08$, $P=0.7$). The correlations between each of these 4 measures from the fights and each of the 3 measures from the following motivation tests (the investigation measure and both of the pushing measures) are displayed in table 1.3a for the winners and table 1.3b for the losers.

For the winners the investigation measure did not correlate with any of the 3 aggressiveness measures or with the durations of the encounters. The pushing measures, however, did significantly correlate with two of the aggressiveness measures – the pushing percentage measure was significantly correlated with the duration of vigorous fighting ($P=0.006$) and marginally correlated with the fight intensity measure ($P=0.07$), while the pushing angle was significantly correlated only with the fight intensity measure ($P=0.02$). There were no significant correlations with the latency to get into a rolling fight or with the durations of the encounters.

Unlike these findings for the winners, for the losers neither the investigation measure nor the pushing measures correlated with the total durations of the encounters or with any of the aggressiveness measures ($P>0.1$ for all).

Table 1.3a (Winners)

	Fight duration	Latency to roll	Rolling duration	Intensity
Investigation	-0.085	0.005	-0.269	-0.174
Pushing proportion	0.173	0.086	0.546**	0.374[#]
Pushing angle	-0.269	-0.275	0.153	0.461*

Table 1.3b (Losers)

	Fight duration	Latency to roll	Rolling duration	Intensity
Investigation	0.319	0.108	0.114	0.098
Pushing proportion	0.148	-0.106	0.244	-0.152
Pushing angle	0.268	0.051	0.298	-0.024

The correlations between the variables from the fights and the variables from the motivation tests.

Discussion

The primary aim of this paper was to present a novel method for the study of motivation and to demonstrate how this method could be used for the study of aggressive motivation in male hamsters. Although getting through the high-resistance door during training turned out to be quite challenging for some of the subjects, the minimal training we put them through was sufficient for all of them to successfully perform the task with the door at the highest level of resistance. Subjects from the various groups did not differ in their ability to perform the task, suggesting that pushing ability is unlikely to be a confounding variable when comparing the different groups on the amount of pushing during the motivation test. During those tests, roughly half of the subjects who tried to get into a goal box succeeded; importantly, this was also true for each group individually (again supporting the independence of fighting ability and task performance). Consequently, we quantified the level of motivation displayed by the subjects by using the amount of effort they put into getting into either goal box, regardless of whether or not they succeeded in doing so. Trials were stopped 1 minute after the first major push towards either of the goal boxes, a rule that effectively minimized the occurrence of subjects trying to enter both goal boxes (only 2 subjects out of 60 did that) without actually turning the task into a forced choice. It is important to note that subjects were not trained to push the doors in the

testing apparatus, they never encountered any type of reward there and the aggressive motivation test (with the conspecific stimulus) was the first and only time that the subjects got the opportunity to push through either of the doors in the testing apparatus. Thus, the training that the subjects went through (in a different apparatus, with a single goal-box), could not have influenced their choice for one goal box over the other during the motivation test. The training may have contributed to the fact that only three subjects (5%) did not try to get into either goal box, but it could be argued that the presence of a male conspecific is a far more salient stimulus than the memory of a few sunflower seeds, suggesting that the influence of the training over the subjects' behavior during the motivation test was minimal at most.

Our interpretation that subjects that pushed towards the male stimulus were driven by aggressive motivation is supported by the fact that subjects initiated a rolling fight with the stimulus male as soon as they got into the goal box with it. In addition, the positive correlations found between the measures of fight vigorousness and the amount of pushing for the stimulus later performed by the winners of those fights further support our supposition that our pushing measures provide a valid quantification of aggressive motivation.

Given the evidence that our measures of pushing towards a stimulus appear to be appropriate measures of aggressive motivation, this method can offer new insight into proximity-induced inter-male aggression. Critically, our design did not include any of the factors commonly suggested to be the cause of aggressive behavior. That is, subjects were not defending themselves or a territory, they did not experience either pain or frustration, and they did not stand to gain anything tangible from fighting with the stimulus male (an identical "territory" was available in the form of the empty goal box). Thus, the aggressive motivation exhibited by our subjects had to have been elicited purely by the proximity of another male, a situation to which

28

they could have responded either with approach (aggression) or avoidance (by standing in another corner of the apparatus or entering the empty goal box). All these reasons provide strong support for our assertion that the subjects that tried to enter the stimulus goal box were motivated purely by a desire to fight, with no goal beyond the fight itself.

It is important to point out that we do not suggest that subjects expressing aggressive motivation under the circumstances presented in our design did so due to some internal drive that built up and required a release, as suggested by Lorenz (1966). Instead, our framework closely follows that of incentive motivation, in which the exposure to a stimulus is necessary in order to elicit the corresponding motivation (Bindra 1974; Berridge 2004). Accordingly, we suggest that the exposure to a male conspecific is what triggers the aggressive motivation in subjects tested with our method. In that sense our design is qualitatively different from operant methods, in which the unconditioned stimulus is not present while the task is performed. Interestingly, several of the studies using operant methods to study aggressive motivation have failed to detect such motivation in their subjects without prior aggressive priming (Lagerspetz 1964, Tellegen et al. 1969; Tellegen & Horn 1972). Such a priming effect is known in contexts entirely unrelated to aggression, such as the 'cocktail peanut' phenomenon ("After taking one tidbit without desire and merely to be polite, you suddenly find you want to eat a few more", Berridge 2004, p.190) and it is typically explained using the incentive motivation approach (Berridge 2004).

In order for incentive motivation to be elicited, exposure to an appropriate stimulus is necessary but not sufficient. The other necessary factor is for the individual be in a state that allows the motivation to come about. The present study has tackled this issue by testing the effect of prior fight experience (winning or losing) on subsequent aggressive motivation.

When examining the behavior of males who won a fight on the day before the aggressive

motivation test, we found that significantly more of them tried getting into the stimulus goal box than into the empty goal box. Winners also showed a consistent preference for the stimulus goal box when we specifically looked at both duration of pushing and magnitude of pushing (quantified by the mean angle of the doors). When isolating the effect of winning on motivation towards a conspecific stimulus using a general linear model (with naïve subjects serving as baseline), we found that winning was associated with an increase in both pushing duration and pushing magnitude. When comparing these findings to the literature on the "winner effect", it is important to note that unlike the effect of winning on aggressiveness in subsequent fights, our measure is completely independent from fighting competence or winning ability. While the standard "winner effect" could be explained by suggesting that the winners developed the habit of attacking (e.g. Ginsburg & Allee 1942; Scott & Fredericson 1951), such that repeated wins result in the males becoming more effective fighters compared to naïve subjects (as shown by Oyegbile & Marler 2005), our findings could not be explained in that way. Using our method to test aggressive motivation in winners is more comparable to findings of aggressive motivation using conditioning paradigms, in that those methods test desire to fight rather than fighting ability. However, such paradigms can only test subjects with repeated winning experience (necessary for the operant training), typically using submissive males that do not fight back as fight opponents/incentive stimuli. The "painless victory" experienced by winners in such paradigms has been suggested to increase aggressive motivation through the elimination of the fear components from aggressive encounters, leaving only the supposedly rewarding component of the fight – the defeat of another individual (Potegal 1979). Pairing our subjects against each other, in contrast, resulted in evenly-matched opponents (i.e. balanced fights), yet the winners of such fights still showed heightened aggressive motivation. A possible explanation for this

finding is that although the fights themselves were not completely pleasurable for the winners, they were not highly aversive for them either. If that level of aversiveness is lower than what a naïve subject might expect from a fight, then this may have been sufficient to reduce some of the ambivalence found in naïve subjects, contributing to the more consistent preference for the aggressive choice found in the winners (similar to the fear-reduction mechanism proposed by Potegal 1979). An alternative explanation is that since our winners were not randomly assigned but rather self-selected (an unavoidable side-effect of using evenly-matched fight opponents), it is possible that the males that had a higher level of aggressive motivation to begin with were also the ones who were more likely to win the fights. This alternative could potentially be negated by the finding that mice showed no clear correlation between aggressiveness and winning in their first fight, unlike the clear correlation between these two factors found after repeated fights (Ginsburg & Allee 1941, although these authors stated that they were not ready to draw conclusions on that matter based on the data that they had). Our study did identify correlations between measures of aggressiveness during the fights and subsequent measures of aggressive motivation in the winners of the fights, suggesting that more vigorous fights were associated with higher aggressive motivation in the winners. However, there is no way to determine the direction of the causality in those correlations, since the course of a fight is determined by the behaviors of both the winner and the loser (the loser determines how long it takes it to retreat, and whether it ends the fight by fleeing the cage or just distances itself from its opponent). Thus, our current data is not sufficient to determine whether winning a single fight resulted in increased aggressive motivation or that the more aggressive males were the ones who won the fights; we suspect that the answer is likely to be a bit of both.

Turning our focus to the males who lost the fight prior to the motivation test, we found that these

subjects did not show a consistent preference for either the stimulus goal box or the empty goal boxes, as assessed by the number of subjects that tried to enter each goal box and by the amount of pushing each subject performed on each of the flap doors (using our two pushing measures). Additionally, when isolating the effect of losing on motivation towards the male stimulus using GLR analysis, we found that it was associated with a significant decrease in both of the pushing measures, compared to naïve subjects. These results suggest that losing a fight was associated with decreased aggressive motivation and thus a lack of preference for the stimulus over the empty goal box. These findings are quite different from those of typical "loser effect" studies, since such studies typically address the increase in fear and submissiveness in addition to the decrease in aggressiveness in losers within an agonistic encounter. In such a context, it has been suggested that the losers have learned to react submissively to a conspecific, possibly through a fear conditioning mechanism (i.e. Ginsburg & Allee 1942; Scott & Fredericson 1951). The other paradigm used to study the effect of losing, investigation through a partition, is also used primarily to evaluate fear and avoidance of conspecifics by the losers (Avgustinovich et al. 1997), likely resulting from a similar fear conditioning mechanism. In contrast to this typical "loser effect" found following the loss of multiple fights (often to bigger and/or more aggressive opponents), we found that the loss of a single evenly-matched fight did not result in any clear fear or avoidance of a conspecific stimulus. This was demonstrated by measuring the amount of time spent investigating the conspecific stimulus compared to the investigation of the empty goal box. This analysis revealed that males in all behavioral groups, regardless of experience, showed a strong preference towards investigating the stimulus and that the degree of preference did not differ between the groups. Additionally, subjects with losing experience did not differ from the naïve subjects or even from the winners in the time spent investigating the stimulus animal.

32

These results indicate that although losers clearly showed diminished aggressive motivation, this was not due to general fear or avoidance of the stimulus. Thus, it is possible that the loss of a single fight is not sufficient to produce fear of a conspecific, while still having a clear negative effect on the motivation to engage in further fights. This interpretation suggests an independence between the approach and avoidance factors underlying aggression (along the line of the pugnacity and fear factors hypothesized by Seward 1945). Accordingly, the lack of correlations between measures from the fights and measures of approach towards the stimulus during the motivation test could be attributed to the fact that for the losers, the measures taken from the fights are likely to be channeled into fear, which is not what our motivation method was designed to quantify[4]. Then again, the reduced aggressive motivation expressed by the losers could also simply be caused by a tendency of less aggressive subjects to be the ones that lose the fights. This lack of random assignment is unavoidable when testing the effect of losing to an evenly-matched opponent, since the loser of such an encounter cannot be predicted in advance. Thus, as we concluded in our analysis of the winners, our data does not allow us to determine the direction of causality between losing a fight and demonstrating decreased aggressive motivation on the following day. We once again propose that this could be due to a combination of the effects of both experience and individual differences. Either way, our inability to conclusively determine the source of the effects found for the winners and the losers does not undermine the validity of our method for quantifying aggressive motivation.

Unlike the clear effects found for winning and losing, familiarity with the conspecific stimulus had no effect on any of our measures – winners tested with the opponent they had beaten did not differ from winners tested with a novel conspecific and losers tested with the opponent who beat

---

[4] The amount of time spent investigating the stimulus can suggest a lack of fear, but it is not suitable for quantifying degrees of fear. Entering the empty goal box is not applicable either, since it could simply reflect a calm deliberate choice for avoidance.

them did not differ from losers tested with a novel conspecific. These results may seem surprising, since familiarity has been one of the primary stimulus properties suggested to affect the potency of the stimulus at eliciting aggression (Marler 1976). Additionally, it is possible that the familiar opponents differed in their behavior compared to the novel conspecifics, since all the familiar opponents had the same recent experience (either winning or losing, depending on the group), while the novel stimuli were chosen randomly and had no fighting experience in the days prior to the test. Therefore, the lack of an effect of opponent familiarity suggests that in our method the particular properties of the stimulus did not seem to matter, given that the stimuli were all adult male conspecifics (which are the top properties for an aggression-provoking stimulus suggested by Marler 1976). As to the lack of a familiarity effect specifically, it seems that a single fight may not be enough for male hamsters to form a long-term memory of their opponent, although it may also be that such a memory was not sufficient to produce a noticeable change in the subjects' behavior when tested with our method.

One key advantage of our motivation method is that it allowed us to assess aggressive motivation of naïve males, an issue that has not been previously addressed. In our experiment the majority the naïve subjects (75%) pushed hard in order to enter the stimulus goal box. This percentage is considerable, even though it was not statistically different from the small number (33%) of males that tried entering the empty goal box. Naïve subjects showed a consistent preference for the stimulus goal box over the empty goal box on the pushing percentage measure, but only a non-significant trend on the pushing angle measure. Overall, the important discovery regarding aggressive motivation of naïve subjects is that many of them showed clear signs of moderate aggressive motivation, demonstrated by their effort to get to the stimulus male. Like subjects from the other groups, all naïve subjects that got into the stimulus goal box (4 total) immediately

engaged in a rolling fight with the stimulus male, suggesting that despite their lack of fighting experience, these subjects were pushing in order to fight. This finding is particularly noteworthy considering that naïve subjects never experienced a fight or its consequences – either positive or negative. Additionally, none of the subjects ever experienced the outcome of getting into the stimulus goal box, since the motivation test was the first and only time they encountered a conspecific behind a flap door[5]. Thus, the door-pushing task is intended to test whether a male conspecific could be an unconditioned trigger for aggressive motivation, rather than whether aggression is reinforcing (which is the matter addressed by operant conditioning paradigms). This is an entirely new angle on the study of aggressive motivation, especially when using subjects whose aggression levels have not been artificially increased through repeated training with submissive opponents. It appears that for male hamsters, exposure to a novel male conspecific can be sufficient to induce the willingness to exert effort in order to fight. Furthermore, although this willingness seems to be sensitive to prior fight experience, increasing or decreasing due to winning or losing respectively, it is particularly interesting that the baseline level of aggressive motivation, found in males without any fight experience, seems to be moderate even when the only aggression trigger is the proximity to a male conspecific.

Finally, since most animal models of aggression are expected to ultimately provide insight into the factors underlying human aggression, it is worth pointing out how our method is relevant to that. Nelson & Trainor's (2007) discussion on the limits of animal models for understanding human aggression states that "Rather than comparing overt aggressive behaviours between species, a more effective strategy might be to focus analyses on the motivational systems that influence the decision to engage in aggression" (p. 537, box 1). This decision is clearly the focus

---

[5] What actually happened was that they were taken out of the apparatus and returned to their cages almost immediately. Additionally, if they had been allowed to fight, most subjects tested with novel males would have lost since these stimuli were typically slightly older than the subjects, giving them an advantage.

of our method. More importantly, these authors go on to suggest that "Pathologically aggressive individuals do not fight more furiously in a situation when anyone would fight; they fight in situations in which virtually nobody else would fight" (Nelson & Trainor 2007, p. 537, box 1). This is where our approach to aggressive motivation differs from approaches that use subjects that were trained to fight. It has been suggested that the reason such training yields heightened aggression is precisely because it teaches the subjects that aggressive behavior has no painful negative consequences (Potegal 1979). The pathological aggression resulting from such training is entirely different from that of an individual choosing to fight with complete disregard to the potential consequences, in a situation in which the consequences may very well be adverse. We propose that studying aggressive motivation in the golden hamster using our new method could yield new, important insight into the factors underlying the aspect of pathological aggression characterized by such disregard of consequences.

Chapter 2: behavioral and theoretical factors underlying sexual motivation

Introduction

Sexual motivation has long been recognized as a case of incentive motivation (Bindra 1974; Toates 1986; Ågmo 1999), critically depending on the presence of an external stimulus in order to be elicited. Although it is often considered to be one of the primary motivations (Bindra 1974; Toates 1986), clear differences are apparent when comparing the motivation to mate with the more commonly studied motivations to eat or drink. An individual deprived of food or drink experiences physical discomfort, with prolonged deprivation being physiologically detrimental and eventually resulting in death. Sexual behavior, on the other hand, is not necessary for an individual's physical well-being and generally its prolonged absence has no harmful consequences (Beach 1956). Thus, unlike the case with food or drink, there is no physiological need to engage in sexual behavior in order to maintain some sort of homeostasis but instead this motivation must be elicited by a sexually attractive stimulus (Beach 1956; Beach 1976).

When assessing the aptness of various methods for quantifying sexual motivation, it is crucial to pinpoint what types of behaviors performed by the animals reflect this motivation. In his seminal 1956 paper Beach proposed that male sexual behavior could be divided into an appetitive component, referred to as the "sexual arousal mechanism" (SAM), and a consummatory component, referred to as the "intromission and ejaculatory mechanism" (IEM). The SAM was defined as the arousal mechanism necessary for the male to make contact with the female, bringing it over the copulatory threshold so that mounting and intromissions would occur (Beach 1956). Although some researchers use the SAM (particularly the latency to mount) as reflecting sexual motivation (e.g. Pfaff & Ågmo 2002), as an arousal mechanism it is likely to be more closely tied to the physiological responsiveness of the males rather than to actual motivation (for an analysis of the difference between sexual arousal and sexual motivation see Sachs 2007).

Here we view motivation as "the process that causes organisms to seek goals or goal objects, and is measured by the willingness of organisms to work (expend energy) to gain access to those goals" (Sachs 2007, p.576). Thus sexual motivation is what causes a subject to "respond to a sexual stimulus by actively seeking contact with a sexual partner" (Hetta & Meyerson 1978, p. 63).

Methods used to study sexual motivation can be divided into two broad categories: investigation methods and operant methods. The first category includes methods characterized by allowing the male to investigate a receptive female through some form of a perforated barrier, permitting olfactory, auditory and visual signals but no physical interaction. When a male subject is presented with both a receptive female and a non-sexual stimulus (either a non-receptive female or a male) at the same time in order to compare the male's relative interest in these stimuli then this is referred to as a "preference method" (Hetta & Meyerson 1978; Vega Matuszczyk & Larsson 1993; Ågmo 2003; Ballard & Wood 2007). An alternative design involves presenting each stimulus separately and comparing the different conditions (Ågmo et al. 2004; Amstislavskaya & Popova 2004). In both of these designs the main behavioral measure is the time spent investigating the stimuli (i.e. time spent next to the perforated barrier). One variant of this approach was used by Lopez et al. (1999), who, instead of measuring investigation time, used a runway to test how fast male rats would run in order to investigate a receptive female, a non-receptive female or a male. Overall, a major advantage of using investigation as a measure of motivation is that it allows the assessment of sexual motivation of naïve subjects. However, a clear drawback is that investigation methods do not allow the subjects to actually gain access to and physically interact with the females, making them not entirely in line with our definition of sexual motivation.

In contrast to the investigation methods, the second category of methods does allow males to interact and copulate with receptive females, and uses various conditioned responses to study their motivation to do so. The simplest of these conditioned response methods is the runway method, which measures the increase in running speed towards a receptive female over successive trials (Sheffield et al. 1951; Beach & Jordan 1956). Another type of task using approach behavior is the obstruction method, in which subjects are required to endure electric shocks on their way to the female, measuring how many subjects endure it (Moss 1924) or how many times they do so (Stone et al. 1935). A somewhat more cognitively challenging task is the T-maze test, which measures the percentage of trials in which the males correctly choose the maze arm containing the receptive female and the males' running speed, and how both of these measures increase over successive trials (Kagan 1955; Whalen 1961). Another task relying heavily on learning is the lever pressing task, which requires rats to learn the association between pressing a specific lever and the presentation of a receptive female (Schwartz 1956; Jowaisas et al. 1971) or a conditioned stimulus associated with a receptive female (Everitt et al. 1987), with the rate of acquisition and performance of the lever pressing response reflecting the sexual motivation of the subjects. Finally, a more recent task for evaluating sexual motivation is "level searching", which measured the rate of level changes performed by a male in a bilevel chamber prior to the introduction of a receptive female, given past mating experience in that chamber (Mendelson & Pfaus 1989; Van Furth & Van Ree 1996). Unlike the other operant tasks, the level-changing behavior does not actually produce access to a female, since the female is presented after a specific amount of time regardless of the male's behavior. Nonetheless, this is a conditioned response and it was interpreted by the authors as being performed in order to gain access to a female (Mendelson & Pfaus 1989). Overall, one attribute almost all the operant

paradigms have in common is that the male performs a particular task in order to gain access to a female without actually being exposed to her. Therefore, such paradigms cannot discriminate between the motivation to copulate and the desire to merely be in the proximity of a female (this distinction is important because it was shown that male rats are willing to run in a runway in order to investigate a female without being able to actually copulate, Lopez et al. 1999). Another issue with testing sexual motivation without the presence of a female is that, as stated earlier, sexual motivation must be triggered by the appropriate incentive (or, in the words of Frank Beach: "The quasi-romantic concept of the rutting stag actively seeking a mate is quite misleading", 1956, p.5). Only two of the operant studies, both using lever pressing chambers, avoided this issue by keeping the female stimuli confined in the chambers, where the male subjects could see, hear and/or smell them (Jowaisas et al. 1971; Everitt et al. 1987). However, given the nature of the lever pressing task, investigation of the female actually conflicted with task performance, so the males had to learn to inhibit their instinctive approach behaviors in order to perform the task that would ultimately give them access to the female. This relates to another important issue, which is that the rate of performance on any operant task is dependent not only on the level of motivation but also on the strength of the association between the operant action and the outcome (Dickinson & Balleine 2002). The necessity of repeated experience of the consequences of the behavior (i.e. sexual interactions) prevents operant methods from testing the sexual motivation of sexually naïve animals. Moreover, it prevents the examination of sexual motivation towards females who are not receptive, since such females would not allow a sexual interaction to occur.

In this paper we demonstrate how our new method for measuring motivation can be used to study sexual motivation. Our method shares aspects with both the investigation methods and the

operant methods: as in the investigation methods, our subjects were allowed to investigate the female stimulus through a transparent, perforated barrier, but, in addition, the barrier was a high-resistance flap-door that the subjects could push in order to gain access to the female. The subjects' motivation was quantified by measuring the amount of pushing they performed on the door leading to the female, independent of investigation time. It is important to point out, however, that our task was not an instrumental task in the way such tasks are typically defined (involving a learned action-outcome contingency, e.g. Dickinson & Balleine 1994), since each subject was tested with a conspecific stimulus only once and thus they never had the opportunity to learn about the outcome of successfully getting in contact with the female stimulus. This allowed us to examine the sexual motivation of sexually naïve subjects and also to focus on the sexual motivation of males towards non-receptive females, which is one important difference between studying the sexual motivation of hamsters and that of rats.

Almost all of the studies of male sexual motivation described above have used rats as their model animal. In rats, the female exhibits "proceptive behavior" meant to attract the male, involving "ear-wiggling, darting and hopping" (Beach 1976). Sexually naïve male rats do not copulate with a female that is in estrus and exhibits lordosis but does not exhibit these proceptive behaviors (Beach 1942), suggesting a fairly low baseline level of sexual motivation. Sexual motivation in the golden hamster (*Mesocricetus auratus*), on the other hand, is an entirely different story. Although the findings as to whether or not male hamsters are capable of differentiating between the odors of estrus and diestrus females have been inconsistent (Landauer 1978; Johnston 1980; Huck et al. 1989), no studies have demonstrated preferences by male hamsters when tested with actual females (Johnston 1977; Landauer 1978; Johnston 1980; Carmichael 1980). Male hamsters have been found to pursue and attempt mating with intact females throughout their

41

estrus cycle (Payne & Swanson 1970; Takahashi & Lisk 1983) and they will even mount a docile male that had its tail smeared with vaginal secretions, regardless of whether the secretions were taken from a female in estrus or in diestrus (Lisk et al. 1972). Thus, according to Beach's suggestion that "a tendency to attempt coitus with partners other than the estrus female was indicative of a high level of sexual motivation" (Beach 1956, p.10), it seems that the sexual motivation of male hamsters is much higher than that of male rats. The other side of this high level of sexual motivation in male hamsters is the responses of female hamsters to males' pursuit when they are not receptive. Female hamsters exhibit high levels of aggression on their non-receptive days in response to both males and females (Payne & Swanson 1970). Since male hamsters are sexually interested in females throughout their estrus cycle, males do not initiate attacks on intact females regardless of their estrus state (Payne & Swanson 1970; Payne 1974). Therefore, most aggressive interactions between a male and a female are won by the female (Payne & Swanson 1970). It has been proposed that this is the way females communicate their lack of receptivity to males (Payne & Swanson 1970) and that this is how males learn to avoid females when they are not receptive (Carmichael 1980). It has been shown that under highly specific circumstances, males that got to interact with both estrus females and with diestrus females preferred the odors of the estrus female over the diestrus female (Johnston 1980; Huck et al. 1989). However, to the best of our knowledge, no attempts have been made to look at the effect of either of those experiences alone on sexual motivation towards non-receptive females, so we chose to address these questions here.

Materials and Methods

Overall Design

We used 3 behavioral groups to investigate the sexual motivation of male hamsters towards non-receptive females. The stimuli for all 3 sexual motivation groups were unfamiliar females in diestrus II (2 days after estrus). The first group consisted of 13 males with no sexual or aggressive experience, and will be referred to as group NNF (Naïve males tested with a Novel Female). The second group consisted of 12 males that lost a fight with a female (interaction with female in diestrus II), and will be referred to as group LNF (Losers tested with Novel a Female). The third group consisted of 12 males that got to mate (interaction with female in estrus), and will be referred to as group MNF (Mated subjects tested with a Novel Female).

Additional analysis was performed to investigate the general effects of losing a fight on subsequent motivation. For this analysis we used two groups from the previous chapter's aggressive motivation study: the naïve males (tested with a novel male), i.e. group NNM, and the losers that were tested with a novel male, i.e. group LNM. Here they were compared to the two corresponding sexual motivation groups (NNF and LNF). Finally, to get a better sense of the nature of the interest of male hamsters in diestrus females, we compared aspects of male-male fights to the male-female fights to determine whether these fights were qualitatively different.

