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Investigation of potential applications of odorant receptor (OR) 37 ligands in mice and animal welfare refinement.

Dandri Aly Purawijaya

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Doctor of Philosophy in the Faculty of Life Sciences.

School of Physiology, Pharmacology and Neuroscience.

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Abstract

Members of the evolutionary conserved odorant receptor (OR) 37 family of mouse olfactory receptors are known to have an unusual direct projection to the paraventricular nucleus (PVN) of the hypothalamus, via the main olfactory bulb. Previous research has shown that the mixture of three OR37 ligands were effective in reducing the activation of the neurons in the PVN following novel cage exposure, similar to conspecifics' natural secretion. This effect suggests that the OR37 ligands may play a role in reducing the stress axis response by a potential social buffering effect via olfactory cues. This study investigates the acute exposure of the OR37 ligands' effect and shows that the reduction of the PVN activation could be done in different anxiogenic tests without affecting the behaviours and the endocrine responses.

Laboratory conditions and environments have been known to be a source of stressors for mice: by socially isolating the mice, which potentially reduces the amount of olfactory cues exposure, by routine handling, and by an ambient laboratory temperature that does not reflect mice's thermoneutral zone. This investigation then explores the chronic application of the OR37 ligands as a potential enrichment for socially isolated mice and showed a buffering effect on the social isolation-induced temperature change without other behavioural changes due to the lack of social isolation-induced changes. Finally, this investigation explores other husbandry refinements including mice preference towards housing temperature to group nesting size preference, and mouse stress responses to tail-handling. The investigation on the housing temperature preference showed a negative correlation to the group nesting size preference, while the tail-handling responses did not show a change in affect but to overt behaviours associated with anxiety-related state. Although further studies will be needed before drawing any significant conclusions, this thesis provides preliminary data towards better understanding of the OR37 system and of animal welfare refinement.

Dedication and Acknowledgements

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To my grandfather, who gave me the push to send the email to Pete and applying for the scholarship, while he was in the hospital:

"Sekolahlah kamu setinggi-tingginya, supaya kamu bisa menjadi contoh dan memotivasi keluarga besar kita." [Go for the best education you can have, so that all your siblings and cousins will be motivated and also pursue higher education, for them and their kin]

- Djakaria Purawijaya, PhD

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations* and *Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .... DATE:..........................

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Abbreviations

3Rs	Replacement, Reduction, and Refinement
ABC	avidin-biotin complex
ACTH	adrenocorticotropic hormone
AOB	accessory olfactory bulb
AOS	accessory olfactory system
ASPA	Animals (Scientific Procedures) Act
ATP	adenosine triphosphate
AVP	arginine-vasopressin
BAOT	bed nucleus of accessory tract
BNST	basal nuclei of stria terminalis
BNST	bed nucleus of the stria terminalis
cAMP	adenosine 3'-5'-cyclic monophosphate
CBBT	Cotton Bud Biting Test
cGMP	cyclic guanosine monophosphate
CI	confidence interval
CNG	cyclic-nucleotide gated
CORT	corticosterone
CPP	conditioned place preference
CRH	Corticotropin Releasing Hormone
CVS	Chronic Variable Stress
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
EC3	extracellular three
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
GC-D	guanylyl cyclase-D
GG	Grueneberg ganglion
HPA	hypothalamic-pituitary-adrenal
IEG	Immediate Early Gene
JC	Juxtaglomerular Cells
LAL	Long Attack Latency
MC2R	melanocortin-2-receptors

MeA	medial amygdala
MePD	posterior dorsal part of the medial
MOB	main olfactory bulb
MOE	main olfactory epithelium
MOS	main olfactory system
MUP	major urinary proteins
NG	necklace glomeruli
NSFT	Novelty Suppressed Feeding Test
OMP	olfactory marker proteins
OR	Odorant Receptor
OSN	olfactory sensory neurons
OXY	oxytocin
PBS	phosphate-buffered saline
PEA	phenylethylamine
PMCoA	posteromedial cortical amygdala
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
RIA	Radioimmunoassay
RIT	Resident-Intruder Test
SAL	Short Attack Latency
SBT	2-sec-butyl-4,5-dihydrothiazole
SEM	standard error of the mean
SO	septal organ
SOT	Standard Opponent Test
TAAR	trace amine-associated receptors
TBS	tris-buffered saline
TBST	0.1% triton-x in TBS
TMT	trimethylthiazoline
UPP	Unconditioned Place Preference
VMHyp	ventromedial nuclei of hypothalamus
VNO	Vomeronasal organ
VSN	vomeronasal sensory neurons
ZM	Zero Maze Test

Chapter 1: General Introduction

Improving the welfare of laboratory rodents is a key objective within the context of the Animal (Scientific Procedure) Act 1986 and the 3Rs (Replace, Reduce, Refine). The nature of the laboratory environment and scientific needs place a number of limitations on the environment for laboratory rodents. The need to house large numbers of animals in an environment where high standards of hygiene can be maintained and the animals can be easily observed for routine health and wellbeing does not come without compromising on animal welfare. However, there are potential ways in which the environment and routine management of these animals may be refined and this thesis seeks to explore some of these. The majority of the studies undertaken focus on the potential use of olfactory chemosignals to reduce stress and enhance the welfare of the laboratory mice through provision of putative social buffering pheromones. The final series of studies looks into the improvement of animal welfare through refinement of mice housing temperature and their handling method, which can provide objective measures of