The experimental design, apparatuses, and training & testing procedures were the same as those described in the previous chapter.

Animals

Subjects were male golden hamsters 3-4 months old from our laboratory colony (derived from Charles River stock), weaned at one month of age and housed individually in solid-bottom polycarbonate cages (45x25x16 cm) containing sani-chip bedding with food and water *ad libitum*. Animals were maintained on a reversed 14:10-h light/dark cycle. Subjects had no sexual

or aggressive experience prior to this experiment.

Female golden hamsters, 3-6 months old, were used as aggressive opponents or as mating partners. Additional females of the same age were used as stimuli in the motivation tests. All females were intact and naturally cycling; their estrus cycles were determined prior to the beginning of the experiment. Estrus tests were performed for all females in the colony by placing an older male in their cage once a day until lordosis was elicited (signifying estrus). Given golden hamsters' reliable 4-day cycle (Lisk, 1985), this allowed us to calculate the specific day of the cycle for each female. Females used for aggressive or sexual encounters did not have any prior aggressive or sexual experience, and each of those females was used only once. Since the females used for the mating experience were intact, most of them got pregnant as a result of that interaction, and were allowed to carry the pregnancy to term.

All research was conducted with approval from the Cornell University Institutional Animal Care and Use Committee.

Procedures

Aggressive encounters

Subjects in the female aggression condition went through a single encounter. A male and a female on diestrus-II were placed in the clean cage and allowed to interact with each other freely. These interactions lasted until the male lost the fight and fled by jumping out of the cage. Females typically win fights with males (Payne and Swanson, 1970), but to ensure the male always lost, the female opponent was always slightly bigger and older. If the female left the cage or no aggression was observed for 10 minutes then the female was switched for another one (this happened in 2 out of the 12 encounters).

The male-male fights used to contrast with the male-female fights were carried out in a manner

identical to the encounters described above. The only difference was that those fights were balanced, meaning that there was no age or size discrepancy between the male subjects, so the loser of the encounter was not pre-determined (it could have been either of the males).

<u>Sexual encounters</u>

A male subject and a female in estrus were placed together in a clean cage and allowed to mate over a single session. The sessions were not limited in time and lasted until one of the hamsters left the cage, similar to the aggressive encounters.

Data collection and analysis

Data collection from the training and motivation testing was the same as in the previous chapter. The scoring of the sexual encounters was complicated by the fact that sexually inexperienced male hamsters often mount from incorrect orientations (Lisk et al. 1972; Landauer et al. 1978) and they are also prone to "pseudo-intromit" without actual vaginal penetration (Rabedeau 1963). Therefore, we decided not to score the sexual encounters for mounts or intromissions. We did, however, note for each encounter whether or not the female attacked the male towards the end of the encounter.

Both the male-female fights and the male-male fights (from the aggressive motivation study) were scored for the same behavioral measures. These measures were: investigation time (defined as the hamster's nose pointing at its partner within a 1cm distance), both by the winner (always the female in the case of male-female fights) and by the loser (always the male in the case of male-female fights); the latency to initiate vigorous aggression (a "rolling fight", defined as rapid rolling with kicking and boxing, see Floody & Pfaff 1977 for a more detailed description); the total duration of vigorous aggression (rolling fights and chases combined); and the total duration of the encounter (from first contact until the loser jumped out of the cage).

Results

Where applicable, mean values are presented as M±SE. All statistical tests were performed using a significance threshold of $P < 0.05$.

Pushing ability

We evaluated the ability of the subjects in the 3 sexual motivation groups to get through a weighted door, using the latency measures taken for the final two steps of the training (with the door weighed at level 3). Using the shorter of these two latencies for each subject, we found that subjects varied greatly on their ability to perform the door-pushing task, with latencies ranging between 7sec and 122sec (M=45±4.7). Since the distribution of these values was somewhat skewed, they were log transformed (yielding M=1.56±0.05) and then these scores were used to test whether the males from the 3 groups differed in their pushing ability. A one-way ANOVA did not detect a significant difference between the groups on this measure, $F_{2,34}=1.567$, $P=0.22$, so it was not used in further analysis.

Qualitative analysis

As a secondary comparison of subjects' ability to get through the doors, we examined the number of subjects that successfully got into either goal box during the motivation test and compared this to the number of subjects that pushed hard (angle greater than 0.5 radians at least once) towards either goal box but did not manage to get into either of them. The number of subjects in each of these categories for each of the 3 sexual motivation groups is shown in table 2.1. Using an exact binomial test for each of the 3 groups showed that the number of subjects that got in and the number of subjects that tried but did not succeed was not significantly different for any of the groups. Fisher's exact test showed no significant difference on this

measure between the groups ($P=1$).

Table 2.1

Group	Got in	Pushed hard
NNF	4	8
LNF	3	4
MNF	4	6

Number of subjects that got into either goal box ("got in") or tried to get into either goal box but didn't manage to ("pushed hard"). NNF – naïve subjects tested with a novel female (13 subjects); LNF – losers to a female tested with a novel female (12 subject); MNF – mated subjects tested with a novel female (12 subjects).

Next, we compared the number of subjects that tried getting into each of the goal boxes for each of the 3 groups (see table 2.2). Exact binomial tests for each group between the number of subjects that tried to get into the stimulus goal box and the number that tried to get into the empty goal box showed a highly significant difference for the naïve subjects ($P=0.0005$) but no significant difference for either of the other groups ($P=0.13$ and 0.34 for groups LNF and MNF respectively). Fisher's exact test showed a nearly significant difference between the 3 groups on the distribution of the number of subjects that tried getting into the stimulus goal box, the number that tried getting into the empty goal box, and the number of subjects that did not try for either, $P=0.054$.

Table 2.2

Group	Stimulus	Empty	Neither
NNF	12	0	1
LNF	6	1	5
MNF	7	3	2

Number of subjects that were interested in getting into the stimulus goal box, empty goal box, or neither of them. NNF – naïve subjects tested with a novel female (13 subjects); LNF – losers to a female tested with a novel female (12 subjects); MNF – mated subjects tested with a novel female (12 subjects).

Quantitative analysis

We compared the performance of the 3 sexual motivation groups on our 3 behavioral measures: the investigation measure (the percentage of the trial spent near the door without pushing it) and the two door-pushing measures (the percentage of the trial spent pushing the door and the mean angle of the door while being pushed). We looked only at investigation and pushing towards the

stimulus goal box, because none of the naïve subjects pushed towards the empty goal box (see figures 2.1b and 2.1c).

For each of the 3 behavioral measures we first ran a one-way ANOVA to compare the 3 groups. If there was a significant difference between the groups, we followed it with t-tests for the two a priori comparisons, between the naïve group and each of the other two groups (NNF&LNF and NNF&MNF), using the Bonferroni adjusted alpha levels of 0.025 per test.

The percentage of time that males spent investigating the female stimulus did not differ between the 3 groups, $F_{2,34}=0.09$, $P=0.92$ (see figure 2.1a), so this measure was not investigated further. The percentage of the trial spent pushing, however, did differ significantly between the 3 groups, $F_{2,34}=3.7$, $P=0.035$. Post-hoc comparisons showed that the naïve males spent a larger percentage of the trial pushing towards the female stimulus (M=0.52±0.05) than either losers (M=0.31±0.07) or mated subjects (M=0.32±0.07), t_{23} = 2.55 and 2.44 respectively, $P=0.018$ and 0.023 respectively (see figure 2.1b). Finally, the mean door-pushing angle did not differ significantly between groups, $F_{2,34}=1.68$, $P=0.2$, but it did show a slight trend in the expected direction, with naïve subjects (M=0.26±0.02) pushing slightly harder than the losers (M=0.2±0.03) and the mated subjects (M=0.19±0.03) (see figure 2.1c).

To find out why mating caused decreased sexual motivation on one of our motivation measures we investigated whether the fact that half of the males that mated were attacked by the female at the end of their encounter could have been a contributing factor. We found that mated subjects that were not attacked spent a significantly larger percentage of the trial pushing for the female stimulus than the mated subjects that were attacked, $t_{10}=2.73$, $P=0.021$, although they did not push harder, $t_{10}=1.03$, $P=0.33$.

These findings suggest that the decline in sexual motivation found for the mated group could be

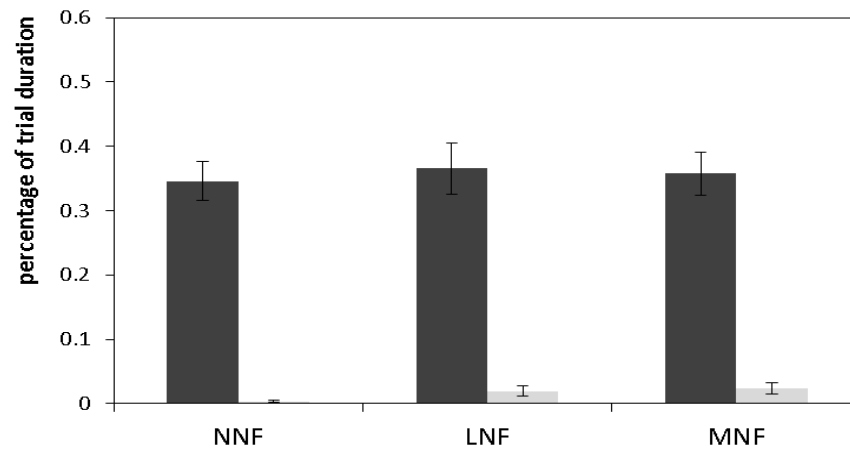Figure 2.1a: the percentage of the motivation trial spent investigating each door.

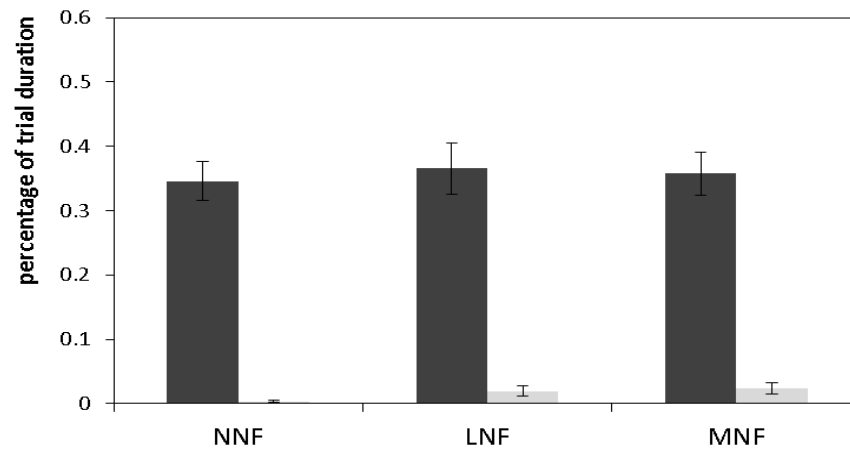Figure 2.1b: the percentage of the motivation trial spent pushing each door.

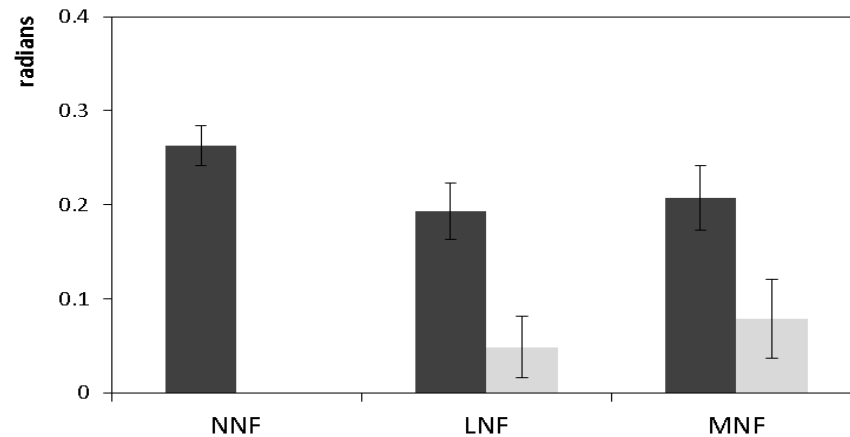Figure 2.1c: the mean angle of each door while being pushed.

Figure 2.1: dark grey bars – stimulus goal box; light grey bars – empty goal box; NNF – naïve subjects tested with a novel female; LNF – losers tested with a novel female; MNF – mated subjects tested with a novel female.

attributed to the attacks experienced by half of the mated subjects. To test this hypothesis we used a two-way ANOVA on the 3 behavioral measures for the 3 groups. For each of the 3 behavioral measures we tested for a potential effect of mating (independent of being attacked), a potential effect of being attacked (independent of mating, for both the LNF group and the attacked subjects from the MNF group), and a potential interaction between mating and being attacked (testing whether the effect of being attacked was different depending on whether it came at the end of a mating session or whether it was a fight with a diestrus II female). Being attacked caused a significant reduction in the percentage of the trial spent pushing for the female stimulus, $F_{1,34}=13.37$, $P=0.00085$, but mating alone did not have a significant effect, $F_{1,34}=1.9$, $P=0.18$ and the interaction was not significant, $P=0.49$ (see figure 2.2a). Moreover, this analysis revealed that being attacked did cause a significant decrease in the mean angle of the door while pushing, $F_{1,34}=4.63$, $P=0.039$, but once again there was no significant effect of mating, $P=0.55$, and no significant interaction, $P=0.99$ (see figure 2.2b).

Comparison of sexual and aggressive motivations

In order to further investigate the effect of losing to a female, we used two of the groups from the sexual motivation study, groups NNF and LNF, together with the 2 corresponding groups from the aggressive motivation study – NNM (naïve subjects tested with novel male stimuli) and LNM (losers to males tested with novel male stimuli). First we verified that subjects from the two studies did not differ significantly on pushing ability by comparing their log-transformed pushing ability measure; a one-way ANOVA between the 4 groups yielded $F_{3,45}=0.29$, $P=0.83$.

We used a two-way ANOVA to examine the effect of being tested with a female compared to being tested with a male, the effect of losing a fight, and whether there was an interaction between the two factors (a difference in the effect of losing to a male versus losing to a female).
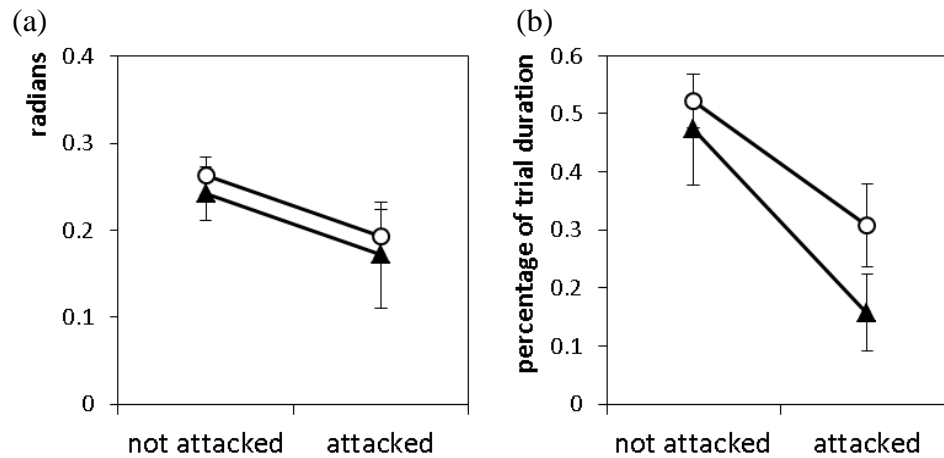
Figure 2.2: open circles – subjects without mating experience; black triangles – subjects with mating experience. (a) percentage of the trial spent towards the stimulus; (b) mean angle of the door while it was being pushed.
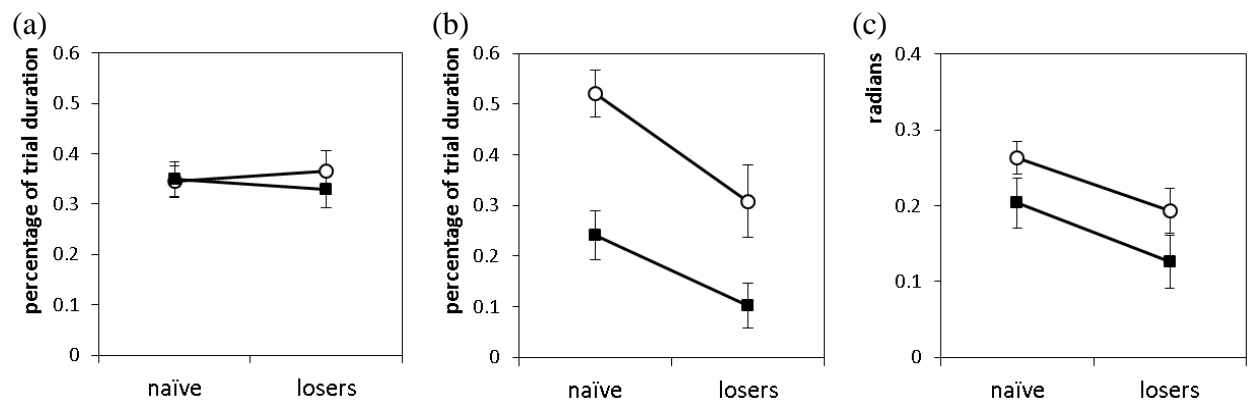

Figure 2.3: open circles – subjects tested with a female; black squares – subjects tested with a male. (a) percentage of the trial spent investigating the stimulus; (b) percentage of the trial spent towards the stimulus; (c) mean angle of the door while it was being pushed.

Interestingly, when we examined the percentage of the trial spent investigating the stimulus, this measure was not affected by whether the stimulus was male or female, $F_{1,46}=0.22$, $P=0.64$, or by the experience of losing, $F_{1,46}=0.00001$, $P=0.997$, and the interaction was not significant either, $P=0.57$ (see figure 2.3a). The same two-way ANOVA analysis on the door-pushing measures did identify significant effects of the factors tested (male/female, losers/naïve). Being tested with a female stimulus resulted in a significantly higher percentage of the trial spent pushing towards the stimulus, $F_{1,46}=21.19$, $P=0.000032$, while losing resulted in a significant decrease on this measure, $F_{1,46}=11.09$, $P=0.0017$, and once again there was no significant interaction, $P=0.49$ (see figure 2.3b). Finally, being tested with a female resulted in an only marginally larger mean door angle, $F_{1,46}=3.38$, $P=0.072$, while losing still resulted in a significant decrease on this measure, $F_{1,46}=7.12$, $P=0.01$, with the interaction being highly non-significant, $P=0.91$ (see figure 2.3c).

Comparisons between male-female fights and male-male fights

To verify that males' motivation towards diestrus II females was in fact sexual and not aggressive, despite the fact that the decrease in motivation they showed due to losing was identical to what was found in losers of a male-male fight that were tested for aggressive motivation, we compared male-male encounters to aggressive male-female encounters to determine whether they are in fact qualitatively different. Except when said otherwise, all following comparisons were performed using the Welch-modified t-test, since the equal variance assumption could not be made. Starting from the overall encounter durations, we found that male-female encounters were significantly longer (M=452.6±43.9) than male-male encounters (M=161.4±33.2), $t_{20.48}=5.29$, $P=0.00003$ (figure 2.4a). This may be in part due to the fact that the initiation of a rolling fight took significantly longer in male-female encounters (M=260.25±35.75) than in male-male encounters (M=72.25±14), $t_{14.29}=4.9$, $P=0.0002$ (figure
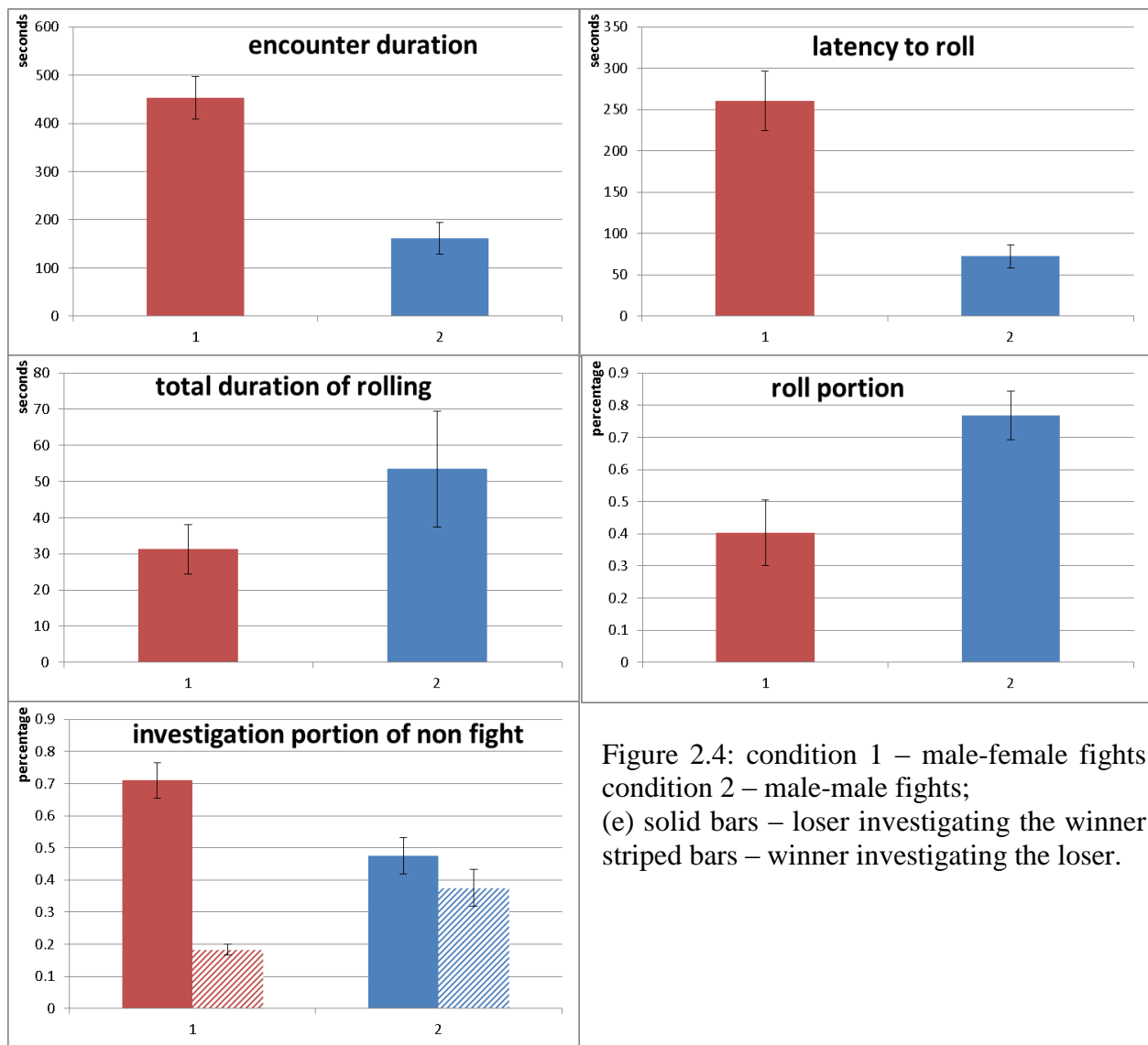
Figure 2.4: condition 1 – male-female fights; condition 2 – male-male fights;
(e) solid bars – loser investigating the winner; striped bars – winner investigating the loser.

2.4b). The total time spent in a vigorous fight did not differ significantly between the two conditions, $t_{14.9}=1.28$, $P=0.22$, although the trend was actually for less vigorous fighting in male-female encounters (M=31.25±6.84) than in male-male encounters (M=53.5±16) (figure 2.4c). Correspondingly, the percentage of the time spent fighting vigorously from the initiation of fighting was significantly lower in male-female encounters (M=0.4±0.1) than the male-male encounters (M=0.77±0.08), $t_{20.27}=2.88$, $P=0.009$ (figure 2.4d). Finally, we examined the non-aggressive interest of the subjects in their opponents and vice versa, by analyzing the time that they spent investigating one another as a percentage of the portion of the encounter that was not spent fighting (i.e. investigation time divided by (encounter duration minus fight duration)). We found that the investigation by the losers was significantly higher when the encounter was with a female (M=0.71±0.056) than with a male (M=0.48±0.057), $t_{22}=2.93$, $P=0.008$. We also found that the investigation by the opponent was significantly lower when the opponent was a female (M=0.18±0.017) than when it was another male (M=0.38±0.058), $t_{12.9}=3.18$, $P=0.007$ (figure 2.5). When comparing the percentage of the encounter that the loser spent investigating the winner to the winner's investigation of the loser for each of the two fight conditions, we found that in the male-female condition the difference was highly significant, $t_{13.1}=9$, $P<0.000001$, while in the male-male condition it was not significant, $t_{22}=1.23$, $P=0.23$. A two-way ANOVA verified that there was a significant interaction between status (winner/loser) and fight type (male-male/male-female), $F_{1,44}=18.15$, $P=0.0001$.

Discussion

Previous studies have shown that sexually naïve male hamsters do not discriminate between females who are in estrus and females who are diestrus (Johnston 1977; Landauer 1978;

Carmichael 1980; Johnston 1980), and thus are sexually attracted to and attempt to mate with females regardless of their receptivity (Payne & Swanson 1970; Takahashi & Lisk 1983). Using our new motivation method we were able to show that sexually naïve males are not only interested in spending time investigating diestrus females through a perforated barrier, but are also willing to exert considerable effort in order to interact with them. This is of particular interest since diestrus female hamsters do not allow males to mate with them and they are even prone to be aggressive towards conspecifics (Payne & Swanson 1970).

Taken together with the finding that non-receptive female hamsters typically avoid males by hardly approaching them and by withdrawing whenever approached (Steel 1979), our findings on the differences between male-male fights and male-female fights support the idea that male-female fights result from a combination of high sexual interest by the male and failure of avoidance by the female. Similar to the findings by Payne & Swanson (1970), we found that in male-female fights the males spent most of their time investigating the females and following them, while the females showed very little interest in the males and mainly tried to avoid them. Such a discrepancy was not found in male-male fights, where both winners and losers spent about half of the non-fighting time investigating their opponent (see figure 5). Accordingly, during encounters between a male and a diestrus female it took significantly longer for fights to develop than in encounters between pairs of males, since male hamsters are inhibited from attacking intact females (Payne & Swanson 1972), whereas the females appeared to attack primarily because of their failure to avoid the males (a couple of females actually climbed out of the encounter cage rather than initiate a fight). This interpretation of the cause for male-female fights is supported by Johnston's 1980 finding of aggression being very infrequent in encounters between males and diestrus females, since the fighting arena used in that study was about 4 times

larger than the cages used for fighting by Payne & Swanson (1970) and in the current study. Other studies of male-female fights did not use neutral arenas and thus are not comparable. Even after a rolling fight was initiated there was an important difference in the manner in which the encounter progressed – although the total duration of active aggression was not significantly different between the two types of fights, this aggression was far more fragmented in the male-female fights, meaning that the aggression ceased during the encounter without the male fleeing the cage. Together with the longer attack latency, male-female fights were about 3 times longer than male-male fights. Therefore we conclude that, although females are more aggressive than their male opponents in male-female fights, this is because the males are interested in sex rather than a fight, and are only aggressive in response to the females' attacks. When comparing the behavior of the females in such fights to the behavior of the male winners in male-male fights it is clear that females are in fact not very aggressively motivated, and their aggression is mostly meant to convey their lack of receptivity (as suggested by Payne & Swanson 1970).

Previous studies on sexual attraction of male hamsters have been primarily focused on the males' ability to detect females that are receptive, investigating the effects of experience (sexual or aggressive) only to find how it affects that ability (e.g. Landauer et al. 1978; Carmichael 1980; Johnston 1980). Unlike these studies, we investigated the effects of sexual or aggressive experiences on sexual motivation towards diestrus females directly, rather than the ability of males to discriminate between estrus and diestrus. When comparing our naïve males to males that had either a single mating interaction or lost a single fight with a female, we found that both groups showed lower sexual motivation than the naïve subjects on our pushing duration measure. This result was noteworthy for the sexually experienced group, since prior studies have shown that sexual experience does not result in avoidance of diestrus females (e.g. Landauer et al. 1978;

56

Carmichael 1980, with the former study finding that sexual experience increased the males' interest in both estrus and diestrus females) and fatigue is unlikely to play a role 24 hours after the sexual encounter. However, upon further examination of the sexual encounters we found that half the mated subjects were attacked by the female at the end of the encounter, leading to a brief rolling fight, while the other half of the mated subjects were not attacked. These two subgroups differed significantly on the pushing duration measure but not on the pushing angle measure. This suggests that the post-mating attacks may be the source of the difference between the naïve controls and the entire mated group, since those groups also differed only on the pushing duration measure. When we teased apart the effect of mating and the effect of being attacked using a two-way ANOVA, we found that mating experience on its own indeed had no significant effect on sexual motivation. The brief rolling fight at the end of the sexual experience led to the exact same reduction in sexual motivation that an entire fight with a female in diestrus did, which is somewhat surprising. This lack of a clear difference between the effect of being attacked by a diestrus female and the effect of being attacked by an estrus female on the males' sexual motivation towards a diestrus female supports the idea that any decrease in that motivation was unlikely to be due to an ability to discriminate between estrus and diestrus females.

The decrease in sexual motivation (identified by both pushing measures) resulting from an attack by a female was not surprising, although this may be the first study to clearly demonstrate such an effect. The two previous studies that tested male hamsters that interacted with diestrus females did not compare them to naïve males (Johnston 1980; Huck et al. 1989), since the focus of these studies was to look for estrus discrimination in male hamsters. Moreover, the subjects in these studies went through several encounters with diestrus females in addition to several sexual

experiences, so neither of the studies was attempting to address the effect of a single aggressive encounter with a female (Johnston 1980; Huck et al. 1989). To further investigate why being attacked by a female, regardless of context, resulted in a decrease in sexual motivation, we compared our findings on losing and sexual motivation to our findings from the previous aggression chapter. That comparison highlighted the fact that although the experiences of males that lose to a female are very different from the experiences of males that lose to another male (as discussed earlier), both of these experiences produced the exact same reduction in both of our pushing measures (see figure 2.3 b&c), despite the fact that in one case the pushing measures were representing sexual motivation and in the other case they were representing aggressive motivation. Thus we conclude that for males, being attacked by a conspecific of either sex results in a reduction in their motivation towards conspecifics of that sex, likely through a similar stimulus-devaluation mechanism. Nonetheless, our comparison between sexual and aggressive motivations did identify a clear difference between them, namely that sexual motivation was significantly stronger than aggressive motivation and that the difference in magnitude was the same for both naïve subjects and for subjects with the respective losing experiences. The finding that for male hamsters a female is a more attractive incentive than a male was expected, since it has already been shown that even naïve male hamsters have a clear preference for a female stimulus over a male stimulus (Landauer et al. 1978; Ballard & Wood 2007), regardless of whether the female was in estrus or in diestrus (Landauer et al. 1978). There was, however, a major difference between our design and the previous studies, namely that in these studies the subjects were presented with both male and female stimuli at the same time (Landauer et al. 1978; Ballard and Wood 2007), whereas we tested our subjects either with a male stimulus or with a female stimulus. This is crucial, since in the aggression study many of the naïve males

were actively interested in interacting with the male stimuli, exhibiting clear aggressive motivation. Thus, we conclude that not only do male hamsters prefer to interact with a female over interacting with a male, but moreover that, while they show both sexual and aggressive motivation, their sexual motivation is significantly greater than their aggressive motivation. Moreover, this difference in magnitude between the two motivations is maintained even after the respective stimulus (either male or female) is devalued through the corresponding losing experience.

The unique properties of our method for assessing motivation yielded an additional unexpected finding – the amount of time that a subject spent investigating the stimulus animal was not influenced by the different experiences that the subjects had or even by the sex of the stimulus animal. This finding is obviously not directly comparable to any studies in which investigation was the only measure of motivation, but it does raise an important concern regarding the validity of using investigation time as a measure of motivation. In our study, we suspect that investigation was used by the subjects only to get a sense of what the stimulus was (general interest), but the motivation itself was expressed by the decision of how much to push the door in response to that stimulus. Thus, a male stimulus is not necessarily less interesting than a female stimulus, it just elicits less desire for actual physical interaction. This distinction is something that methods using permanence near the stimulus as their "active approach" behavior could not make. However, the fact that males do show a clear difference in investigation time of males and females when tested with such methods (e.g. Hetta & Meyerson 1978; Vega Matuszczyk & Larsson 1993; Ågmo 2003; Ballard & Wood 2007) suggests that in the absence of a means to get to interact with the stimulus, the increased motivation would get translated into an increase in the time spent in proximity to the stimulus. Nevertheless, using investigation time as a measure of

motivation may not be sensitive enough to detect variations in magnitude, and it seems to be primarily used to test whether sexual motivation is present or absent (e.g. Ågmo 2003; Ballard & Wood 2007).

Finally, it is essential to discuss the differences between conditioned and unconditioned motivation and how the methods used to study them affect the phenomenon actually being addressed. There does not seem to be a single definition of what "motivation" really is, but Beck (1978) provided a description approximating a definition, stating that "Motivation is broadly concerned with the contemporary determinants of choice (direction), persistence, and vigor of goal-directed behavior" (Beck 1978, p. 24). We believe that our method satisfies all of these requirements, given that the animals are free to choose to push either of the two identical doors in the apparatus (direction), they can push for varying amounts of time (persistence) and they can push at varying degrees of force (vigor). What makes our method qualitatively different from other methods for measuring motivation, however, is how the goal of such behavior is defined. Dickinson and Balleine (1994) have discussed this topic, and suggested that "By characterizing an action as being 'directed' at a goal, we mean that performance is mediated by knowledge of the contingency between the action and the goal or outcome […] Our conception of goal directedness also requires that the outcome of the action should be represented as a goal for the agent at the time of performance" (Dickinson & Balleine 1994, p. 1). These authors go on to argue that behaviors that are unconditioned, such as approach towards reward cues, are not "goal oriented" since such actions do not reflect an action-outcome contingency but rather a "Pavlovian control of approach to stimuli associated with the outcome" (Dickinson & Balleine 1994, p. 2). This is where our conceptualization of motivation differs, since we believe that exerting great effort in order to get into contact with a stimulus, without this action ever having

been associated with the stimulus or with any outcome of an interaction with the stimulus under these circumstances, is still a form of goal-oriented behavior. Nevertheless, this type of action clearly belongs to an entirely different category of behavior than conditioned motivation. This is not only because the behavior itself does not rely on any previously-learned action-outcome contingency, but, since our subjects only get tested once, there was no way for them to know what the outcome of getting into the stimulus goal box would be. As far as we know, our paradigm is the first to assess the behavior of gaining access to a goal object in an unconditioned setting. We propose that this approach is particularly suitable for the study of sexual motivation, as opposed to the more commonly studied ingestive motivational systems.

As stated in the introduction, an important distinction between sexual motivation and motivations towards food or water is that there is no physiological state of being "sex-deprived", and unlike with hunger or thirst, there is no way for this state to cause any physical discomfort (e.g. Beach 1956). This makes sexual motivation a far more distinct case of incentive motivation, since its emergence relies exclusively on the external stimulus. This means that in contrast to the various procedures used to create a state of need in the study of ingestive motivations, sexual motivation cannot be elicited independently of the sexual incentive. Thus, the sexual motivation exhibited by a male performing an instrumental action to gain access to a female must depend heavily on the saliency of the male's mental representation of a female. This is likely aided by the Pavlovian association between the various apparati used for such tests and the sexual experiences (as proposed by Pfaus et al. 2001 and Ågmo 2003), since we have no way of knowing whether rodents are even capable of an entirely self-generated mental representation. Consequently, the strength of motivation exhibited by males in instrumental paradigms corresponds both to the strength of the learned action-outcome contingency, and on the saliency

of the mental representation of the female. Rats can still be trained to perform various instrumental tasks despite these complications (Sheffield et al. 1951; Kagan 1955; Beach & Jordan 1956; Schwartz 1956; Whalen 1961; Jowaisas et al. 1971), even under a second-order reinforcement schedule (Everitt et al. 1987), although it is unclear to what degree the rats' level of performance reflects their "true" levels of sexual motivation. In contrast, our task was designed to be as straightforward as possible in order to reflect the most undiluted levels of sexual motivation possible – the only learning it requires is that doors could be pushed to get to through them, which takes a very minimal amount of training[6]. The fact that our subjects were directly exposed to the female ensures the highest possible levels of saliency (e.g. Toates 2009, suggests that sexual motivation increases as the distance from the female decreases); the direct relationship between our pushing task and access to the female eliminates the need for the subjects to learn that contingency. Additionally, the potent unconditioned incentive properties of female stimuli allow this method to tackle motivation entirely devoid of a representation of an outcome, showing that sexual experience is not required for the expression of sexual motivation (unlike what was suggested by Pfaus et al. 2001). Motivations towards ingestable incentives may simply be unable to approach that type of question.

In conclusion, studying unconditioned sexual motivation in male hamsters provides a unique opportunity – not only are they far more sexually motivated than rats are, but they rely far less on conditioning for their sexual performance (Ballard & Wood 2007). Moreover, the fact that male hamsters lack the ability to discriminate between estrus and diestrus females provides an

---

[6]   We highly doubt that the training with the sunflower seeds was a significant contributor to the pushing towards the stimulus, not only since the training was minimal and took place in a different apparatus, but also because the subjects were not even attracted much to the sunflower seeds during training. It is possible that the pushing towards the empty goal box was driven by the training, but given the high potency of a female as a stimulus, causing a huge difference between the interest in the stimulus goal box and the empty one, it seems unlikely that a general conditioning of "pushing doors is good" factored into our motivation assessment.

interesting case of an apparent outcome-insensitivity, while remaining sensitive to stimulus-devaluation (as we have shown in subjects who were attacked by females). We believe that this new avenue of study could further our understanding not only of sexual motivation, but of the nature of motivation at large.

Chapter 3: comparing the neural activation in male-male vs. male-female fights

Introduction

Understanding the way in which activity of neural regions translates into behavior has been one of the main goals of the field of neuroscience, and when it comes to a framework for the neural mechanisms underlying social behavior, one of the most commonly accepted views is the "Social Behavior Network" (Newman, 1999). This theory proposes that rather than having specialized brain regions each of which controls one aspect of social behavior, such behaviors are instead governed by a set of overlapping regions that comprise the "social behavior network" (SBN). The SBN regions include the medial extended amygdala (including the BNST), lateral septum (LS), medial preoptic area (MPO), anterior & ventromedial nuclei of the hypothalamus (AH&VMH), and the midbrain periaqueductal gray (PAG). All of these areas are reciprocally interconnected, and each was found to play a key role supporting at least two different social behaviors (Newman, 1999). The theory suggests that it is the pattern of relative activation throughout that entire network of regions that determines the specific behavior produced by the individual, with a distinguishable pattern for each social behavior that changes dynamically as various behaviors follow one another (Newman, 1999). This idea has never been directly evaluated, however.

The foundation for the SBN theory relies on studies that have focused on the main social behaviors that mammals engage in – sexual behavior (in males and in females), aggression (mostly inter-male), and maternal behavior. Direct support for that theory requires a direct comparison of the neural activation associated with two distinct social behaviors across the SBN regions. Two such studies concentrating on male social behavior have utilized immediate early gene (c-Fos) expression following either mating or inter-male aggression (Kollack-Walker &

Newman, 1995; Veening et al., 2005). Kollack-Walker & Newman (1995) used golden hamsters and found overlapping activation in the medial amygdala, LS, VMH, most of the BNST, and several other non-SBN regions, while finding that the MPOA (plus one of the BNST sub-nuclei) was activated only by mating and the AH and PAG (plus another of the BNST sub-nuclei) were only activated by fighting. Veening et al. (2005) used rats and thus focused on a somewhat different assortment of sub-regions in the amygdala, BNST and hypothalamus, yielding a significant difference in activation between mating and fighting in almost all the investigated regions (the few exceptions included a sub-region of the medial amygdala and a sub-region of the MPOA), yet the authors still interpreted their results as supporting a partial overlap in the neural mechanisms underlying the two behaviors. Overall the results of these studies are consistent with the idea of similar yet distinct patterns of neural activation for different social behaviors, though the role played by the overlapping mechanism is a little hard to interpret since the behaviors composing male sexual behavior are quite different from the ones involved in aggressive behavior.

The current study was designed to test the SBN theory more directly, utilizing the aggressiveness of non-receptive female hamsters towards males (see discussion in previous chapter), by comparing the neural activation of males who fought and lost to a female to the neural activation of males who fought and lost to another male. Those two conditions are behaviorally comparable since male-female fights include the same behavioral components as male-male fights (Payne & Swanson, 1970; Previous chapter), although cognitively the experiences are expected to be quite different between interacting with a female and with a male. The neural activation associated with a male-female agonistic interaction has not been previously investigated, as far as we know, so we used c-fos immunoreactivity to assess it, in the SBN regions comparable to the ones

assessed by Kollack-Walker & Newman (1995), whose study also used male golden hamsters. We hypothesized that if the neural mechanisms underlying sex and aggression, or more specifically sexual and aggressive motivation, share some key features, then comparing them in a situation in which the overt behaviors are highly comparable should highlight those commonalities. Ultimately, finding that the neural activation pattern associated with losing to a female should falls somewhere in between the activation patterns associated with male-male aggression and with male sexual behavior would provide the first direct evidence to support the SBN theory.

Materials and Methods

Animals

Subjects were male golden hamsters 3-7 months old from our laboratory colony (derived from Charles River stock), weaned at one month of age and housed individually in solid-bottom polycarbonate cages (45x25x15 cm) containing sani-chip bedding with food and water *ad libitum*. Animals were maintained on a reversed 14:10-h light/dark cycle. Due to subjects availability we had to use a few subjects with prior aggressive experience, but in all of those cases the experience was minimal and occurred at least a month prior to the current study.

Male and female opponents were also 3-7 months old, age-matched to the subjects. Females were on diestrus-2, with their estrus timing determined prior to the current study by using a stud male to elicit lordosis (a procedure used for all female hamsters in the lab).

Fighting arena

All aggressive encounters took place in neutral clean cages with clean bedding, identical to that used in the hamsters' home cages (45x25x15 cm). There was no top on the cage during the encounters so that the loser could escape by jumping out.

Procedure

Behavioral

Subjects were assigned either to a male-male fight condition (n=7), a male-female fight condition (n=7) or a control condition (n=6). All experiences were carried out during the first half of the hamsters' dark cycle, under dim light. To begin the experiment, subjects were placed in a neutral cage, divided by a removable wire mesh. For the two fight conditions, an opponent (male or diestrous female) was placed on the other side of the mesh divider. The divider was removed as soon as the two hamsters faced each other across the mesh, which was when the fight timing was initiated. Fights lasted until one of the hamsters escaped from the cage, and that hamster was the subject of the following neural analysis. Since this study focused on neural activation in males, in the case of male-male fights either male could have lost and thus become the subject, while in the case of male-female fights we relied on the finding that males typically lose such interaction (Payne & Swanson, 1970), although in one of the 7 pairs the male actually won and thus was removed from the analysis. The control group was exposed to the same conditions as the fighting groups only without an opponent, meaning that they experienced handling, exposure to a clean cage, and moved freely in that cage until they climbed out of it, thus controlling for the non-social factors of the design.

Following the behavioral experience, all subjects were promptly returned to their cages, which were placed in a dark room until the neural processing phase.

Neural

An hour following the end of the behavioral phase, subjects were deeply anesthetized using 2.5cc-5cc of 30% urethane (dosage estimated depending on the size of the hamster), and then were perfused using a 9% saline solution followed by a 4% paraformaldehyde solution. Brains

were then extracted and post-fixed in 4% paraformaldehyde for an hour, followed by a 30% sucrose solution for at least 24 hours (until sinking).

For immunohistochemistry processing, brains were first frozen and sectioned into 40μm slices using a microtome, and every third slice was placed free-floating in staining trays (Nason Machine, Fort Bragg, CA) filled with KPBS solution. To stain for c-Fos activation, the slices were washed in KPBS before and after being incubated in 20% normal goat serum (Vector Laboratories, CA), and were then incubated in primary antibody solution (1:1000 rabbit polyclonal antiserum, SC-253, Santa Cruz Biotechnology, CA) for 69 hours on a shaker at 4ºC. The sections were then washed again in KPBS before being labeled using biotinylated goat antirabbit secondary antibody (1:500, Vector Laboratories) for 1 hour, and were subsequently washed in Tris solution followed by 1 hour incubation in avidin–biotin complex (ABC kit, Vector Laboratories). Finally, labeling was visualized using DAB solution (SIGMAFAST™, Sigma) for 7 minutes, preceded and followed by washes in the Tris solution.

The stained slices were mounted on gelatin-coated slides, air-dried and coverslipped.

Data-acquisition

Behavioral

Fight durations were measured using a timer. Additionally, the fights were recorded using a Sony DCR-TRV900 digital video camera. The fights were scored using a stopwatch for the total time the subject (loser) spent investigating his opponent (defined as the hamster's nose pointing at its partner within a 1cm distance), the total duration of rolling fight (defined as rapid rolling with kicking and boxing, see Floody & Pfaff 1977 for a more detailed description), and the total duration of chasing (the loser being chased by the winner, following rolling fights).

Unfortunately, one of the fight tapes, including 2 male-male fights and 2 male-female fights was lost, so for those fights we did not have the detailed behavioral analysis data, only the total duration of the encounters.

<u>Neural</u>

Since the purpose of this study was to address the social neural network hypothesis, the analysis focused on the regions proposed to underlie that network (Newman, 1999):

* Medial amygdala (4 subdivisions: MeAV, MeAD, MePV, MePD),

* Bed nucleus of stria terminalis (5 subdivisions: BSTam, BSTai, BSTav, BSTpm, BSTpi),

* Lateral septum (the ventral subdivision LSv, which was the only one included in the Kollack-Walker & Newman 1995 study),

* Medial preoptic area and medial preoptic nucleus,

* Anterior hypothalamic nucleus,

* Ventromedial nucleus of the hypothalamus,

* Midbrain periaqueductal gray (2 dorsal subdivisions: DMPAG and DLPAG).

Stained slices containing the regions of interest were selected based on landmarks and with guidance from the Hamster Brain Atlas (Morin & Wood, 2001). Five slices were chosen for analysis for each subject: Bregma 0.8mm (for BSTam, BSTai, BSTav, LSv), Bregma 0.2mm (BSTpm, BSTpi, MPN, MPO), Bregma -0.9mm (MeAV, MeAD, AH), Bregma -1.8mm (MePV, MePD, VMH), and Bregma -4.6mm (DMPAG, DLPAG). Slides were viewed using a Nikon eclipse microscope with a $10\times10\text{mm}^2$ square grid eyepiece reticle, at 20x magnification (such that each grid square cover a surface of 0.0025mm^2). For each region, stained neurons were counted blind to the experimental condition, once for each subject within a small sample area guaranteed to be within the boundaries of the region: 10 grid squares (for BSTpi, BSTpm, and

LSv), 12 squares (MPN), 20 squares (VMH) or 6 squares (all other regions). For standardization purpose, an activation density measure was obtained by dividing the number of stained neurons counted for each region by the surface area counted, so all activations levels are expressed as #/mm^2.

Results

Behavioral

The distributions of encounter durations and their components for the male-male and male-female fight conditions are shown in table 3.1. Due to the small sample sizes and severely skewed distributions of the measures, they were log-transformed for the analysis.

As shown in the previous chapter, male-male fights were once again much shorter than male-female fights (P=0.03 for the log-transformed values), and male losers investigated a female opponent significantly more than they investigated a male opponent (P<0.005). However, the two groups did not differ either on rolling duration or on chase duration.

Table 3.1

	Male-Male		Male-Female		P-value
	Mean	SD	Mean	SD	
Encounter duration	226.71	202.05	593.67	345.64	
log-transformed	2.19	0.39	2.68	0.29	0.03
Investigation duration	49.00	24.99	534.25	261.57	
log-transformed	1.63	0.24	2.65	0.28	0.002
Rolling duration	51.40	67.00	74.25	62.55	
log-transformed	1.44	0.43	1.66	0.45	NS
Chase duration	7.00	1.9	5.75	5.21	
log-transformed	0.83	0.12	0.60	0.42	NS

Durations (in seconds) of encounters and their components, for comparison between male-male fights and male-female fights. Note that the encounter durations are for 7 m-m fights and 6 m-f fights, while the components are only for 5 m-m and 4 m-f of them. The P-values are from Welch-modified t-test for unequal variances.

Neural

<u>Comparison between 3 groups</u>

Table 3.2

Region	Controls	Losers to Males	Losers to Females	P-value
Lateral septum				
LSV	353 ± 143.6	886 ± 196.6	667 ± 206.7	
BNST				
BSTAM	244 ± 78.3^a	1067 ± 256.6^b	$667 \pm 233.5^{a,b}$	0.044
BSTAI	156 ± 93.8	1038 ± 346.4	844 ± 247.5	0.077
BSTAV	167 ± 70.4^a	657 ± 124.7^b	633 ± 105.8^b	0.008
BSTPM	93 ± 36.8^a	$789 \pm 250.2^{a,b}$	1200 ± 237.1^b	0.007
BSTPI	67 ± 26.7	314 ± 99.3	220 ± 59.1	0.081
Amygdala				
MeAV	711 ± 224.2	1200 ± 258.6	800 ± 154.9	
MeAD	267 ± 104.7	933 ± 301.0	656 ± 214.6	
MePV	478 ± 152.4	1248 ± 408.6	1533 ± 377.9	
MePD	400 ± 132.2	952 ± 300.2	1089 ± 297.0	
Hypothalamus				
MPN	511 ± 147.0	1371 ± 463.9	878 ± 132.7	
MPO	256 ± 118.5	800 ± 227.7	800 ± 230.9	
AH	333 ± 118.0	743 ± 216.5	700 ± 165.8	
VMH	113 ± 43.1^a	677 ± 183.9^b	$377 \pm 135.6^{a,b}$	0.037
PAG				
DMPAG	178 ± 65.9	590 ± 194.3	544 ± 110.8	
DLPAG	111 ± 65.9	476 ± 224.2	211 ± 86.8	

Activation density in each subnucleus (M±SE) and P-value from a one-way ANOVA between the 3 groups (listing only P-values under 0.1). For regions showing an overall group effect, the letters ([a],[b]) indicate the significantly different groups according to a Tukey post-hoc test, with a significant difference indicated by different letters.

The mean activation density for each region for each of the control, losers-to-males, and losers-to-females groups is shown in table 3.2. The one-way ANOVA analysis revealed significant or marginally significant differences between the groups in all sub-nuclei of the BNST and in the VMH, but not in any of the other regions. The Tukey post-hoc analysis showed that those differences were always between the control group and either the losers-to-males group (BSTAM, VMH), the loser-to-females group (BSTPM), or both losers groups (BSTAV). A closer look at the data found that although the mean activation levels for the losers were higher

71

than that of the control group in essentially all regions, the variance in neural activation within each of these groups was quite high. Since unlike previous studies (Kollack-Walker & Newman, 1995; Veening et al., 2005) here the duration of the encounters was not fixed, we decided to proceed by examining whether this variability might be related to the variability of the encounter durations.

Correlations between neural activation and behavior

Correlating the encounter duration (log-transformed) with the activation density in the investigated brain regions for each of the two losers groups revealed an unexpected yet quite striking pattern. To fully visualize it, and all the other correlation patterns from this study, we generated color-coded correlation matrices using C++, with deeper colors representing greater r values, and different significance levels (determined by the r value and the group size, using thresholds from a table) denoted by different hues. As could be seen in figure 3.1, for losers to males the correlations were fairly homogenous and distinctly negative (indicating reduced activation for longer encounters), with pearson's r ranging from -0.55 to -0.83 $(M(r)^7$=-0.68±0.02). Although those correlation values are all moderate to high, due to the small sample size only the correlations for the BSTAM, MPO and AH were significant (MeAV, MePV, MePD, MPN, VMH and DLPAG were marginally significant, with r values greater than -0.7).

For losers to females, however, the pattern was quite different, with correlations being much more diverse and mostly positive, ranging from 0.94 all the way down to -0.61 $(M(r)=0.31±0.11)$, and they were significant only in the BSTAI and DMPAG.

When comparing the correlations between the two groups within each region by analyzing the interaction between group and encounter duration using a mixed two-way ANOVA (whether longer encounters had a different effect depending on whether they were with males or females),

---

[7] M(r) stands for the mean of the correlation coefficient (pearson's r) values across the 16 regions.
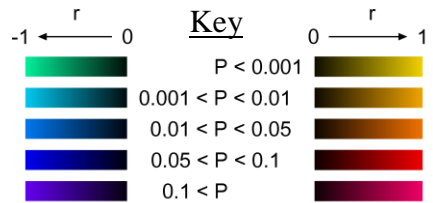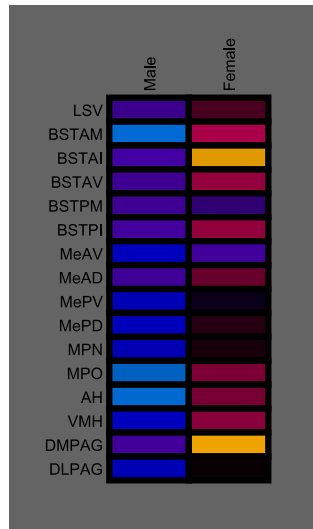
Figure 3.1: visual representation of the correlations between c-Fos activation in the SBN regions and encounter durations for losers to males (left column) and losers to females (right column). The deeper the color the greater the r value, with different significance levels (determined by the r value and the sample size, using thresholds from a table) denoted by different hues (one scale for positive correlations and another for negative, see Key).

finding a significant interaction in the BSTAM, BSTAI, MPO, AH, VMH and DMPAG (marginally significant interaction was found in the BSTAV and BSTPI), out of the 16 regions.

To further determine the source of the difference in the correlation patterns between the two groups, we proceeded to test for correlations between the c-Fos activation and the components of the encounters – investigation, rolling fight, and chase (the log-transformed measures were used in this analysis as well). The visual representation of this analysis is shown in figure 3.2a and 3.2b. Since the sample sizes in this part of the analysis (5 and 4 animals) were too small for meaningful assessment of the significance of each region separately, we instead evaluated all 16 regions in aggregate for each of the three behavioral measures, for each of the two groups[8]. For the losers to males, the correlations between the neural activation and both investigation time and rolling duration exhibited patterns similar to the correlations with overall encounter duration, with investigation time yielding correlations ranging from -0.53 to -0.99 (M(r)=-0.73±0.03) while rolling yielded correlation ranging from -0.47 to -0.85 (M(r)=-0.64±0.03). Chase duration, however, exhibited quite a different tendency and mostly positive correlation values, correlations ranging from -0.34 to 0.71 (M(r)= 0.31±0.09); a two-tailed t-test for the distribution of correlations between neural activation and chase duration revealed that the mean of those correlations was significantly different from 0 with a p-value=0.005, suggesting that those correlations were in fact mostly positive and not just randomly distributed.

For the losers to females, the correlations between fight components and activation level yielded a pattern which could be found in figure 3.2b. The correlations between investigation time and neural activation yielded a pattern very similar to the correlations with overall encounter duration, ranging from 0.96 to -0.42 (M(r)=0.47±0.09); analyzing these values using a two-tailed

---

[8] small samples are more likely to yield extreme values by random chance, but if all findings are due to random chance then the values would be expected to still center around 0, a deviation from which suggesting an overall effect.
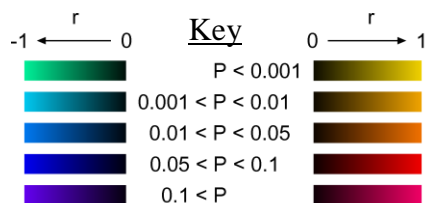
(a) male-male   (b) male-female

Key
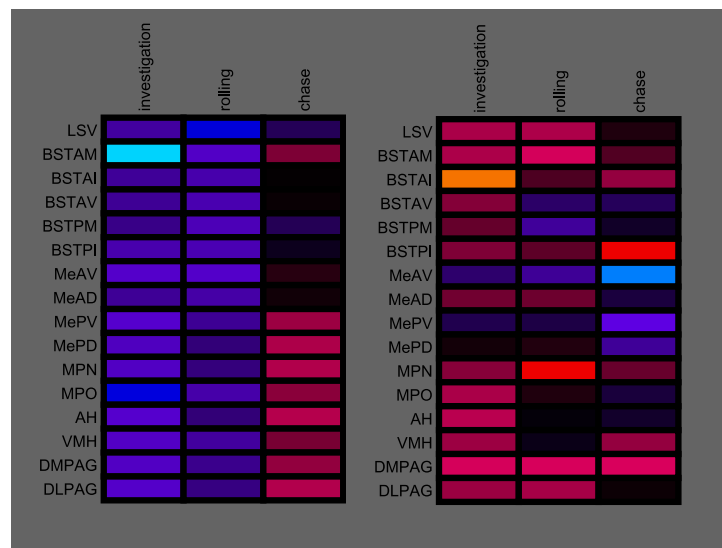
Figure 3.2: visual representation of the correlations between c-Fos activation in the SBN regions and behavioral measures from the fights (log-transformed durations of investigation, rolling, and chase) for losers to males (a) and losers to females (b).

75

t-test revealed that these too were significantly different from 0, with a p-value=0.0002. The distribution of correlations for rolling and chasing, on the other hand, were more balanced between positive and negative values, ranging from 0.93 to -0.6 (M(r)=0.2±0.13) for rolling and from 0.93 to -0.99 (M(r)=0.01±0.14) for chasing, suggesting that these values could have been due to random chance.

<u>Functional connectivity</u>

Since functional connectivity patterns are considered integral to the analysis of the SBN (Goodson & Kabelik, 2009), we concluded our study by examining whether the correlation in activation between the SBN regions for the two fight conditions differed the way the correlation patterns between the behavioral measures and the neural activation did. A visual representation of this analysis is shown in figure 3.3. As expected from the homogeneity found in the previous analysis, the losers-to-males group showed only positive correlations between the various brain regions, with r's ranging from 0.004 up to 0.98 (M(r)=0.61±0.02). The correlations for the losers-to-females group were mostly positive although not nearly as strong and far more diverse, ranging from -0.73 to 0.94 (M(r)=0.22±0.04). Running the same analysis for the control group revealed a correlation pattern much more similar to that of the losers-to-males group, with stronger and almost exclusively positive correlations, ranging from -0.45 to 0.99 (M(r)=0.59±0.03).

Discussion

The current study aimed to test the social neural network hypothesis by comparing the pattern of neural activation within the network associated with two behaviorally similar but contextually different male social behaviors – an aggressive encounter with a male and an aggressive

(a) male-male   (b) male-female   (c) control
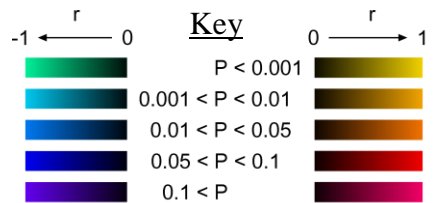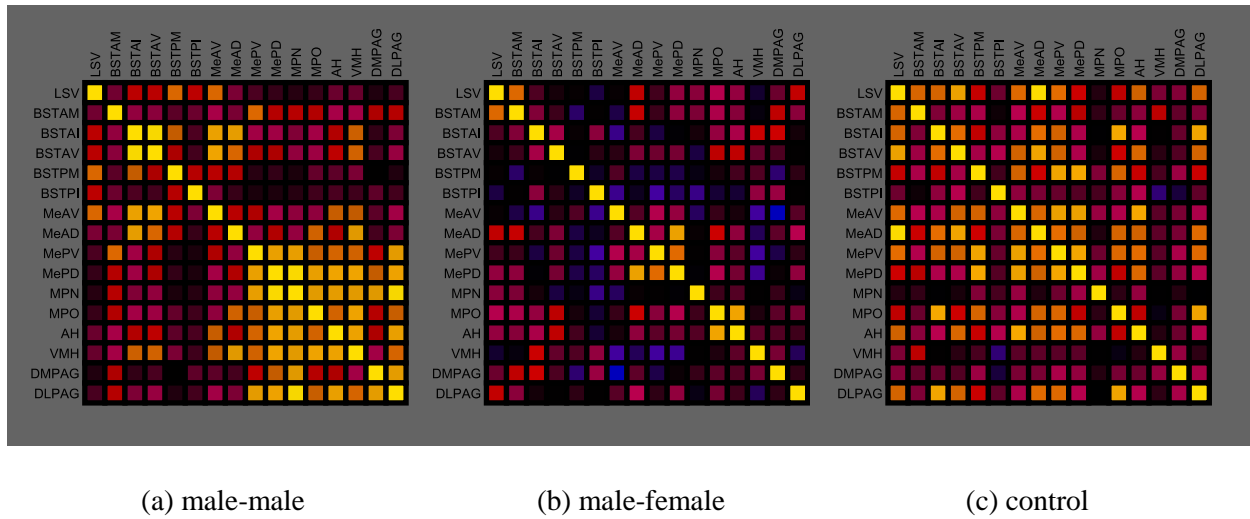
Key

Figure 3.3: functional connectivity matrices (a visual representation of the correlations between levels of c-Fos activation across the various regions, with each square representing a correlation between two regions) for each of the 3 groups.

encounter with a diestrus female, both resulting in a loss. No other study (that we know of) has taken this approach to unravel the neural mechanisms underlying social behaviors, since prior studies have focused on whether different social behaviors might share some common neural substrates, such as by comparing male-male aggression to mating (Kollack-Walker & Newman, 1995; Veening et al., 2005). The initial analysis comparing the two losers groups and a control group did not find any difference between the losers groups in any of the SBN regions. This might seem like strong support for overlapping mechanisms if it weren't for the fact that only 4 of the 16 regions showed a significantly higher c-Fos expression than the control group for either of the two experimental groups. This was inconsistent with the findings of previous studies, which found a significant difference from the control group in nearly all corresponding regions (Kollack-Walker & Newman, 1995; Veening et al., 2005). This inconsistency is most likely due to the methodological differences between the current study and the aforementioned ones.

One variation here was that subjects from our control group were not simply left in their home cages or picked up and immediately placed back, but rather they were placed in a neutral clean cage and allowed to explore it and eventually climb out of it, giving them a novel yet non-social experience to contrast to the other two groups' novel social experiences. Had this change in control group produced higher activation in our control group than the controls in the previous studies, thus making them less different from the two experimental groups, this might have undermined the idea that the analyzed regions were specifically involved in social behavior. However, this was not the case – the levels of activation in our control group were not clearly different from those in the handled control group from the comparable Kollack-Walker & Newman (1995) study in any of the analyzed regions, unlike the difference between handled and unhandled controls reported in many of those regions in the follow-up to that study (Kollack-

Walker et al., 1997). This suggests that the different experience of our control groups compared to the controls from prior studies was unlikely to have been the cause of the discrepancy in our findings.

A more significant variation in our experimental design was in the way the interactions were conducted. Unlike other studies, in which all subjects were put through a predetermined duration of interaction (10 minutes in Kollack-Walker & Newman, 1995, 30 minutes in Veening et al., 2005), our subjects were free to end the fights whenever they wanted, simply by jumping out of the cage (something that none of them had any difficulty doing). This led to a wide variance in the duration of the interactions experienced by our subjects, which in turn led to the wide variance in levels of neural activation in our experimental groups, causing the lack of significant difference between the experimental groups and the control group.

On the other hand, verifying the relationship between the encounter durations and c-Fos expression in the SBN regions revealed an unexpected difference between the two groups, with the losers to males showing only negative correlations between encounter durations and neural activation, while the losers to females showed mostly positive correlations, which is noteworthy in two separate ways.

One is the fact that across all the SBN regions, the longer a male-male fight lasted, the lower the levels of c-Fos activation were. This pattern has actually been found before, in two different studies that evaluated correlations between IEG activation and aggression, one focused on mice (Haller et al., 2006) and the other on birds (Goodson et al., 2005). The Haller et al. (2006) study tested mice bred for high and low aggression with a non-aggressive intruder stimulus, and found negative correlations between investigation duration and c-Fos expression in the central amygdala, BNST and PAG (correlations in other regions were not reported), although those

regions showed higher activation in the high-aggression strain (correlations between neural activation and offense within the high-aggression strain were not reported). Those findings were interpreted as suggesting that increased activation of the central amygdala lowers the occurrence of non-aggressive investigations and thus promotes more abnormally aggressive interactions, without mention of involvement of other regions. Although the central amygdala was not investigated in the current study, this interpretation seems unlikely in light that our study found negative correlations between neural activation in various other regions (including the BNST) and not only investigation but also the highly aggressive rolling fights. Goodson et al. (2005) used an entirely different paradigm, testing non-breeding male song sparrows in a simulated territorial intrusion paradigm (presenting the subjects with a song playback and a male decoy in an adjacent cage), and finding negative correlations between approaches to the stimulus and IEG expression in septal and hypothalamic regions. The authors interpreted these correlations as suggesting that increased activation likely indicates social aversion rather than aggressive motivation (Goodson et al., 2005). However, our breakdown of the fights to investigate the contribution of each fight component to the correlations, revealed that it was actually investigation time and to a slightly lesser extent time spent rolling, both demonstrating approach/aggression (when interacting with a male), that showed moderate to strong negative correlations with c-Fos expression across the SBN regions. Running away from the winner, demonstrating active aversion, showed much weaker correlations, although these correlations were predominantly positive. This suggests that the negative correlations are more strongly associated with aggressive motivation than with social avoidance, a matter that will be further addressed in the following chapter.

The more important discovery in the context of the SBN theory, though, is how the males that lost to females showed quite a different pattern of correlations than those of the losers to males – mostly positive rather than exclusively negative. Even when focusing on the clearly agonistic components of the interactions, neither rolling nor being chased by a female winner showed any clear pattern of association with the c-fos expression (in contrast to the consistent patterns found in the male-male group). Moreover, the pattern of correlations between the c-fos expression and the time spent investigating the female opponent showed the opposite pattern than investigation of a male opponent did, being mostly positive rather than strongly negative. This supports our interpretation that the motivation of males towards a diestrous female is not aggressive but rather sexual, however it casts serious doubt on the idea regarding the role that the SBN regions play in support of social behavior. The previous interpretation of overlapping activation between social behaviors such as fighting and mating as reflecting a general socially-induced arousal (Kollack-Walker & Newman, 1995), for example, would suggest that both groups show the same correlation pattern between neural activation and investigation. Additionally, an elaboration on the original SBN theory has suggested that it is the pattern of activation across the network that determines the behavioral output, meaning that different behaviors are associated with different levels of relative activations of the various SBN regions (Goodson & Kabelik, 2009). A functional connectivity analysis to identify such patterns of relative activation revealed that while for male-male aggression all the SBN regions appeared quite coordinated, showing positively correlated c-fos activation levels between each other, losing a fight to a diestrous female resulted in a pattern of correlations among those same regions that appeared virtually random. Based on the finding that the non-social control experience resulted in a primarily positive connectivity pattern resembling that of the losers to males, it appears that rather than an inter-male aggressive

experience leading to a highly coordinated activation pattern across the SBN, it is the male-female fight experience that leads to uncoordinated activation levels across those regions. One possibility for the source of this effect is that the neural system underlying sexual motivation and the neural system underlying the expression of aggressive behavior are not congruent, perhaps due to the fact that males are inhibited from attacking females (Payne & Swanson, 1970). This interpretation, however would contradict the suggestion that the overlap in regions showing c-Fos activation following fighting or mating reflect an overlapping system of general social arousal. This matter is critical for the conceptualization of the SBN theory, and it will be further discussed in the final chapter.

Chapter 4: Neural mechanisms underlying aggressive motivation

Introduction

In order to achieve a thorough understanding of the neural mechanisms underlying aggression, it is useful to distinguish between the mechanisms underlying the motivation to engage in the aggressive interaction, and the mechanisms responsible for carrying out the behavioral pattern involved in the fighting behavior. The previous chapter identified correlations between neural activation in various regions and fight duration, plus likely correlations with durations of investigation and of rolling fights, which could potentially correspond to aggressive motivation. However, since those activations were taken following an entire fight and the measures were influenced not only by the behaviors of subjects but also their opponents, it was impossible to draw firm conclusions regarding the meaningfulness of these findings.

Besides the c-fos studies discussed in the previous chapter, most previous studies investigating the neuroanatomical mechanisms of aggression used an entirely different approach, focusing on identifying regions whose stimulation leads to attack behavior. These studies have primarily centered on an area in the hypothalamus that they labeled the "hypothalamic attack area", roughly corresponding to the anterior and ventromedial hypothalamic nuclei (e.g. Kruk et al., 1982; Lammers et al., 1987), and subsequently it has been suggested that this area (or either of the nuclei comprising it) is responsible for aggressive motivation (e.g. Adams, 2006; Lin et al., 2011). However, an alternative view has been that activation of this area merely releases the motor patterns involved in attack behavior rather than generating aggressive motivation in the subjects (Kruk, 1991). This issue has not been conclusively settled since the analysis of aggression via the expression of attack behavior could not really tell whether or not this behavior was driven by an active desire to fight (as discussed in the first chapter).

A recent study took a somewhat different approach, using fMRI to investigate the neural activation associated with piloerection resulting from being presented with a male intruder (Ferris et al., 2008). This autonomic response was argued to reflect aggressive motivation since it is typically followed by an attack on the intruder, which in that study was prevented by their being fixed in place for the fMRI scan. This study found an increased volume of activation and increased activation intensity in a variety of regions, both cortical and subcortical, including the lateral hypothalamus but not the anterior or ventromedial hypothalamus (Ferris et al., 2008), which may reflect the fact that the subjects did not get to carry out the act of attacking the intruder. Overall, though, this design completely eliminates the choice aspect of aggressive motivation, instead placing them in a situation that inevitably elicits aggression (including the presence of their female mate, in addition to the intruder) while physically preventing them from doing anything about it.

Finally, although a variety of behavioral methods have been designed specifically to investigate aggressive motivation (as described in the first chapter), as far as we know none of these methods have been used to try to identify the neuroanatomical mechanisms underlying this motivation, instead focusing only on neurochemical manipulations (e.g. Almeida et al., 2005; Couppis and Kennedy, 2008; Fish et al., 2002 & 2005).

The goal of the current study was to investigate the patterns of c-fos activation associated with our novel aggressive motivation test design (as discussed in chapter 1) as a different angle towards assessing the neuroanatomical mechanism underlying aggressive motivation. Since the test allows subjects to act on their aggressive motivation by exerting effort in order to gain access to an unfamiliar male conspecific, while also being free to choose not to do so (and possibly pushing to enter an identical empty goal box instead), the levels of activation correlating with

varying degrees of aggressive motivation could be identified. Since it has been shown that prior winning experience is associated with heightened aggressive motivation while losing experience is associated with low aggressive motivation when tested on the day following the fight experience (chapter 1), the current study used subjects from these two conditions in addition to ones with no fight experience. The main purpose of that design was to make for broader and more robust distribution of aggressive motivation levels, but it also allowed for comparing the neural mechanisms between those three conditions.

Importantly, since the neural mechanisms of aggressive motivation have never been evaluated in such a way, we decided to take the exploratory route and investigate all regions that could potentially be involved, either due to the aggression aspect or due to the motivation aspect. This included most subdivisions of regions suggested to be involved in the social behavior network (all sub divisions of the amygdala, BNST, lateral septum, hypothalamus, and the PAG), in addition frontal cortical regions and regions associated with the dopamine motivation system (Goodson & Kabelik, 2009).

Methods

Behavioral component

The behavioral design, animals, apparatus, and behavioral procedures were identical to those described in chapter 1, only using 3 groups instead of 5 (naïve, winners tested with a novel male, losers tested with a novel male), and with only 6 subjects per groups. The behavioral data collection was slightly altered for the neural test, such that subjects whose tests were ended a minute after the first strong push (the condition meant to avoid them getting frustrated) occasionally took a bit longer than a minute before being taken out, in order not to disrupt any pushing that they were in the middle of.

In addition to the 3 aggression groups, this study also included a control group, that went through the exact same procedure as the other groups only instead of being tested with a male conspecific in the test phase, both goal boxes of the apparatus were left empty, to evaluate the neural activation for simply pushing on the doors without an aggressive context.

Neural component

Procedure

The neural procedures (brain acquisition, slicing, and immunohistochemistry staining) were the same as those described in chapter 3.

Data acquisition

With the guide of the Hamster Brain Atlas (Morin and Wood, 2001), 8 sections from each brain were selected for analysis, as closely as possible corresponding to the plates of Bregma 3.8mm, 2.6mm, 0.8mm, 0.2mm, -0.9mm, -1.8mm, -3.5mm, and -4.9mm (see figure 4.1). These sections were chosen such that each of the regions selected for analysis could be clearly identified in at least one of them. The selected sections from each brain were scanned at x20 magnification into digital images using a ScanScope (Aperio Technologies Inc.), and were then analyzed through the ImageScope analysis software (Aperio Technologies Inc.) On each section, all the analyzed regions were first circled based on the landmarks and boundaries from the Hamster Brain Atlas (see figure 4.1 for a visual representation), then the stained neurons within each region were counted and divided by the area of the region to provide an activation density measurement (in $\#/mm^2$), with the densities from the left and right sides averaged to produce the average density for the region. Regions that were counted on two separate slices were analyzed as two separate regions (e.g. LH(5) and LH(6), denoting LH from slice 5 and LH from slice 6). All counting was done blind to the experimental condition.

Figure 4.1: a sample slice for each of the 8 slices analyzed, illustrating the way the regions were circled (stained neurons were counted within each of the circled areas, with their number divided by the surface of the area to provide the activation density).

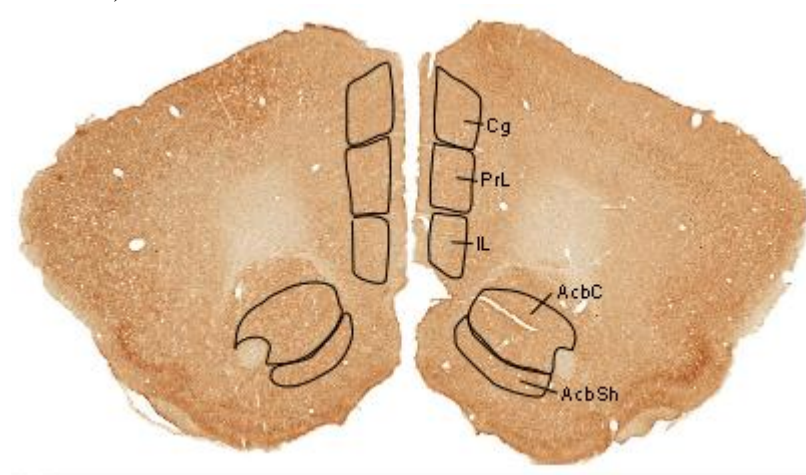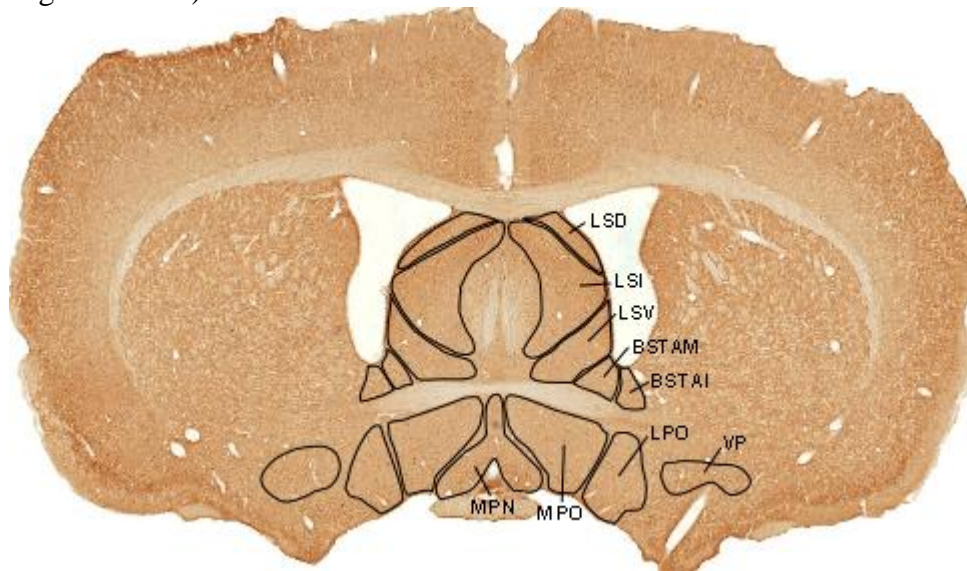Slice #1 (Bregma 3.8mm):
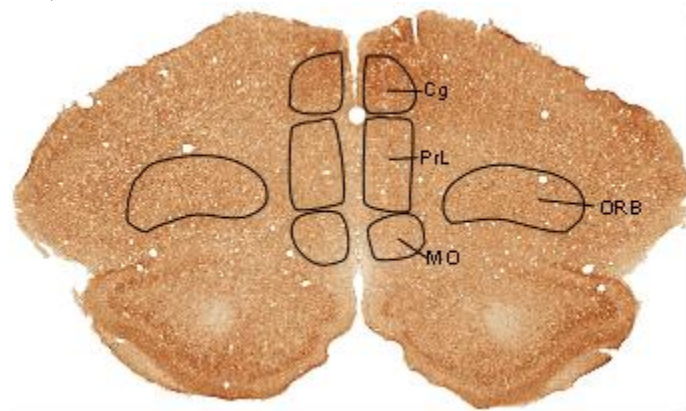
Slice #2 (Bregma 2.6mm):

Figure 4.1(cont.):

Slice #3 (Bregma 0.8mm):
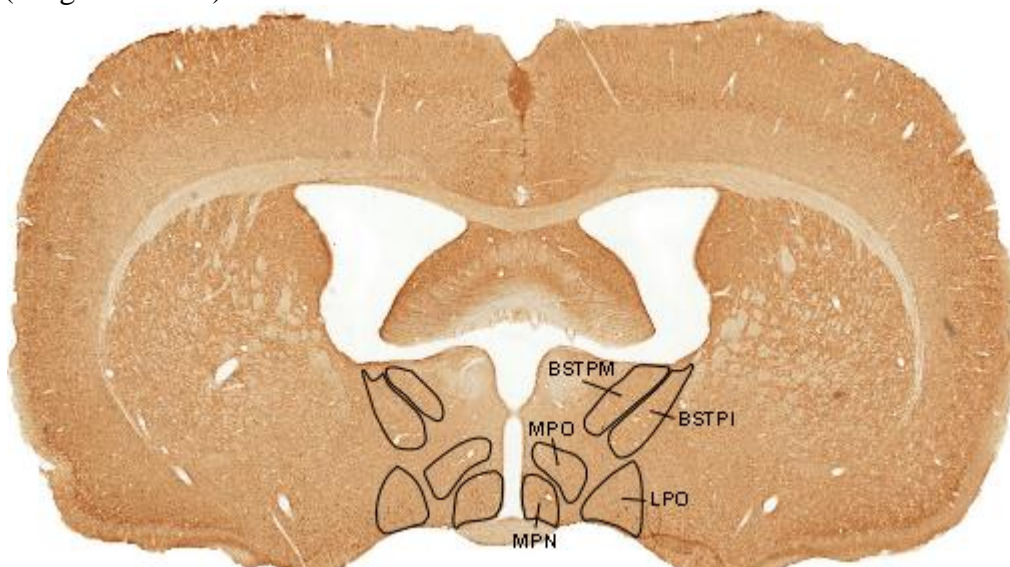

Slice #4 (Bregma 0.2mm):

Figure 4.1(cont.):
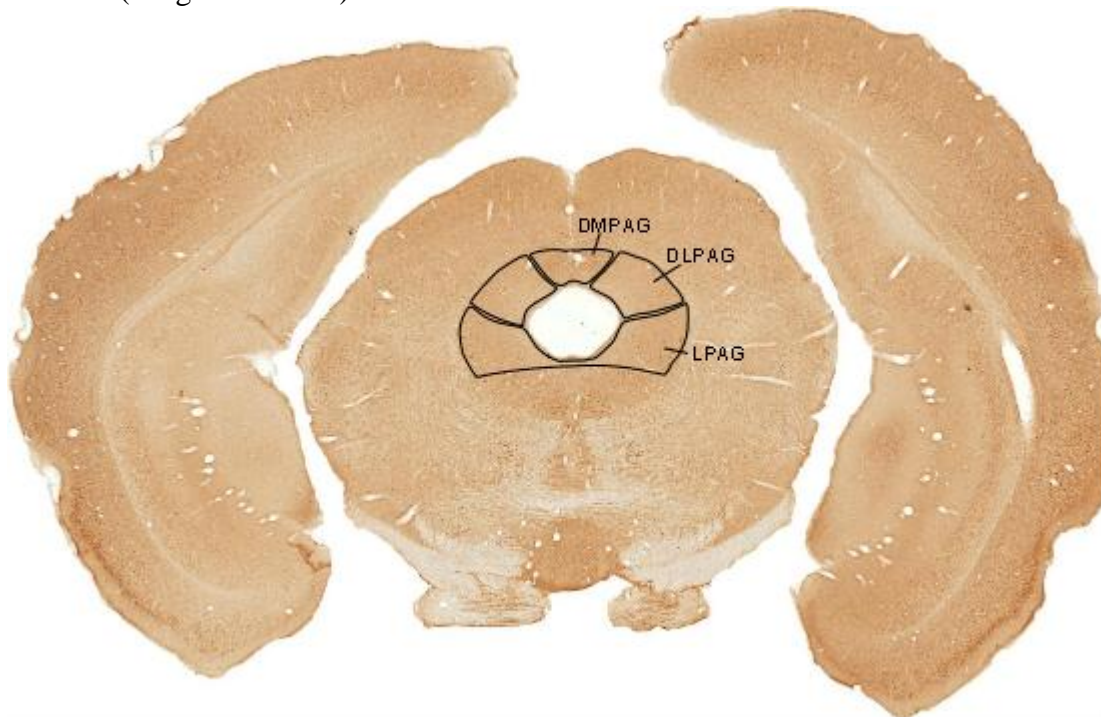
Slice #5 (Bregma -0.9mm):

Slice #6 (Bregma -1.8mm):

Figure 4.1(cont.):

Slice #7 (Bregma -3.5mm):

Slice #8 (Bregma -4.9mm):

Investigated regions (also see figure 4.1)

Frontal cortex: ORB, MO, Cg, PrL, IL

Ventral striatum: AcbC, AcbSh, VP

Lateral Septum: LSD, LSI, LSV

Bed Nucleus of the Stria Terminalis: BSTAM, BSTAI, BSTPM, BSTPI

Amygdala: medial: MeAV, MeAD, MePV, MePD; cortical: ACo, PLCo, PMCo; basolateral: BLA, BLP; central (Ce)

Hypothalamus: MPN, MPO, LPO, Pa, AH, LH, VMH, SuM

Periaqueductal Gray: DMPAG, DLPAG, LPAG.

Results

Behavioral

Special note: one of the fighting pairs was fairly non-aggressive so their encounter did not escalate to a rolling fight, but winner and loser could still be determined based on the less vigorous aggression they engaged in, and the loser did escape the fighting cage to end the encounter. This atypical pair was not excluded from this study (unlike the behavioral study) since the focus was on levels of aggressive motivation rather than on effects of winning and losing, so they made for an interesting special case.

The behavioral measures taken during the motivation test for the three aggression groups can be seen in table 4.1. It is clear that while the mean pushing towards the aggressive opponent has the same rank order as in the behavioral study (highest for the winners and lowest for the losers), this trend is far from significance due to the small sample size in the current study. Additionally, the overall range of aggressive motivation measures ended up being quite similar between the three groups, such that the effect of the group (prior experience) on the neural activation could be

evaluated independently from the effect of aggressive motivation levels. While only 8 subjects (out of 18) pushed towards the empty goal box, all but 3 subjects pushed at least a little bit towards the male stimulus − 2 of these were from the losers group, and the third was the winner of the non-aggressive fight. These 3 subjects plus 3 others ended up getting into the empty goal box, while only 4 subjects successfully entered the stimulus goal box (and were immediately separated to prevent the occurrence of a fight).

Table 4.1

	Naïve			Winners			Losers		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Towards male stimulus:									
Investigation time (%)	0.37	0.08	0.25−0.47	0.25	0.11	0.09−0.40	0.43	0.16	0.09−0.56
Pushing time (%)	0.27	0.21	0.01−0.56	0.38	0.19	0−0.58	0.21	0.24	0−0.67
Pushing angle (rad)	0.22	0.10	0.09−0.33	0.27	0.13	0−0.38	0.18	0.15	0−0.38
Towards empty side:									
Investigation time (%)	0.03	0.03	0−0.08	0.03	0.02	0.01−0.07	0.01	0.01	0−0.04
Pushing time (%)	0.06	0.06	0−0.15	0.04	0.08	0−0.23	0.03	0.03	0−0.09
Pushing angle (rad)	0.18	0.18	0−0.38	0.11	0.15	0−0.36	0.18	0.19	0−0.46
Total duration (sec)	107.8	29.9	56−153	106.7	42.3	34−180	89.7	43.1	25−137

Behavioral measures taken during the motivation tests for the 3 aggression groups. The motivation measures were described in detail in chapter 1.

All subjects from the control group entered one of the empty goal boxes, taking an average of 93.6 seconds (26–160 range, SD=44.74), which was comparable to the aggression groups.

Neural

General considerations

For the 18 subjects from the aggression group, not all regions could be counted for all subjects for various technical reasons, but since for each region there were at most 2 data points missing then the analysis for each region was simply carried out omitting the missing points. One exception to this was the PMCo, which could not be counted for a considerable number of

subjects, so this region was not included in the analysis. For the control group no data points were missing besides for the PMCo, so that region was omitted from their analysis too.

Of the regions selected for analysis, several were found to exhibit no c-fos expression in any of the aggression subjects. These inactive regions were: AH, VMH, BLA, BLP, and Ce, and they were not investigated further.

Activation levels across the separate behavioral conditions

The mean activation density for each region for the control group and each of the three aggression groups can be found in table 4.2. When testing for an effect of being tested with a conspecific stimulus by comparing the activation of the control group to all aggression groups combined, a significant difference was only found for 2 of the 36 regions included in the analysis. The control group showed lower activation than the aggression condition groups in the ORB and higher activation in the LH(6). These differences, however, may not mean much and may simply be due to random variations that result from analyzing this many regions. Furthermore, a one-way ANOVA comparing the 3 aggression groups (disregarding the control group) found no difference in activation between them for any of the regions. Overall, these negative findings could be attributed to the wide variance in activation within each of the groups, a finding which was anticipated given the variances in the behavioral measurements found within each group. A correlational analysis was used to test whether the neural activation levels indeed corresponded to respective levels of the behavioral measures exhibited during the motivation test.

Table 4.2: Mean c-fos activation density (# neurons/mm^2)

	Controls	Naïve	Winners	Losers
Frontal cortex				
ORB	14 ± 6.1*	48 ± 18.6	32 ± 15.8	28 ± 13.8
MO	40 ± 12.1	51 ± 25.3	28 ± 17.3	44 ± 14.4
Cg(1)	8 ± 4.3	14 ± 7.3	14 ± 10.7	12 ± 3.7
Cg(2)	17 ± 13.5	17 ± 16.9	36 ± 26.7	28 ± 12.4
PrL(1)	26 ± 9.5	26 ± 13.8	32 ± 25.3	37 ± 25.8
PrL(2)	49 ± 20.4	39 ± 23.7	40 ± 20.8	60 ± 23.2
IL	44 ± 14.9	26 ± 13.3	41 ± 22.5	63 ± 22.6
Ventral striatum				
AcbC	10 ± 4.9	18 ± 10.3	7 ± 4.1	16 ± 6.6
AcbSh	3 ± 1.1	2 ± 1.4	4 ± 3.8	11 ± 7.6
VP	2 ± 1.1	5 ± 2.4	4 ± 2.3	3 ± 2.0
Lateral septum				
LSD	32 ± 12.3	43 ± 21.5	43 ± 17.1	36 ± 10.9
LSI	11 ± 5.7	15 ± 5.7	10 ± 4.3	19 ± 6.5
LSV	19 ± 4.9	24 ± 11.3	21 ± 7.7	47 ± 18.7
BNST				
BSTAM	52 ± 21	21 ± 8.2	29 ± 13.7	28 ± 8.6
BSTAI	9 ± 4.2	14 ± 6.0	20 ± 6.4	21 ± 9.6
BSTPM	5 ± 3.7	5 ± 2.5	3 ± 3.7	15 ± 12.5
BSTPI	5 ± 3.3	4 ± 1.6	0 ± 0	9 ± 5.7
Amygdala				
MeAV	44 ± 11.1	44 ± 18.2	21 ± 9.2	47 ± 27.2
MeAD	27 ± 12.8	15 ± 10.3	10 ± 3.9	18 ± 9.4
MePV	27 ± 7	21 ± 11.7	12 ± 5.5	23 ± 11.5
MePD	16 ± 5.2	10 ± 7.1	12 ± 4.7	7 ± 3.5
ACo	63 ± 21.3	49 ± 21.7	29 ± 11.5	44 ± 25.0
PLCo	9 ± 4	7 ± 3.7	9 ± 6.7	9 ± 7.3
Hypothalamus				
MPN(3)	39 ± 14.8	30 ± 11.1	29 ± 9.7	36 ± 11.9
MPN(4)	61 ± 22.9	20 ± 6.9	68 ± 42.6	70 ± 24.5
MPO(3)	22 ± 10.6	24 ± 6.8	21 ± 7.8	37 ± 15.3
MPO(4)	16 ± 6.0	7 ± 2.3	7 ± 5.7	15 ± 9.4
LPO(3)	29 ± 9.9	27 ± 6.5	19 ± 11.1	29 ± 8.7
LPO(4)	34 ± 9.1	24 ± 8.5	15 ± 7.2	30 ± 8.8
Pa	193 ± 112.2	100 ± 29.1	143 ± 65.5	249 ± 58.5
LH(5)	80 ± 27.9	54 ± 25.9	31 ± 17.7	72 ± 31.0
LH(6)	118 ± 43.6*	43 ± 28.9	20 ± 14.4	39 ± 23.5
SuM	120 ± 35.5	152 ± 24.6	117 ± 27.8	156 ± 30.4
PAG				
DMPAG	15 ± 8.5	9 ± 5.5	1 ± 0.8	44 ± 29.7
DLPAG	10 ± 6.3	5 ± 2.6	5 ± 3.6	19 ± 9.1
LPAG	16 ± 6.4	8 ± 1.7	14 ± 7.3	25 ± 10.3

Activation density in each subnucleus (M±SE) for each of the 4 groups.

* : significant difference between control and aggression.

<u>Correlations between neural activation and behavior</u>

Pushing towards a male

To test for the neural activation corresponding to aggressive motivation, the c-fos activation levels of all the 18 subjects from the aggression condition (the three groups combined) were correlated with the three behavioral measures directed towards the male stimulus (investigation time, pushing time, and pushing angle). The color-coded correlation matrix produced by our C++ code (using colors to visualize r values and significance levels, as described in the previous chapter) could be seen in figure 4.2. Correlations between the investigation percentage and c-fos activation across the 36 analyzed regions were all non-significant with a slight positive tendency (with a mean of r=0.12), ranging from -0.34 to 0.46 (with a mean of 0.12). Correlations between the pushing measures and c-fos activation were predominantly negative, with Pearson's r ranging from -0.77 to 0.15 (with a mean of -0.3) for pushing percentage and from -0.72 to 0.27 (with a mean of -0.29) for pushing angle. The neural correlations for pushing percentage reached significance for 9 regions: MO, LSV, BSTAM, BSTAI, and MeAD, LPO(4), LH(5), LH(6), SuM, all with moderate to high correlations (r=-0.48 and below), of which the SuM is most note-worthy with an r=-0.77 (P=0.0003). The neural correlations for pushing angle reached significance for 10 regions: LSV, BSTAM, BSTAI, MeAD, and MePV, LPO(3), LPO(4), LH(5), LH(6), SuM, also all with moderate to high correlations (r=-0.5 and below), of which the BSTAM was note-worthy with r=-0.72 (P=0.0008).

For another visual representation of these correlation findings, the activation in the regions showing the most significant correlations (BSTAM, LH(5), LH(6), and SuM) was plotted against each of the pushing measures, illustrating what the negative correlations actually look like (figure 4.3).
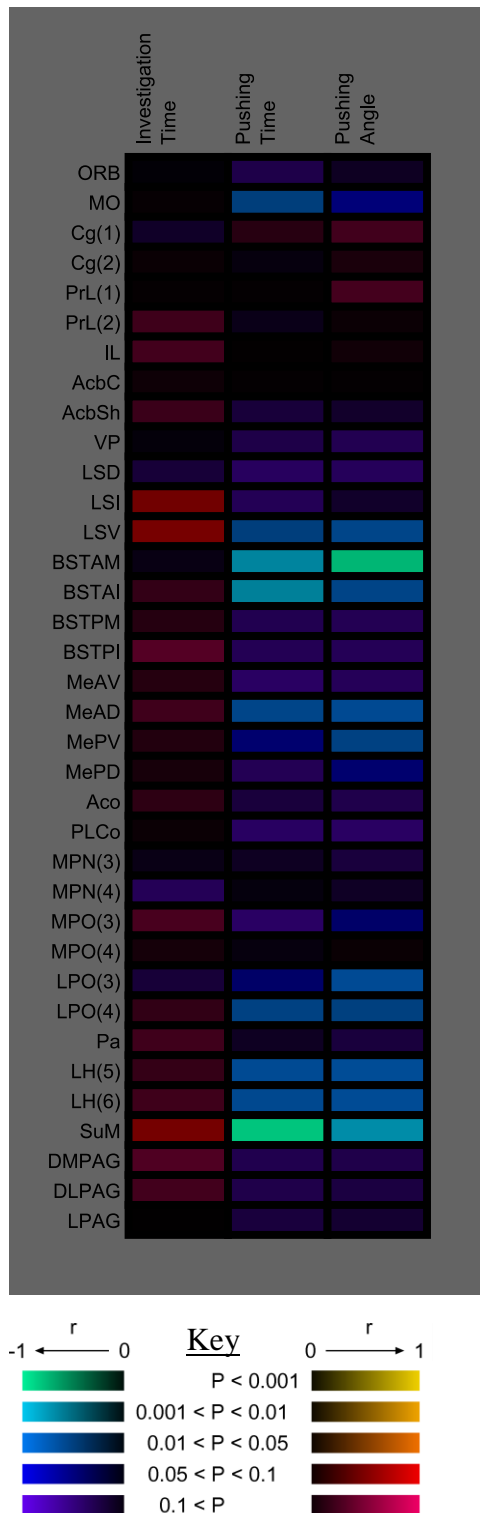
Figure 4.2: visual representation of the correlations between c-Fos activation in the investigated regions and the 3 behavioral measures (pushing time, investigation time, and pushing angle) towards a male stimulus. The deeper the color the greater the r value, with different significance levels (determined by the r value and the sample size, using thresholds from a table) denoted by different hues (one scale for positive correlations and another for negative, see Key).
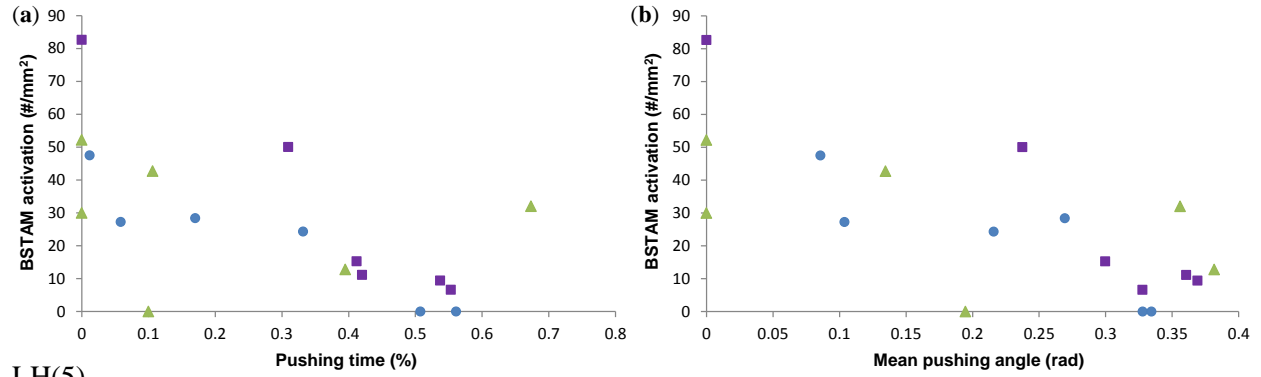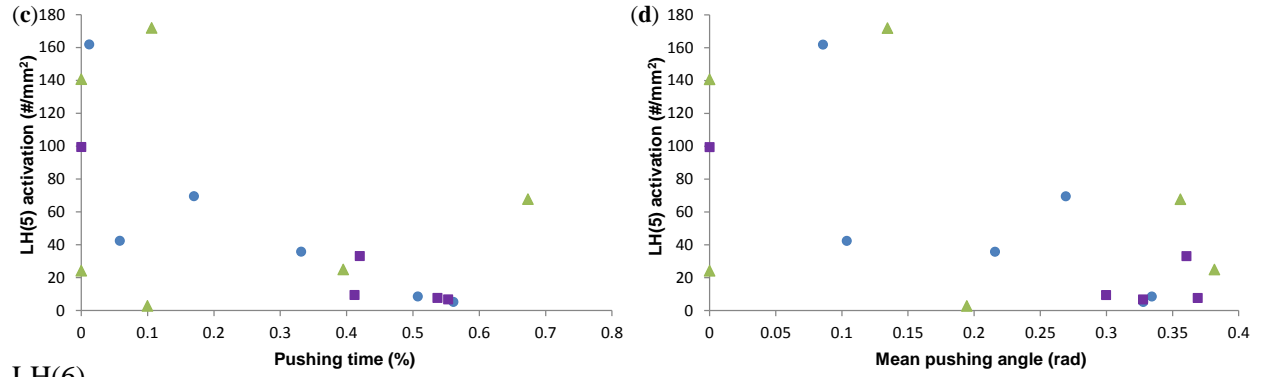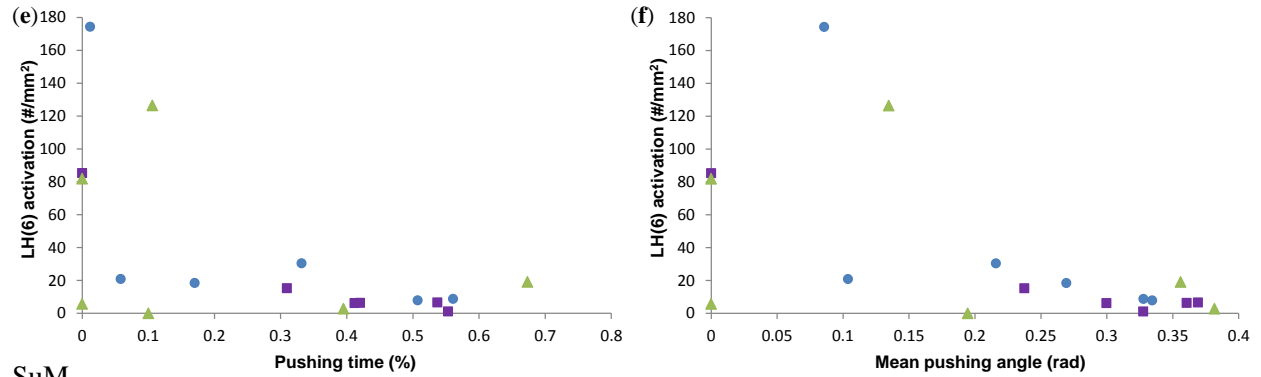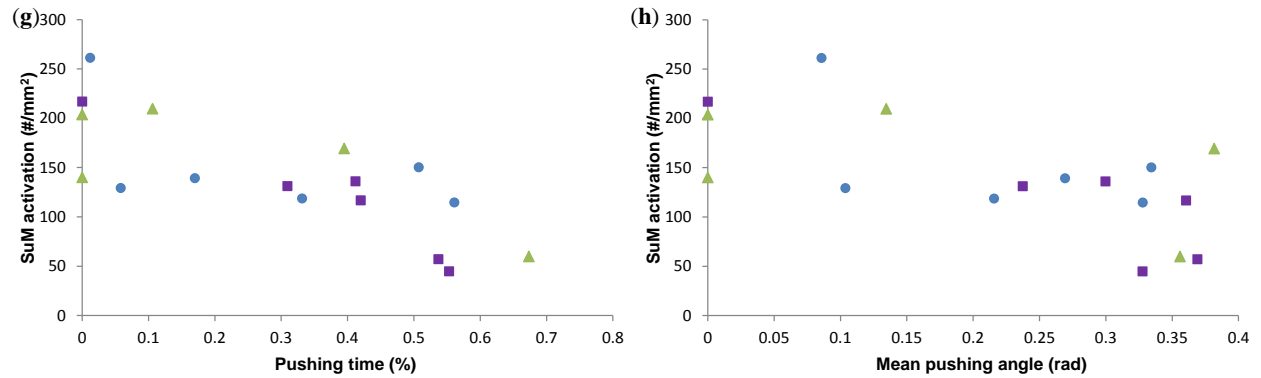
BSTAM

Figure 4.3: scatterplots of c-fos activation density as a function of pushing time and mean pushing angle, for each of the 3 groups, in the BSTAM (a&b), LH(5) (c&d), LH(6) (e&f), and SuM (g&h).

97

Pushing away from a male

Since motivation to gain access to an aggressive opponent yielded primarily negative correlations, meaning that lower c-fos activation was associated with increased levels of pushing, it raised the question of whether it was due to increased activation associated with a desire to get away from the male stimulus. Since 8 of the 18 aggression subjects actively retreated from the stimulus by pushing towards the empty goal box, evenly distributed between the 3 groups (3 naïve, 2 winners, and 3 losers), these subjects were used to investigate the contribution of this retreat motivation to the pattern of neural activation. First a student's t-test was used to compare the mean activation of subjects that pushed towards the empty goal box and of those that did not (see table 4.3), revealing that activation was indeed higher in subjects that pushed towards the empty in nearly all the regions, reaching significance in 8 of them (MO, BSTAM, BSTAI, MeAV, MeAD, MePV, LH(5), LH(6)). Subsequently, a correlational analysis was performed on the behavior towards the empty goal box (investigation and pushing), using only the 8 subjects that pushed towards it. As could be seen in figure 4.4, pushing towards the empty goal box did not yield a consistent correlation pattern as did pushing towards the stimulus, with Pearson's r ranging from -0.35 to 0.57 (with a mean of 0.09) for investigation time, from -0.38 to 0.8 (with a mean of 0.17) for pushing time, and from -0.45 to 0.89 (with a mean of 0.09) for pushing angle. A handful of these correlations reached significance, all of them strong and positive (r>0.7): for the pushing percentage measure significance was reached for the LSD and BSTAM (the latter with r=0.8), while for the pushing angle measure significance was reached for the ORB (r=0.82) and for all the 3 ventral striatum regions, the AcbC, AcbSh, and VP (with r=0.89, 0.74 & 0.83 respectively).

Table 4.3: aggression subjects that pushed towards the empty goal box vs. those that did not.

	No push for empty (N=10)	Pushed for empty (N=8)	P-Value
Frontal cortex			
ORB	30 ± 9.9	45 ± 15.6	
MO	23 ± 9.1	65 ± 18.7	0.041
Cg(1)	16 ± 6.6	9 ± 3.9	
Cg(2)	30 ± 16.2	22 ± 12.0	
PrL(1)	35 ± 18.2	26 ± 10.4	
PrL(2)	47 ± 18.0	46 ± 17.2	
IL	46 ± 18.1	41 ± 12.6	
Ventral striatum			
AcbC	16 ± 7.2	12 ± 3.6	
AcbSh	5 ± 4.3	6 ± 3.8	
VP	4 ± 1.9	5 ± 1.5	
Lateral septum			
LSI	13 ± 4.5	17 ± 4.1	
LSD	30 ± 8.4	55 ± 16.8	
LSV	21 ± 7.6	42 ± 13.7	
BNST			
BSTAM	15 ± 5.4	40 ± 8.5	0.017
BSTAI	12 ± 4.2	27 ± 6.3	0.041
BSTPM	2 ± 1.3	15 ± 8.9	
BSTPI	1 ± 0.9	8 ± 4.2	0.078
Amygdala			
MeAV	19 ± 7.0	60 ± 20.2	0.049
MeAD	5 ± 2.1	26 ± 7.8	0.006
MePV	9 ± 2.7	32 ± 10.0	0.019
MePD	7 ± 2.4	13 ± 5.7	
ACo	33 ± 12.7	51 ± 19.4	
PLCo	3 ± 1.7	14 ± 6.5	
Hypothalamus			
MPN(3)	27 ± 7.9	37 ± 8.9	
MPN(4)	47 ± 16.7	57 ± 26.4	
MPO(3)	20 ± 5.5	36 ± 11.0	
MPO(4)	9 ± 3.4	11 ± 7.1	
LPO(3)	18 ± 5.7	33 ± 7.4	
LPO(4)	16 ± 4.9	31 ± 7.4	0.079
Pa	151 ± 41.5	181 ± 53.6	
LH(5)	22 ± 7.1	90 ± 24.1	0.009
LH(6)	10 ± 3.0	64 ± 24.0	0.017
SuM	117 ± 12.5	168 ± 25.7	0.071
PAG			
DMPAG	4 ± 2.5	36 ± 22.3	
DLPAG	6 ± 2.3	14 ± 7.3	
LPAG	12 ± 3.9	20 ± 8.3	

Activation density (M±SE) and P-value from a student's t-test (only P-values under 0.1 are noted).
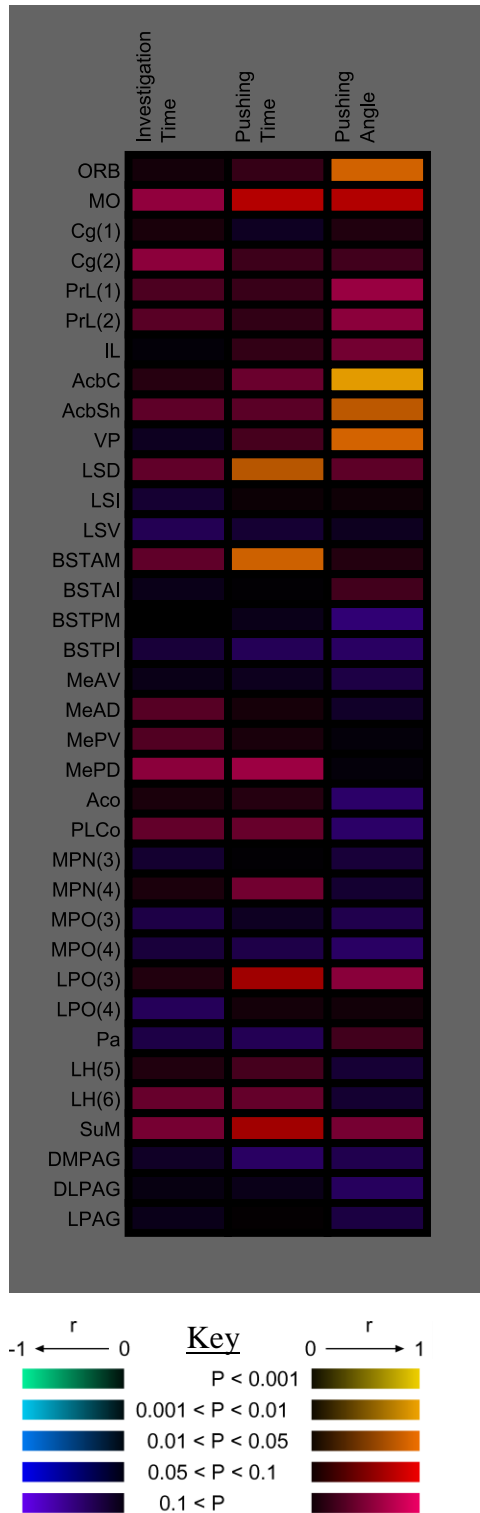
Figure 4.4: visual representation of the correlations between c-Fos activation in the investigated regions and the 3 behavioral measures (pushing time, investigation time, and pushing angle) towards the empty goal box opposite the male stimulus for the 8 subjects that pushing towards the empty goal box.

Pushing in the absence of a conspecific stimulus

Since pushing towards the empty goal box yielded such a different neural correlations pattern from that of pushing towards a male opponent, it was important to test whether either of those patterns corresponded simply to the act of pushing on a weighed door in the apparatus, independent of any aggressive context. This was done by running the same correlational analysis between the behavioral measures and the c-fos activation for the 6 control subjects, a visual representation of which could be seen in figure 4.5. For both investigation time and pushing time the correlations were widely distributed, with Pearson's r ranging from -0.65 to 0.91 (with a mean of 0.01) for investigation and from -0.45 to 0.91 (with a mean of 0.1) for pushing. The pushing angle measure, on the other hand, yielded almost exclusively positive correlations, with Pearson's r ranging from -0.17 to 0.87 (with a mean of 0.46). Due to the small sample size, only a couple of the strong correlations reached significance for each of the behavioral measures (see in figure 4.5).

Correlations within each of the aggression groups

To test whether the correlation patterns associated with pushing towards a male stimulus differed between the 3 aggression groups, we ran the same correlational analysis on each of them separately. Surprisingly, this analysis found a striking difference between the 3 groups, as could be seen in figure 4.6. Rather than showing a pattern of correlations roughly similar to that which was found in the combined aggression condition, just with more variability due to the smaller sample size for each of the groups, it turned out that the strengths of the correlations were very different within each of the groups (in addition to the increased variability). While the correlations with the pushing measures were still mostly negative within each of the groups, they were mostly weak-to-moderate for the naïve subjects, with average Pearson's r of -0.36 for
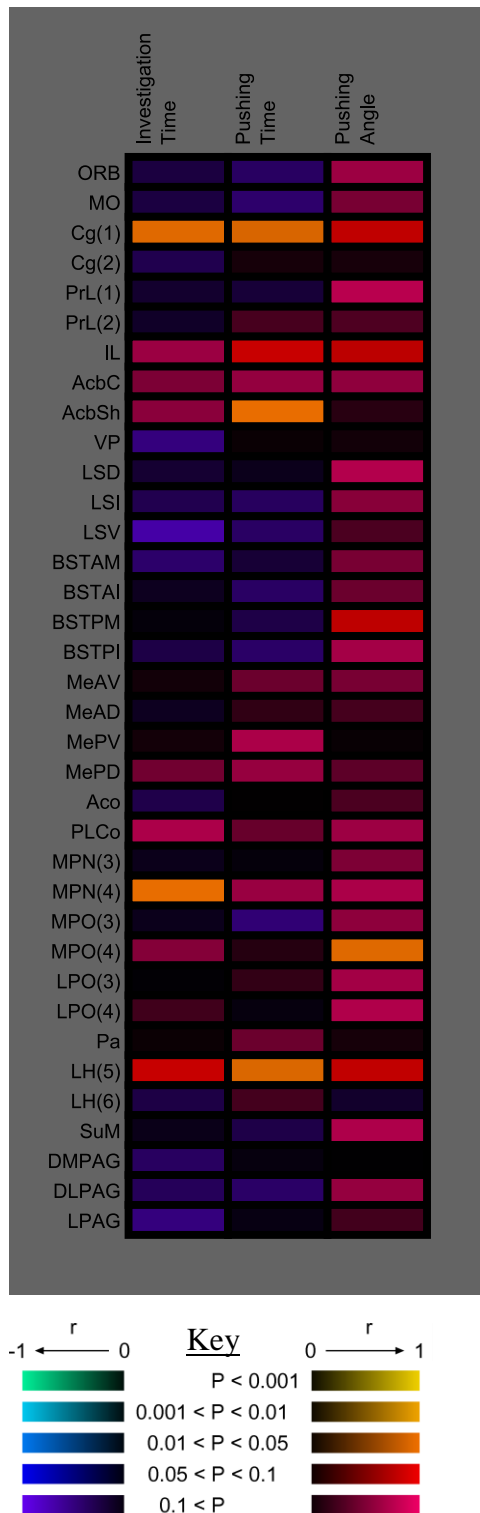
Figure 4.5: visual representation of the correlations between c-Fos activation in the investigated regions and the 3 behavioral measures (pushing time, investigation time, and pushing angle) for the control subjects (tested without a stimulus).
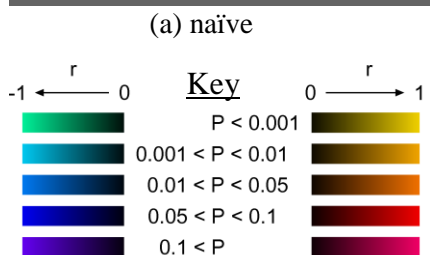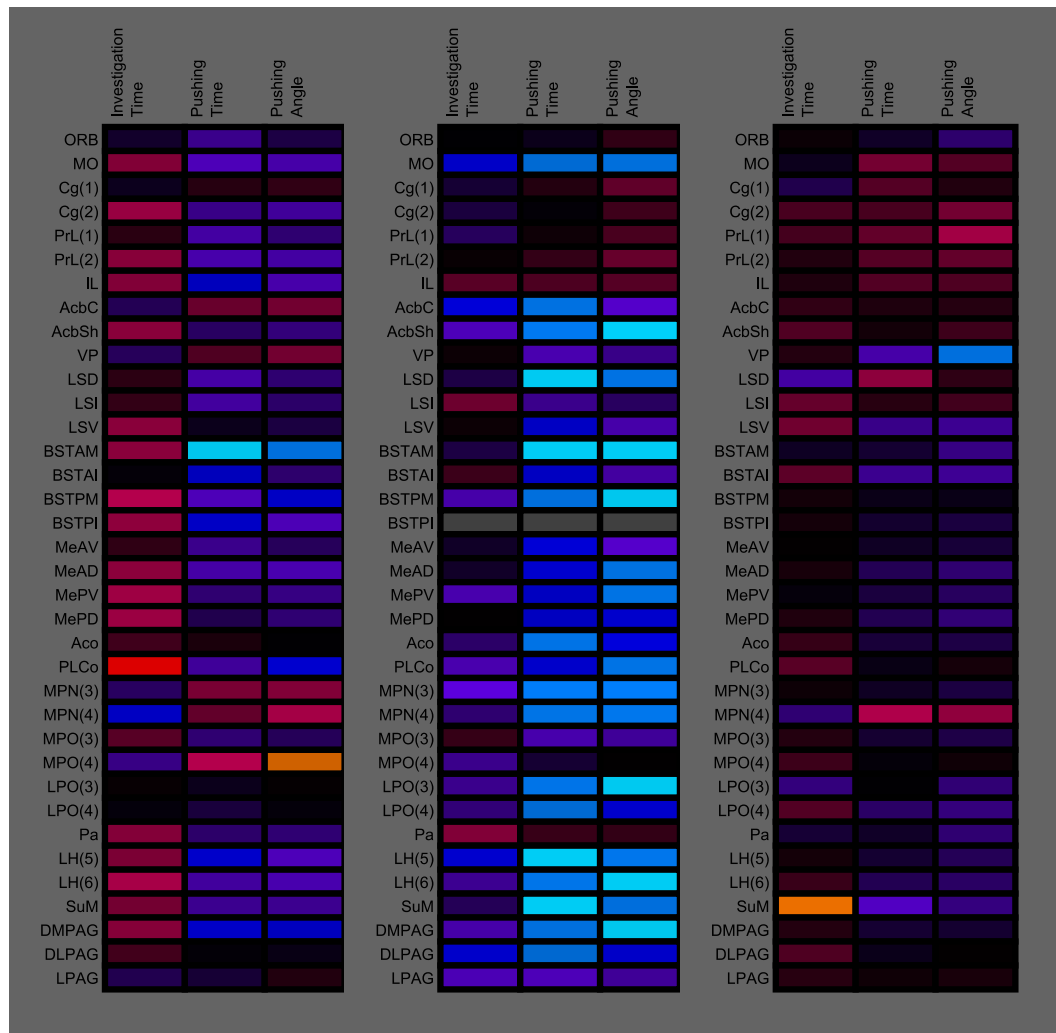
Figure 4.6: visual representation of the correlations between c-Fos activation in the investigated regions and the 3 behavioral measures (pushing time, investigation time, and pushing angle) towards a male stimulus for naïve subjects (a), winners (b), and losers (c).

pushing time and -0.3 for pushing angle; mostly strong for winners, -0.63 for pushing percentage and -0.57 for pushing angle; and very weak and without a clear tendency for losers, with average Pearson's r of -0.07 for pushing percentage and -0.13 for pushing angle. For the investigation percentage the groups showed some difference in the general direction of the correlations, being mostly positive for the naïve subjects with a mean Pearson's r of 0.25, mostly negative for the winners with a mean r of -0.32, and without a clear tendency for the losers with a mean of r=0.11.

Functional connectivity

To get a fuller understanding of the source of the negative correlations between pushing towards a male and neural activation throughout the various analyzed regions, we investigated the functional connectivity patterns between these regions. As could be seen in figure 4.7, the correlations ranged from very weakly negative (r=-0.23, NS) to very strongly positive (r=0.94), with the average correlation of r=0.35. The significant correlations were predominantly localized within the caudal/limbic regions, which for the most part did not correlate with the frontal cortical or striatal regions (the only exception was the MO, which significantly correlated with many limbic regions). The 4 regions that showed the strongest correlations with aggressive motivation (BSTAM, LH(5), LH(6), SuM) all showed significant correlations with one another (r>0.61, P≤0.01 for all), and interestingly they also each significantly correlated with the MO (r>0.62, P<0.01 for all).

A comparable functional connectivity matrix was generated for the control group (figure 4.8), revealing quite a different correlation pattern, without any clear bias in the localization of the stronger correlations but rather having them broadly distributed throughout, despite being of similar overall strength; these correlations ranged from r=-0.57 to r=0.98, with an average of
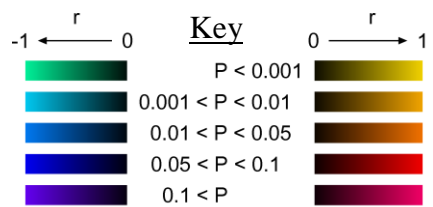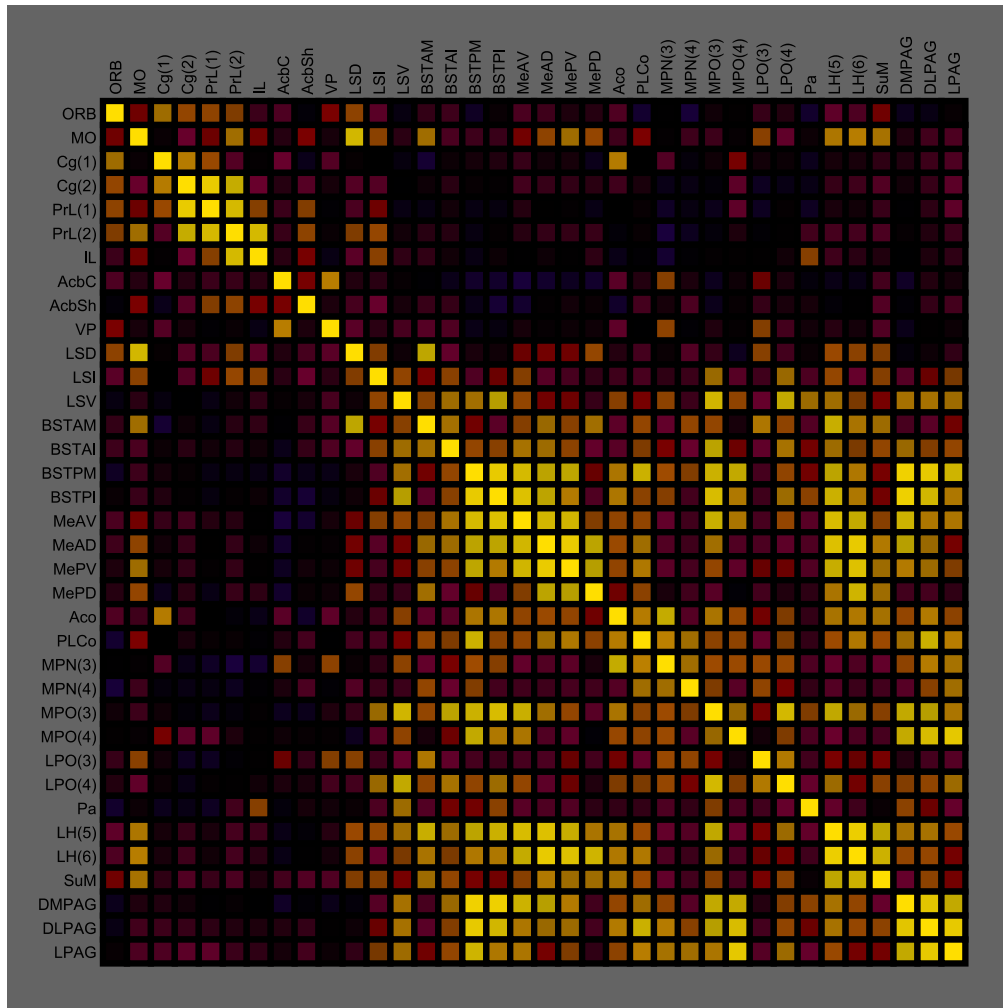
Figure 4.7: functional connectivity matrix (a visual representation of the correlations between levels of c-Fos activation across the various regions, with each square representing a correlation between two regions) for all subjects in the aggressive motivation condition.
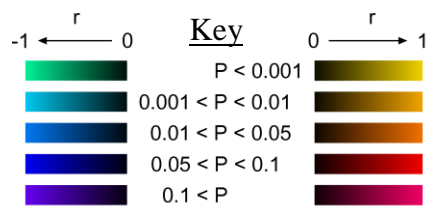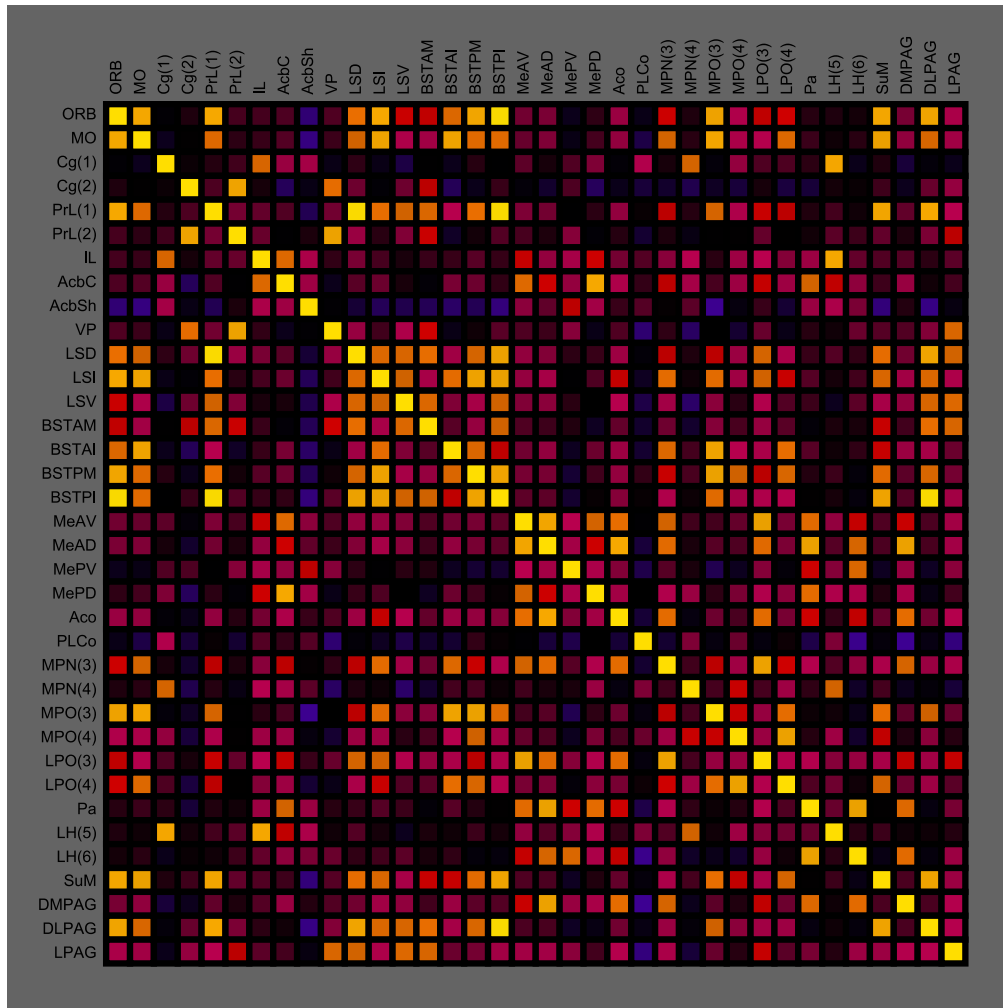
Figure 4.8: functional connectivity matrix (a visual representation of the correlations between levels of c-Fos activation across the various regions, with each square representing a correlation between two regions) for the control subjects (tested in the apparatus without a conspecific stimulus).

r=0.39 (the lower significances and higher variability could be attributed to the smaller sample size).

Discussion

Overall c-fos activation associated with our motivation test was quite low throughout the brain, with activation density 1-2 orders of magnitude lower in this study's subjects than in ones that underwent an actual fight (from the previous chapter). This difference may reflect that a fight is a far more significant experience than working towards a fight without actually undergoing one (only 4 subjects out of 18 got in with the male stimulus, all were taken out immediately). This absence of fighting may also underlie the apparent inactivation of the AH and VMH in this study – both of these regions have been proposed as critical for aggression (Adams, 2006; Lin et al., 2011), but it appears that they are more likely to be involved in the production of attack behavior rather than the motivation to engage in an aggressive encounter. The notable lack of activation in the BLA and CeA, however, was more likely due to the nature of the task itself, which was designed to be as unconditioned as possible (see chapters 1&2 for discussion), since these regions are typically involved in classical and operant conditioning paradigms (Balleine & Killcross, 2006).

Even with the low overall c-fos levels, the activation in a considerable number of regions still showed a significant association with the amount of pushing the subjects exerted on the door leading to the male stimulus. Although two separate and independent measures were used to quantify that pushing behavior, one for pushing duration and the other for pushing angle, most regions exhibiting significant correlations ended up correlating with both measures, supporting our assertion that both of them reflect aggressive motivation. Of the regions showing

associations with aggressive motivation, several are members of the "Social Behavior Network" (SBN, Newman, 1999), namely the LSV, the anterior BST (both medial and intermediate subnuclei), and the medial amygdala (the anterodorsal subnucleus was correlated with both measures, the posteroventral subnucleus correlated only with the pushing angle). In the previous chapter the BSTAM showed a significant correlation with fighting duration (mostly due to the correlation with opponent pursuit/investigation duration), and it yielded the strongest correlations from the group in the current analysis as well, likely implying that it is the SBN region most likely to play a key role in the neural mechanism of aggressive motivation. Prior studies have linked the entire BST to anxiety in general (Walker et al., 2003) and the expression of conditioned defeat specifically (Markham et al., 2009). Although our design did not expose the subjects to the sort of highly aversive stimuli that were used in those other paradigms, the direction of the effect is still compatible, with increased BST (specifically BSTAM) activation associated with decreased aggressive motivation and increased retreat behavior demonstrated by pushing towards the empty goal box.

Beyond the SBN regions, consistent associations with the pushing measures were also found in the lateral hypothalamic area (both the anterior and the posterior parts), and the strongest findings were in SuM nucleus of the hypothalamus, which was also one of the regions expressing the strongest c-fos activation. While some studies have linked the LH to aggression in the past (Kruk et al., 1983; Lammers et al., 1988), a careful inspection of their illustrations suggests that what they referred to as the LH more closely corresponds to the lateral part of the VMH (since most of the stimulations resulting in attacks were ventral to the fornix, not lateral to it). Rather, what we refer to as the LH here is a central component of the medial forebrain bundle (Nieuwenhuys et al., 1982), a major fiber system historically thought to be involved in reward

(Rolls, 1974). The SuM, which showed the strongest associations with aggressive motivation in the current study, connects to the LH via the MFB (Nieuwenhuys et al., 1982), which is in line with the strong correlations we found between these regions. Furthermore, the MFB connects to the BST and plenty of other limbic structures (Nieuwenhuys et al., 1982), likely underlying the correlations between LH activation and the activation of so many other regions found by the current study (figure 4.7). Of the frontal cortical regions, the MO was the only one whose activation correlated with our aggressive motivation measures, as well as with the activity in the LH, SuM, BSTAM, and others. Although not much is known about this region specifically, the strong correlations found in this study suggest that the MO is involved in this system. Ultimately, it appears that unconditioned aggressive motivation taps into the MFB system, and the current findings indicate that the SuM may be driving the effects seen in the other regions through its connections with the LH.

Irrespective of specific regions, the most striking discovery of this study was that none of the investigated regions exhibited a positive correlation between its c-fos activation levels and aggressive motivation. That was quite unexpected, especially given the broad selection of regions, encompassing several neural systems and ranging from the prefrontal cortex to the brainstem. Instead, the pattern that was identified involved a significant portion of the regions exhibiting negative correlations, with increased c-fos activation levels corresponding to lower aggressive motivation. Although c-fos expression is not a direct measure of neural activity (Sheng and Greenberg, 1990), it is still a widely utilized neural marker whose increased activation with relation to a control group is typically used to implicate specific neural regions with specific behavioral contexts (e.g. Kollack-Walker & Newman, 1995; Veening et al., 2005). Thus, a negative relationship between c-fos activation and aggressiveness is noteworthy even

independently from how closely it corresponds to actual neural firing. Importantly, our findings from the control group suggest that this pattern of negative correlations is not an artifact of our door-pushing task, since the subjects pushing in the absence of a conspecific exhibited mostly positive correlations between their pushing levels and c-fos activation. Furthermore, the pattern of negative correlations with aggressiveness levels found in the current study was in line with the pattern found in male-male fights in the previous chapter, and with similar negative correlations found in the literature (Goodson et al., 2005; Haller et al., 2006). Together, this evidence adds up to the conclusion that the inverse relationship between c-fos activation and aggressiveness is a real phenomenon, albeit quite counter-intuitive given the current state of our understanding.

A potential insight for the cause of the negative correlation pattern comes from a very recent study on aggression in humans. Using the point subtraction aggression paradigm (PSAP), a task evaluating aggression in response to provocation by a cheating virtual "opponent", the study found that dopamine levels correlated negatively with reactive (offensive and defensive) responses, but positively with "resilient" responses, i.e. ignoring the "opponent" and producing the response that yields them money (Vernaleken, 2012). Although our design did not offer the subjects any "rewards", subjects that pushed towards the empty goal box showed positive correlations between that pushing and activation in all 3 dopamine-related regions investigated here (AcbC, AcbSh, & VP, see Berridge, 2004). This suggests that the pursuit of aggressive interaction reflects a state where one is driven to react by a provoking stimulus, which comes at the expense of any actual goals that it could potentially pursue. Increased activation in the MFB system appears to inhibit this automatic tendency, freeing the subjects to pursue other goals (such as an alternative territory), which is what the dopamine system is associated with.

This idea is best exemplified by the non-aggressive subject from the winners group, the male that did not engage in a rolling fight with its opponent, and then during the motivation test went for the empty goal box. This subject's lack of aggressive motivation was associated with increased activation in all the regions that showed a significant effect (as could be seen in figure 4.3, where it is the winner with 0 pushing), while the other winners showed diminished neural activation, suggesting they were responding instinctively to the presence of a male without anything inhibiting them. This tendency was not as strong in the naïve group and was nearly non-existent in the losers group, which could perhaps be attributed to their having some anxiety-related factors at play (as discussed in chapter 1), which influenced their responses to the male stimuli through a different mechanism, not identified by the study. Ultimately, aggressive motivation seems to reflect the lack of inhibition from automatically responding to a provoking stimulus. Of course, this phenomenon is only starting to be identified, so more investigation is needed in order to unravel its mechanistic underpinnings.

Chapter 5: Neural mechanisms underlying sexual motivation

Introduction

As a direct test of the social behavior network hypothesis, we used our motivation paradigm to compare the neural activation patterns corresponding to sexual motivation to those found when assessing aggressive motivation. Since the subjects were engaged in the exact same behavior (pushing on doors) for equivalent amounts of time, with the behavior of the conspecific stimulus not being an integral factor of the overall behavioral pattern, this allowed for a more precise evaluation of the potential similarities in the activation patterns of two distinct social behaviors. The females used as stimuli were once again in diestrus, such that their behavior inside the goal box more closely resembles that of the male stimuli, unlike the fixed lordosis posture exhibited by females on estrus under the same circumstances. After investigating the neural activation following the loss to a diestrous female in chapter 3, here we tested the motivation of naïve subjects as well as subjects that lost such a fight. This allowed for the comparison of the neural activation in males under both conditions, in addition to providing a wider distribution of the motivation levels for the correlational analysis and producing a design that closely mimics the aggressive motivation study.

Methods

Behavioral component

The behavioral design, animals, apparatus, behavioral procedures, and behavioral data collection were the same as those described in chapter 2, only using 2 groups instead of 3 (naïve and losers to diestrus females), and with only 6 subjects per groups. Those 2 groups were compared to the 3 aggression groups and control group described in the previous chapter.

Neural component

The neural procedures (brain acquisition, slicing, immunohistochemistry staining, and data acquisition) were the same as those described in chapters 3&4, with the investigated regions being the same as the ones from the previous chapter.

Results

Behavioral

The behavioral measures from the motivation test are presented in table 5.1. It is clear that while the total test durations were similar to the ones found in the aggressive motivation study, the distributions of the behavioral measures within each of the sexual motivation groups is narrower than that found in any of the aggression groups. This lower variance contributed to the significant difference in mean pushing angle between the two groups and marginally significant difference in pushing time, despite their small sample size. A comparison between the motivation levels exhibited by all the subjects that were tested with a female (13 total) and the same measures from all subjects tested with a male (from the previous chapter, 18 total), there was no significant difference in investigation time (P>0.8) or mean pushing angle (P>0.3), but there was a highly significant difference in pushing time (P=0.0056), with subjects tested with females spending a significantly larger portion of their tests pushing towards them. A total of 4 subjects (out of 13) managed to get into the stimulus goal box, 3 of them from the naïve group (a rate similar to that found in chapter 2's behavioral study and to that from the aggressive motivation studies).

Table 5.1

	Naïve			Losers			P-value
	Mean	SD	Range	Mean	SD	Range	
Towards male stimulus:							
Investigation time (%)	0.30	0.05	0.18–0.34	0.41	0.16	0.25–0.73	0.154
Pushing time (%)	0.62	0.09	0.50–0.76	0.44	0.19	0.16–0.67	0.067
Pushing angle (rad)	0.30	0.04	0.23–0.36	0.23	0.06	0.13–0.35	0.046
Towards empty side:							
Investigation time (%)	0	0	0–0	0	0	0–0	NA
Pushing time (%)	0	0	0–0	0	0	0–0	NA
Pushing angle (rad)	0	0	0–0	0	0	0–0	NA
Total duration (sec)	126.9	49.1	59–180	110.7	47.8	39–180	0.59

Behavioral measures taken during the motivation tests for the 2 sexual motivation groups. The motivation measures were described in detail in chapter 1.

Neural

General considerations

As in the previous chapter, there were a few missing counts among the investigated regions for the 13 subjects, but once again there were at most 2 missing for each region (and within each group each region had counts from at least 5 subjects) so once again the analysis was carried out by omitting those. With regard to regions that showed no activation, those were the same as the ones found in the aggressive motivation study (BLA, BLP, Ce, AH, VMH), consequently excluding them from the current analysis as well.

Activation levels across the separate behavioral conditions

The mean activation density for each region for the two sexual motivation groups can be found in table 5.2, showing that despite the significant behavioral differences found between the groups, the vast majority of the investigated regions showed no difference in activation density between them. The differences that were found were mostly in the amygdala, where naïve subjects showed significantly higher activation than losers to females in its anterior medial subnuclei (both dorsal and ventral), and marginally significant trend in that direction in the

Table 5.2: Mean c-fos activation density (# neurons/mm^2)

	Naïve	Losers	P-value
Frontal cortex			
ORB	15 ± 10.1	29 ± 15.0	
MO	30 ± 10.6	24 ± 10.6	
Cg(1)	9 ± 3.7	5 ± 2.7	
Cg(2)	15 ± 6.2	11 ± 9.5	
PrL(1)	20 ± 6.6	9 ± 4.8	
PrL(2)	23 ± 11.6	18 ± 6.8	
IL	27 ± 11.5	14 ± 4.7	
Ventral striatum			
AcbC	13 ± 9.8	9 ± 3.1	
AcbSh	6 ± 2.1	3 ± 2.2	
VP	0 ± 0	1 ± 0.6	
Lateral septum			
LSD	41 ± 21.0	24 ± 9.7	
LSI	14 ± 6.3	7 ± 1.7	
LSV	10 ± 3.7	5 ± 2.4	
BNST			
BSTAM	17 ± 3.4	22 ± 6.6	
BSTAI	16 ± 4.8	7 ± 2.8	
BSTPM	11 ± 6.8	7 ± 3.6	
BSTPI	5 ± 3.6	4 ± 2.5	
Amygdala			
MeAV	53 ± 11.8	15 ± 3.0	0.006
MeAD	18 ± 5.3	3 ± 2.2	0.012
MePV	25 ± 7.3	13 ± 5.1	
MePD	14 ± 4.7	7 ± 2.2	
ACo	16 ± 3.8	17 ± 8.3	
PLCo	34 ± 21.6	6 ± 3.6	
PMCo	25 ± 8.0	6 ± 5.0	0.059
Hypothalamus			
MPN(3)	27 ± 2.1	26 ± 7.9	
MPN(4)	38 ± 16.2	36 ± 18.1	
MPO(3)	17 ± 7.8	21 ± 5.0	
MPO(4)	2 ± 1.1	8 ± 2.6	0.036
LPO(3)	21 ± 7.0	20 ± 5.4	
LPO(4)	30 ± 7.2	24 ± 7.0	
Pa	82 ± 43.7	46 ± 13.9	
LH(5)	31 ± 9.5	25 ± 8.2	
LH(6)	40 ± 21.9	16 ± 4.8	
SuM	116 ± 40.5	91 ± 35.6	
PAG			
DMPAG	6 ± 3.9	8 ± 2.9	
DLPAG	1 ± 0.8	3 ± 1.7	
LPAG	7 ± 2.6	5 ± 2.6	

Activation density (M±SE) and P-value from a student's t-test (only P-values under 0.1 are noted).

PMCo. The only other significant effect was for lower activation in naïve subjects compared to the losers to females in the MPO, but only in its more posterior part.

To find how the neural activation in the sexual motivation condition compares to that in the aggressive motivation condition we compared the activation levels found when combining both of the sexual motivation groups with the levels found when combining the 3 aggression motivation groups (from the previous chapter). This analysis showed that the mean activation for most of the regions was not significantly different, although the mean activation was higher in the aggression condition for all but 4 of the regions; a paired t-test confirmed that the mean activation for the aggression condition was higher than that of the sexual motivation condition (P<0.0005). As to the specific regions, the difference in activation between the two conditioned reached significance in the VP, LSV, and Pa, and was marginally significant in the PRL(2), IL, ACo, DLPAG, and LPAG (with the aggression condition exhibiting higher activation for all of them).

Correlations between neural activation and behavior

Pushing towards a (diestrous) female

To further contrast the neural mechanism associated with sexual motivation with that of aggressive motivation, we conducted the same type of correlational analysis as we did in the previous chapter using the subjects tested with a female, which resulted in a directly comparable correlation matrix (see figure 5.1). Unlike the overall pattern of predominantly negative correlations found across all regions for the pushing measures in the aggression condition, the correlations between activation and the pushing towards a female did not show any clear tendency, with Pearson's r ranging from -0.58 to 0.58 (with a mean of -0.03) for pushing time and from -0.54 to 0.72 (with a mean of 0.07) for pushing angle. A similar distribution of
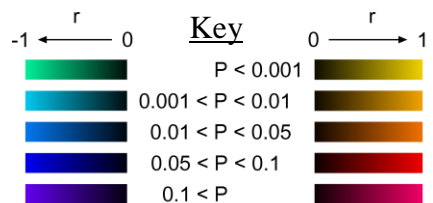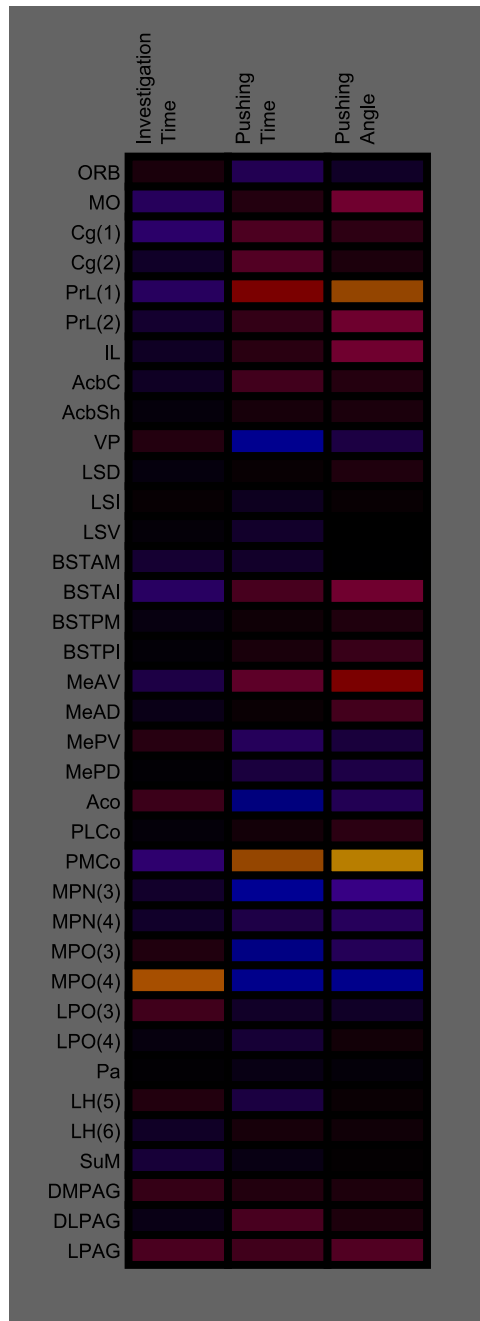
Figure 5.1: visual representation of the correlations between c-Fos activation in the investigated regions and the 3 behavioral measures (pushing time, investigation time, and pushing angle) towards a diestrous female stimulus. The deeper the color the greater the r value, with different significance levels (determined by the r value and the sample size, using thresholds from a table) denoted by different hues (one scale for positive correlations and another for negative, see Key).

correlations was found for investigation time as well, with Pearson's r ranging from -0.43 to 0.65 (with a mean of -0.05). Clearly, only a few of these correlations were strong enough to reach significance, most markedly in the PMCo, which exhibited the strongest correlations with both pushing measures, r=0.58 (with P=0.046) for pushing time and r=0.72 (with P=0.008) for pushing angle. The anterior part of the PrL showed a correlation of 0.58 (P=0.038) with pushing angle, but only a marginal positive trend for pushing time (r=0.48, P=0.09). Lastly, activation in the posterior part of the MPO correlated positively with investigation time (r=0.65, P=0.015), while showing nearly significant negative correlations with pushing time (r=-0.55, P=0.054) and with pushing angle (r=-0.54, P=0.055).

Correlations within each of the groups

To test whether there may have been a qualitative difference in the correlation pattern between the two behavioral groups we ran the same analysis for each of them separately (see figure 5.2). This analysis showed that for the naïve subjects the correlations between neural activation and investigation time were predominantly positive, ranging from -0.51 to 0.81 (with a mean of 0.31), but without a clear tendency for pushing time (ranging from -0.95 to 0.64 with a mean of -0.08) and for pushing angle (ranging from -0.85 to 0.66 with a mean of 0.02). Conversely, the correlation pattern found in subjects that lost to a female did not show any overall tendency for any of the behavioral measures, ranging from -0.62 to 0.59 (with a mean of -0.09) for investigation time, from -0.84 to 0.75 (with a mean of -0.16) for pushing time, and from -0.93 to 0.81 (with a mean of -0.07) for pushing angle.

Of all regions the most notable was the posterior medial amygdala, which showed correlations with pushing behavior for each of the groups. For the naïve group activation in the MePD negatively correlated with pushing time (r=-0.95, P=0.003) and with pushing angle (r=-0.85,
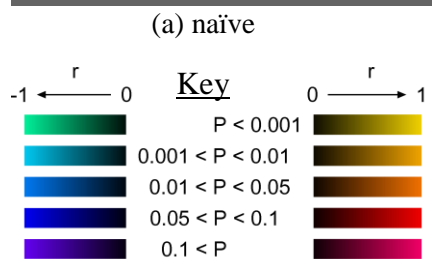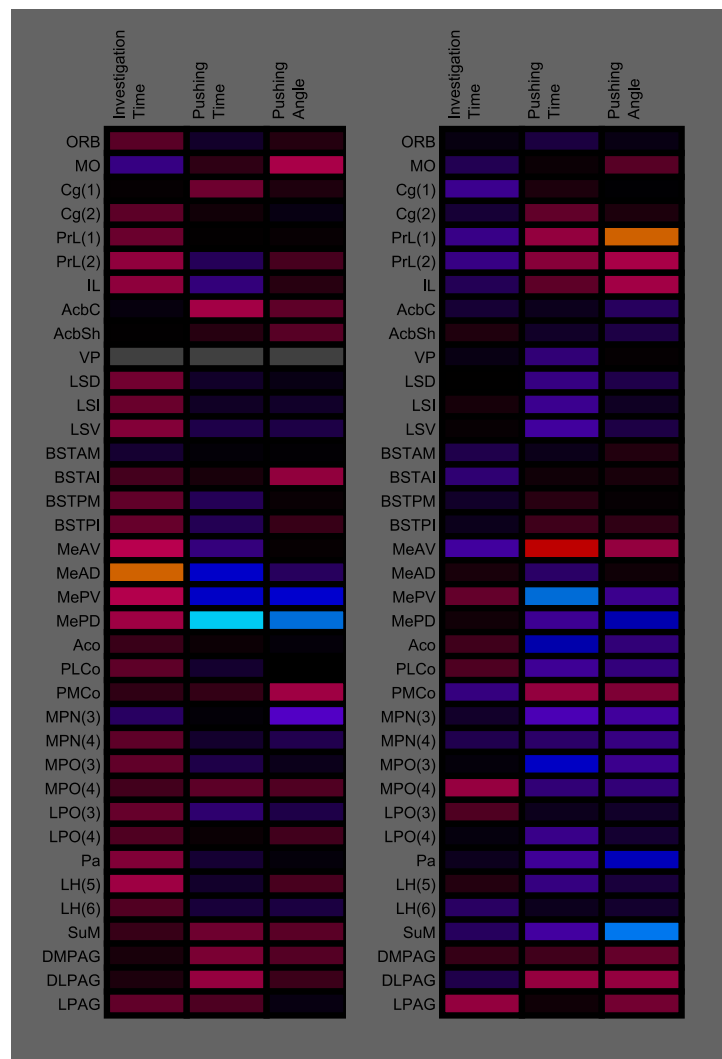
Figure 5.2: visual representation of the correlations between c-Fos activation in the investigated regions and the 3 behavioral measures (pushing time, investigation time, and pushing angle) towards a diestrous female stimulus for naïve subjects (a) and losers to females (b).

P=0.03), while activation in the MePV negatively correlated with these measures only marginally (r=-0.78, P=0.07 for pushing time, r=-0.81, P=0.052 for pushing angle). For the losers to females activation in the MePV negatively correlated only with pushing time (r=-0.84, P=0.017) without any additional significant correlations in the medial amygdala, but significant correlations with pushing angle were found in the anterior part of the PrL (r=0.81, P=0.027) and in the SuM (r=-0.93, P=0.021).

Focus on the PMCo and medial amygdala

Since the most substantial correlations between activation and pushing were found in the PMCo for the two groups combined while for each group individually they were in the MeP(V&D), we plotted each of these correlations in order to take a closer look at them (see figure 5.3). The plots show that while the PMCo was more active in the naïve group than the losers to females, its activation seem to more closely correspond to the level of pushing exhibited by the subjects, which is most apparent in the intermediate pushing levels where the two groups overlap (see figure 5.3 a&b). In contrast, the correlation pattern in the posterior medial amygdala was entirely different, with the range of activation levels being similar between the two groups, while higher activation was associated with decreased pushing within each of them, each within its own range of pushing levels (see figure 5.3 c&d for the MePD and 5.3 e&f for the MeAV).
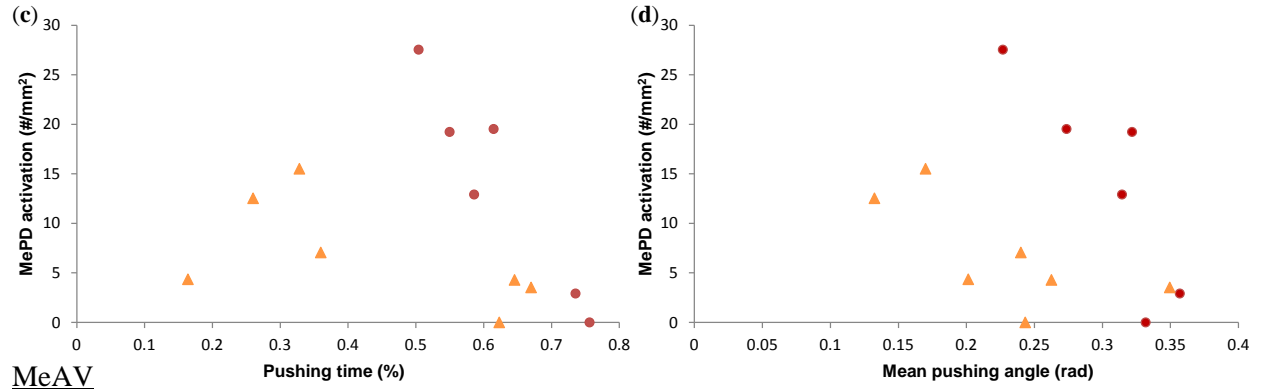
Functional connectivity

We concluded our analysis by constructing a functional connectivity matrix for the sexual motivation subjects, to see whether the way the regions interacted with each other under this condition was similar to what was found in the aggressive motivation condition (previous chapter, figure 5.4). It is clear from figure 4 that like their correlations with the behavior, the correlations between the activation in the various regions was broadly distributed, ranging from
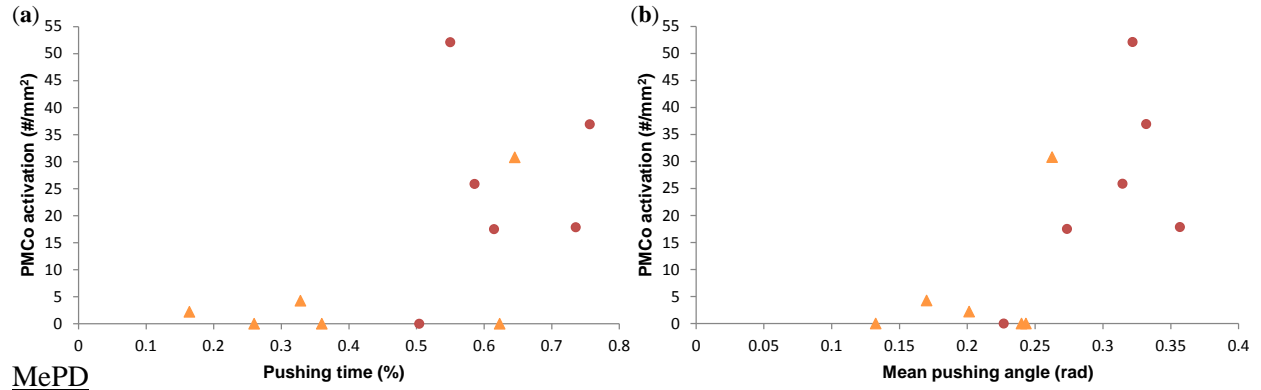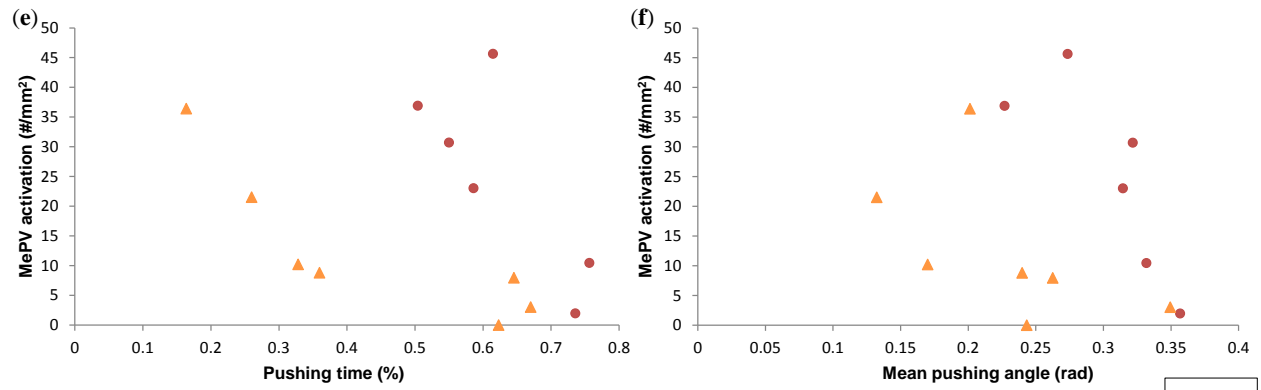
Figure 5.3: scatterplots of c-fos activation density as a function of pushing time and mean pushing angle, for each of the 2 groups, in the PMCo (a&b), MeAD (c&d), and MeAV (e&f).
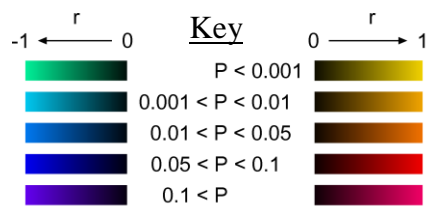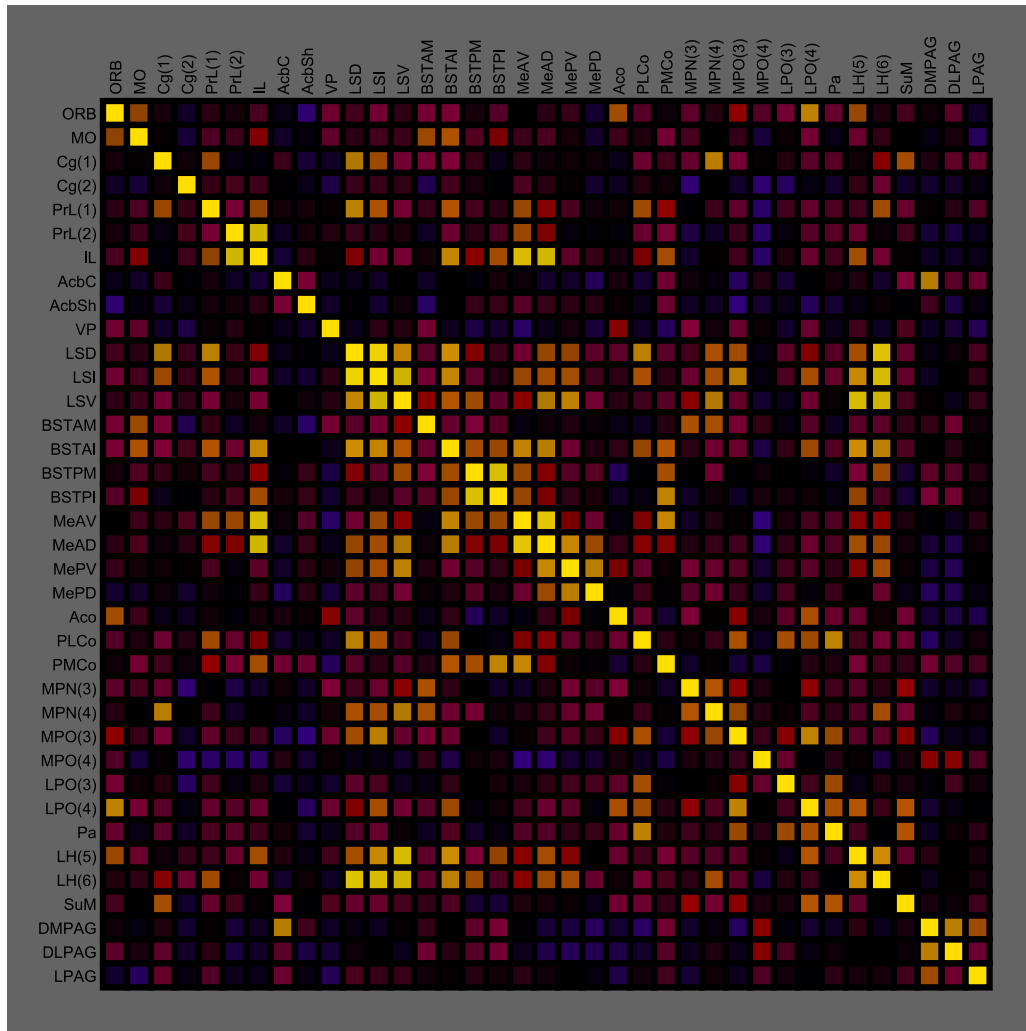
121

Figure 5.4: functional connectivity matrix (a visual representation of the correlations between levels of c-Fos activation across the various regions, with each square representing a correlation between two regions) for all subjects in the sexual motivation condition.

moderately negative (r=-0.47, NS) to very strongly positive (r=0.94). Although many of the correlations reached significance, they were not organized in any overall pattern, unlike the broadly coordinated correlations found in the aggression condition. This was largely due to the fact that correlations were generally weaker in the sex condition, averaging around r=0.21.

Discussion

Testing the neural mechanisms underlying unconditioned sexual motivation using the exact same behavioral paradigm used to test aggressive motivation allowed for the most direct comparison between the two that's been carried out so far, and has yielded mixed results regarding the similarities and differences between these two social motivations.

While subjects tested with a female spent a significantly larger portion of their trials pushing towards her than did subjects tested with a male, the mean activation across the investigated regions tended to be lower in the sexual motivation condition in nearly all of them. This was likely due to the fact that the highest activations in the aggression condition were generally found in the subjects that showed the lowest levels of motivation towards their stimuli – levels of motivation that were hardly displayed by any of the subjects that were tested with a female. Comparing between the two behavioral groups used in the current study, on the other hand, revealed that while subjects that lost to a female tended to exhibit lower levels of sexual motivation than naïve subjects, they also tended to exhibit lower mean activation in most of the investigated regions (see table 5.2), though this trend only reached significance in 2 of the regions (and one region showed a significant difference in the opposite direction). This was entirely unlike the general tendency found in the aggression condition, where the mean activation

for the losers group was higher than those in the naïve group (and even more so the winners group) for most of the investigated region (see table 4.2).

Evaluating the correlations between neural activation and our behavioral measures of sexual motivation the same way we did with aggressive motivation revealed further differences between the two conditions, with the sexual motivation condition not exhibiting anything resembling the overall negative correlations pattern found in the aggression condition, instead showing no pattern at all. The few correlations that did reach significance were all positive, something that was not seen anywhere in the aggressive motivation condition. Further analyzing the correlations found within each of the groups yielded a slightly different picture, since the pattern of correlations for the losers to females did resemble the pattern found in the losers from the aggression condition, including a significant negative correlation between pushing and SuM activation (though on the pushing measure that yielded the weaker correlation in the aggression condition). Meanwhile the naïve group showed even weaker overall correlations, unlike the naïve group in the aggression condition that showed stronger correlations than the losers. The naïve groups in both conditions did have something in common however – they both showed generally positive correlations between investigation time and activation in the various regions, a pattern that was not seen in any of the other groups. This is likely related to their lack of any social experience for an extended period of time prior to the test. As such, mere investigation of a conspecific (regardless of sex) through a barrier was somewhat different for them than for the subjects that got to interact with a conspecific the previous day. However, it is impossible to determine based on the current data what these generally positive (though for the most part non-significant) correlations reflect.

Specific regions showing significant associations with our motivation measures were primarily located in the corticomedial amygdala, specifically in the PMCo and the subnuclei of the medial amygdala. The PMCo was the only region showing a significant positive correlation with pushing behavior, a finding that is made difficult to interpret due to the fact that not much is known of this region beyond its involvement in some aspects of the mating pattern without an effect on preference for female odor (Maras and Petrulis, 2008). The anterior medial amygdala, which projects to the PMCo (Maras and Petrulis, 2010), did not show such positive correlation, although it did exhibit significantly lower activation in the losers-to-females compared to the naïve subjects. This region is known to receive olfactory inputs from the main and accessory olfactory systems and plays a central role in the diminished interest in male odors compared to female odors, leading to the suggestion that it is involved in gauging of the sexual relevance of odors (Maras and Petrulis, 2006). The posterior medial amygdala, which receives input from the anterior subdivision (Maras and Petrulis, 2010), has been implicated in mating experience with the MePD being the subdivision showing increased activation following mating or exposure to female odors (Fernandez-Fewell and Meredith, 1994; Kollack-Walker and Newman, 1996). This region was suggested to be specifically involved in facilitating sexual motivation since lesions of the MeP resulted in decreased attraction of the male subjects to female odors (Maras and Petrulis, 2006). Our findings are consistent with that view since the MeP showed significant correlation with sexual motivation (with the MePD showing the strongest correlations, particularly for the naïve group), meanwhile the losers viewed the diestrous females as less viable prospect, in accordance with their decreased MeA activation. Our findings in the PMCo suggest that it plays a role in sexual motivation as well, however it is unclear what this role may be.

General discussion

Our findings on the neural activation associated with aggressive vs. sexual motivation in our motivation apparatus, taken together with the findings from males that fought with a female or with a male, can be used to evaluate some of the conjectures underlying the social behavior network theory. The central component of that theory, suggesting that several brain regions are associated with each behavior and that each region can be associated with a variety of behaviors, is undisputed, however the way in which regions are associated with behaviors appears to be far more complicated than previously thought. In an aggressive context, a wide variety of regions (not limited to ones included in the original SBN formulation) seem to be activated in consortion, exhibiting broad functional connectivity. When pursuing a female in diestrus, on the other hand, although engaged in a near-identical behavioral pattern, the functional connectivity was far sparser and generally lower throughout, without much overlap with the pattern seen in the aggression condition. Interestingly, the overall connectivity patterns seen in subjects tested for aggressive motivation in our apparatus and in subject that engaged in a complete fight appeared qualitatively (visually) similar, with the same being true for the subjects that pushed for a female compared to the subjects that fought with a female. This is despite the fact that the behavioral output was entirely different between the two contexts, and that overall activation levels were an order of magnitude higher following fights compared to door-pushing.

The functional connectivity findings closely corresponded to the correlations found between the neural activation and the behavioral measures of the respective tests, with the aggression conditions being associated with stronger and more uniform correlations than the sexual motivation conditions. These correlations were, however, almost exclusively negative. Taken together, these findings cast serious doubt on one of the central assertions of the SBN

framework, which proposed that the differences in neural activation patterns between the various social behaviors are specifically linked to the differences in behavioral output (Newman, 1999; Goodson & Kabelik, 2009), with "behavioral 'decisions' [being] generated via the weighting of activity across the nodes" of the network (Goodson & Kabelik, 2009, page 429). This idea was, of course, mostly meant to stand in opposition to the idea that there would be individual regions that determine specific behaviors, instead emphasizing the importance of networks in an overly-simplified manner (Goodson & Kabelik, 2009). Most c-Fos studies are designed as a comparison between the activation following target behaviors and the baseline activation of handled controls, so it is tempting to interpret overlaps in regions showing increased activation in different behaviors as reflecting that increased activation in such regions result in the production of those behaviors. Our findings suggest that what happens in reality is likely to be far more complicated, since (a) increased activation was most often associated with a decrease in the behavior being investigated, and (b) the correlation patterns appear to be more closely associated with the motivational state and/or the nature of the stimulus, rather than with the specific behavioral output. Moreover, the differences observed in the neural correlation patterns between our aggression and sexual studies suggest that the degree of coordination between regions may be highly dependent on how in-line the behavior is with the motivation. It is well established that animals can not be trained to produce any conceivable action for any motivation, that some behaviors are easier or harder to train depending on the species-typical behaviors of the animal (Epstein, 1982). It is possible that fighting and possibly even the exertion of considerable physical effort are contradictory to sexual motivation at the neural level, as it has been proposed that attack and mating are mutually-inhibitory (Anderson, 2012). This would mean that the task selected to examine the neural underpinning of any motivation could have a substantial effect on

the resulting neural activation, regardless of how readily the subjects produce it under the experimental circumstances.

The precise method used is also likely to influence the findings with regard to the neural mechanisms of motivation in general. While all prior investigations into the neural mechanism underlying motivation tended to highlight the importance of the ventral striatum system, namely the nucleus accumbens and/or ventral pallidum (Berridge, 2004), in our study neither aggressive motivation nor sexual motivation showed any involvement of this system. The critical difference in our approach was that we isolated the motivation itself from the conditioning that teaches the individual that producing a specific behavior would cause the incentive to appear. It is possible that the ventral striatum dopamine system is necessary specifically when one has to act without being triggered by the unconditioned incentive stimulus. In our study the subjects that pushed towards an empty goal box were the only ones showing correlations with activation in the ventral striatum, and these correlations were in fact positive, unlike the correlations with pushing towards a stimulus. Unconditioned approach to an incentive stimulus, however, does not seem to require this system, something that has not been previously uncovered since all studies of motivation and its neural underpinnings have used conditioning paradigms where the subjects responded in the absence of the incentive stimulus. Clearly, more studies are necessary to determine whether these findings reflect general principles that apply to different types of unconditioned motivations and under different experimental paradigms.

REFERENCES

Ågmo, A. 2003. Unconditioned sexual incentive motivation in the male Norway rat (Rattus norvegicus). Journal of Comparative Psychology 117(1):3-14

Ågmo, A., Turi, A. L., Ellingsen, E. & Kaspersen, H. 2004. Preclinical models of sexual desire: conceptual and behavioral analyses. Pharmacology Biochemistry and Behavior 78(3):379-404

Amstislavskaya, T. G. & Popova, N. K. 2004. Female-induced sexual arousal in male mice and rats: behavioral and testosterone response. Hormones and Behavior 46(5):544-50

Anderson, D. J. 2012. Optogenetics, sex, and violence in the brain: implications for psychiatry. Biological psychiatry 71(12):1081-9

Archer, J. 1988. The behavioural biology of aggression. Cambridge University Press

Avgustinovich, D. F., Gorbach, O. V. & Kudryavtseva, N. N. 1997. Comparative analysis of anxiety-like behavior in partition and plus-maze tests after agonistic interactions in mice. Physiology & behavior 61(1):37-43

Ballard, C. L. & Wood, R. I. 2007. Partner preference in male hamsters: steroids, sexual experience and chemosensory cues. Physiology and behavior 91(1):1-8

Balleine, B. W. & Killcross, S. 2006. Parallel incentive processing: an integrated view of amygdala function. Trends in Neurosciences 29(5):272-9

Beach, F. A. & Jordan, L. 1956. Effects of sexual reinforcement upon the performance of male rats in a straight runway. Journal of Comparative and Physiological Psychology 49(2):105-10

Beach, F. A. 1942. Analysis of the stimuli adequate to elicit mating behavior in the sexually inexperienced rat. Journal of Comparative Psychology 33(2):163-207

Beach, F. A. 1956. Characteristics of masculine"sex drive". Nebraska Symposium on Motivation, 4:1-32

Beach, F. A. 1976. Sexual attractivity, proceptivity, and receptivity in female mammals. Hormones and Behavior 7(1):105-38

Beck, R. C. 1978. Motivation. New York: Prentice-Hall

Berridge, K. C. 2004. Motivation concepts in behavioral neuroscience. Physiology & Behavior, 81:179-209

Bindra, D. 1974. A motivational view of learning, performance, and behavior modification. Psychological Review 81(3):199-213

Brain, P. & Poole, A. 1974. Some Studies on the Use of "Standard Opponents" in Intermale Aggression Testing in TT Albino Mice. Behaviour 50(1-2):100-110

Carmichael, M. S. 1980. Sexual discrimination by golden hamsters (Mesocricetus auratus). Behavioral and Neural Biology 29(1):73-90

Cheu, J. W. & Siegel, A. 1998. GABA receptor mediated suppression of defensive rage behavior elicited from the medial hypothalamus of the cat: role of the lateral hypothalamus. Brain Research 783(2):293-304

Connor, J. L. 1974. Waning and recovery of conspecific aggression in the house mouse (Mus musculus L). Journal of Comparative and Physiological Psychology 87(2):215-227

Couppis, M. H. & Kennedy, C. H. 2008. The rewarding effect of aggression is reduced by nucleus accumbens dopamine receptor antagonism in mice. Psychopharmacology (Berl) 197(3):449-56

de Almeida, R. M., Ferrari, P. F., Parmigiani, S. & Miczek, K. A. 2005. Escalated aggressive behavior: dopamine, serotonin and GABA. European Journal of Pharmacology 526(1-3):51-64

Dickinson, A. & Balleine, B. 1994. Motivational control of goal-directed action. Animal Learning and Behavior 22:1-18

Dickinson, A. & Balleine, B. 2002. The role of learning in the operation of motivational systems. In: Pashler, H. & Gallistel, R. (Eds.), Stevens' handbook of Experimental Psychology, Third ed. 3:497-533. New York: John Wiley & Sons

Epstein, A. N. 1982. Instinct and motivation as explanations for complex behavior. In: Pfaff, D. (Ed.), The physiological mechanisms of motivation, Springer-Verlag 25-55

Everitt, B. J., Fray, P., Kostarczyk, E., Taylor, S. & Stacey, P. 1987. Studies of instrumental behavior with sexual reinforcement in male rats (Rattus norvegicus): I. Control by brief visual stimuli paired with a receptive female. Journal of Comparative Psychology 101(4):395-406

Fernandez-Fewell, G.D. & Meredith, M. 1994. c-fos expression in vomeronasal pathways of mated or pheromone-stimulated male golden hamsters: contributions from vomeronasal sensory input and expression related to mating performance. The Journal of Neuroscience 14(6):3643-54

Ferris, C. F., Stolberg, T., Kulkarni, P., Murugavel, M., Blanchard, R., Blanchard, D. C., Febo, M., Brevard, M. & Simon, N. G. 2008. Imaging the neural circuitry and chemical control of aggressive motivation. BMC Neuroscience 9:111

Finger, S. 2001. Origins of neuroscience: a history of explorations into brain function. Oxford University Press, USA

Fish, E. W., De Bold, J. F. & Miczek, K. A. 2002. Aggressive behavior as a reinforcer in mice: activation by allopregnanolone. Psychopharmacology (Berl) 163(3-4):459-66

Floody, O. R. & Pfaff, D. W. 1977. Aggressive behavior in female hamsters: the hormonal basis for fluctuations in female aggressiveness correlated with estrous state. Journal of Comparative and Physiological Psychology 91(3):443-64

Frischknecht, H. R., Siegfried, B. & Waser, P. G. 1982. Learning of submissive behavior in mice: A new model. Behavioural Processes 7:235-245

Ginsburg, B. & Alee, W. C. 1942. Some effects of conditioning on social dominance and subordination in inbred strain of mice. Physiological Zoology 15:485-506

Goodson, J. L. & Kabelik, D. 2009. Dynamic limbic networks and social diversity in vertebrates: from neural context to neuromodulatory patterning. Frontiers in Neuroendocrinology 30(4):429-41

Goodson, J. L., Evans, A. K. & Soma, K. K. 2005. Neural responses to aggressive challenge correlate with behavior in nonbreeding sparrows. Neuroreport 16(15):1719-23

Haller, J., Tóth, M., Halasz, J. & De Boer, S. F. 2006. Patterns of violent aggression-induced brain c-fos expression in male mice selected for aggressiveness. Physiology & Behavior 88(1-2):173-82

Hetta, J. & Meyerson, B. J. 1978. Sexual motivation in the male rat: a methodological study of sex-specific orientation and the effects of gonadal hormones. Acta physiologica Scandinavica. Supplementum 453:1-67

Hinde, R. A. 1969. The bases of aggression in animals. Journal of psychosomatic research 13(3):213-219

Huck, U. W., Lisk, R. D., Kim, S. & Evans, A. B. 1989. Olfactory discrimination of estrous condition by the male golden hamster (Mesocricetus auratus). Behavioral and Neural Biology 1(1):1-10

Johnston, R. E. 1977. Sex pheromones in golden hamsters. In: Muller-Schwarze, D. & Mozell, M. M. (Eds.), Chemical Signals in Vertebrates, New York: Plenum Press 225-249

Johnston, R. E. 1980. Responses of male hamsters to odors of females in different reproductive states. Journal of Comparative and Physiological Psychology 94(5):894-904

Jowaisas, D., Taylor, J., Dewsbury, D. A. & Malagodi, E. F. 1971. Copulatory behavior of male rats under an imposed operant requirement. Psychonomic Science 25(5):287-290

Kagan, J. 1955. Differential reward value of incomplete and complete sexual behavior. Journal of Comparative and Physiological Psychology 48(1):59-64

Kelsey, J. E. & Cassidy, D. 1976. The reinforcing properties of aggressive vs. nonaggressive social interactions in isolated male ICR mice (Mus musculus). Aggressive Behavior 2(4):275-284

Kollack-Walker, S. & Newman, S. W. 1995. Mating and agonistic behavior produce different patterns of Fos immunolabeling in the male Syrian hamster brain. Neuroscience 66(3):721-36

Kollack-Walker, S., Watson, S. J. & Akil, H. 1997. Social stress in hamsters: defeat activates specific neurocircuits within the brain. Journal of Neuroscience 17(22):8842-55

Kruk, M. R. 1991. Ethology and pharmacology of hypothalamic aggression in the rat. Neuroscience and Biobehavioral Reviews 15(4):527-38

Kruk, M. R., Van der Poel, A. M., Meelis, W., Hermans, J., Mostert, P. G., Mos, J. & Lohman, A. H. 1983. Discriminant analysis of the localization of aggression-inducing electrode placements in the hypothalamus of male rats. Brain Research 260(1):61-79

Kudryavtseva, N. N. 2004. Lorenz was right! or Does aggressive energy accumulate? Russian Journal of Genetics 40(6):656-562

Kudryavtseva, N. N., Bondar, N. P. & Alekseyenko, O. V. 2000. Behavioral correlates of learned aggression in male mice. Aggressive Behavior 26: 386-400

Lagerspetz, K. 1964. Studies on the aggressive behaviour of mice. Annales academie scientiarum fenniae 131:1-131

Lai, W. S. & Johnston, R. E. 2002. Individual recognition after fighting by golden hamsters: a new method. Physiology & behavior 76(2):225-39

Lammers, J. H., Kruk, M. R., Meelis, W. & van der Poel, A. M. 1988. Hypothalamic substrates for brain stimulation-induced attack, teeth-chattering and social grooming in the rat. Brain Research 449(1-2):311-27

Landauer, M. R., Banks, E. M. & Carter, C. S. 1978. Sexual and olfactory preferences of naive and experienced male hamsters. Animal Behaviour 26(2):611-621

Legrand, R. 1970. Successful aggression as the reinforcer for runway behavior of mice. Psychonomic Science 20:303-305

Leshner, A. I. & Nock, B. L. 1976. The effects of experience on agonistic responding: an expectancy theory interpretation. Behavioral Biology 17(4):561-6

Lin, D., Boyle, M. P., Dollar, P., Lee, H., Lein, E. S., Perona, P. & Anderson, D. J. 2011. Functional identification of an aggression locus in the mouse hypothalamus. Nature 470(7333):221-6

Lisk, R. D., 1985. The estrous cycle. In: Siegel, H. I. (Ed.), The Hamster: Reproduction and Behavior, Plenum Press, New York 23-51

Lisk, R. D., Zeiss, J. & Ciaccio, L. A. 1972. The influence of olfaction on sexual behavior in the male golden hamster (Mesocricetus auratus). Journal of Experimental Zoology 181(1):69-78

Lorenz, K. 1966. On Aggression. New York: Harcourt Brace and World

López, H. H., Olster, D. H. & Ettenberg, A. 1999. Sexual motivation in the male rat: the role of primary incentives and copulatory experience. Hormones and Behavior 36(2):176-85

Maras, P. M. & Petrulis, A. 2006. Chemosensory and steroid-responsive regions of the medial amygdala regulate distinct aspects of opposite-sex odor preference in male Syrian hamsters. European Journal of Neuroscience 24(12):3541-52

Maras, P. M. & Petrulis, A. 2008. The posteromedial cortical amygdala regulates copulatory behavior, but not sexual odor preference, in the male Syrian hamster (Mesocricetus auratus). Neuroscience 156(3):425-35

Maras, P. M. & Petrulis, A. 2010. The anterior medial amygdala transmits sexual odor information to the posterior medial amygdala and related forebrain nuclei. European Journal of Neuroscience 32(3):469-82

Markham, C. M., Norvelle, A. & Huhman, K. L. 2009. Role of the bed nucleus of the stria terminalis in the acquisition and expression of conditioned defeat in Syrian hamsters. Behavioural Brain Research 198(1):69-73

Martinez, M., Salvador, A. & Simon, V. M. 2006. Behavioral changes over several successful agonistic encounters between male mice: Effects of type of "standard opponent". Aggressive Behavior 20(6):441-451

Mendelson, S. D. & Pfaus, J. G. 1989. Level searching: a new assay of sexual motivation in the male rat. Physiology and Behavior 45(2):337-41

Miczek, K. A., Maxson, S. C., Fish, E. W., & Faccidomo, S. 2001. Aggressive behavioral phenotypes in mice. Behavioural brain research, 125(1), 167-181

Miczek, K. A. & O'Donnell, J. M. 1978. Intruder-Evoked Aggression in Isolated and Nonisolated Mice - Effects of Psychomotor Stimulants and L-Dopa. Psychopharmacology 57(1):47-55

Morin, L. P. & Wood, R. I. 2001. A stereotaxic atlas of the golden hamster brain. Academic Press, New York

Moss, F. A. 1924. Study of animal drives. Journal of Experimental Psychology 7(3):165-185

Nelson, R. J. & Trainor, B. C. 2007. Neural mechanisms of aggression. Nature Reviews Neuroscience 8:536-546

Newman, S. W. 1999. The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. Annals of the New York Academy of Sciences 877:242-57

Nieuwenhuys, R., Geeraedts, L. M. & Veening, J. G. 1982. The medial forebrain bundle of the rat. I. General introduction. The Journal of Comparative Neurology 206(1):49-81

Nowack, R. M. & Paradiso, J. L. 1983. Walker's Mammals of the World. John Hopkins University Press, Baltimore, MD

O'Connell, L. A. & Hofmann, H. A. 2011. The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. The Journal of Comparative Neurology 519(18):3599-639

Oyegbile, T. O. & Marler, C. A. 2005. Winning fights elevates testosterone levels in California mice and enhances future ability to win fights. Hormones & Behavior 48(3):259-267

Parmigiani, S. & Brain, P. F. 1983. Effects of residence, aggressive experience and intruder familiarity on attack shown by male mice. Behavioural Processes 8(1):45- 57

Payne, A. P. & Swanson, H. H. 1970. Agonistic behaviour between pairs of hamsters of the same and opposite sex in a neutral observation area. Behaviour 36(4):260-9

Payne, A. P. & Swanson, H. H. 1972. The effect of sex hormones on the agonistic behavior of the male golden hamster (Mesocricetus auratus Waterhouse). Physiology and Behavior 8(4):687-91

Payne, A. P. 1974. The aggressive response of the male golden hamster towards males and females of differing hormonal status. Animal Behaviour 22(4):829-35

Petrulis, A., Weidner, M. & Johnston, R. E. 2004. Recognition of competitors by male golden hamsters. Physiology & behavior 81(4):629-38

Pfaff, D. & Ågmo, A. 2002. Reproductive motivation. In: Pashler, H. & Gallistel, R. (Eds.), Stevens' handbook of Experimental Psychology, Third ed. New York: John Wiley & Sons 3:709-36

Pfaus, J. G., Kippin, T. E. & Centeno S. 2001. Conditioning and sexual behavior: a review. Hormones and Behavior 40(2):291-321

Potegal, M. 1979. The reinforcing value of several types of aggressive behavior: a review. Aggressive Behavior 5:353-373

Potegal, M., Huhman, K., Moore, T. & Meyerhoff, J. 1993. Conditioned defeat in the Syrian golden hamster (Mesocricetus auratus). Behavioral and Neural Biology 60(2):93- 102

Rabedeau, R. G. 1963. Development of sexual behaviour in the male hamster. Canadian Journal of Psychology 17:420-9

Rasa, O. A. E. 1976. Aggression: Appetite or aversion? An ethologist's viewpoint. Aggressive Behavior 2(3):213-222

Rolls, E. T. 1974. The neural basis of brain-stimulation reward. Progress in Neurobiology 3:71-82

Sachs, B. D. 2007. A contextual definition of male sexual arousal. Hormones and Behavior 51:569-78

Schwartz, M. 1956. Instrumental and consummatory measures of sexual capacity in the male rat. Journal of Comparative and Physiological Psychology 49(4):328-33

Scott, J. P. 1966. Agonistic behavior of mice and rats: A review. American Zoologist, 6(4), 683-701

Scott, J. P. & Fredericson, E. 1951. The Causes of Fighting in Mice and Rats. Physiological Zoology 24(4):273-309

Scott, J. P. 1971. Theoretical Issues Concerning the Origin and Causes of Fighting. In: Eleftheriou, B. E. & Scott, J. P. (Eds.), The Physiology of Aggression and Defeat, New York: Plenum 11-42

Seward, J. P. 1945. Aggressive behavior in the rat: II. An attempt to establish a dominance hierarchy. Journal of Comparative Psychology 38:213-224

Sheffield, F. D., Wulff, J. J. & Backer, R. 1951. Reward value of copulation without sex drive reduction. Journal of Comparative and Physiological Psychology 44(1):3-8

Sheng, M., & Greenberg, M. E. 1990. The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4, 477-485

Siegel, A. & Pott, C. B. 1988. Neural substrates of aggression and flight in the cat. Progress in Neurobiology 31(4):261-83

Steel, E. 1979. Male-female interaction throughout the oestrous cycle of the Syrian hamster (Mesocricetus auratus). Animal Behaviour 27(3):919-29

Stone, C. P., Barker, R. G. & Tomlin, M. I. 1935. Sexual drive in potent and impotent males as measured by the Columbia obstruction box method. The Journal of Genetic Psychology 65:461-465

Takahashi, L. K. & Lisk, R. D. 1983. Organization and expression of agonistic and socio-sexual behavior in golden hamsters over the estrous cycle and after ovariectomy. Physiology and Behavior 31(4):477-82

Teitelbaum, P. 1966. The use of operant methods in the assessment and control of motivational states. In: Honig, W. K. (Ed.), Operant behavior: Areas of research and application, New York: Apple ton-Century-Crofts 565-608

Tellegen, A. & Horn, J. M. 1972. Primary aggressive motivation in three inbred strains of mice. Journal of Comparative and Physiological Psychology 78(2):297-304

Tellegen, A., Horn, J. M. & Legrand, R. G. 1969. Opportunity for aggression as a reinforcer in mice. Psychonomic Science 14(3):104-105

Toates, F. 1986. Motivational Systems. Cambridge University Press, Cambridge

Toates, F. 2009. An integrative theoretical framework for understanding sexual motivation, arousal, and behavior. Journal of Sex Research 46(2-3):168-93

Van Furth, W. R. & Van Ree, J. M. 1996. Appetitive sexual behavior in male rats: 2. sexual reward and level-changing behavior. Physiology and Behavior 60(3):1007-12

Veening, J. G., Coolen, L. M., de Jong, T. R., Joosten, H. W., de Boer, S. F., Koolhaas, J. M. & Olivier, B. 2005. Do similar neural systems subserve aggressive and sexual behaviour in male rats? Insights from c-Fos and pharmacological studies. European Journal of Pharmacology 526(1-3):226-39

Vega Matuszczyk, J. & Larsson, K. 1993. Sexual orientation and sexual motivation of the adult male rat. Physiology and behavior 53(4):747-50

Walker, D. L., Toufexis, D. J. & Davis, M. 2003. Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. European Journal of Pharmacology 463(1-3):199-216

Whalen, R. E. 1961. Effects of mounting without intromission and intromission without ejaculation on sexual behavior and maze learning. Journal of Comparative and Physiological Psychology 54:409-15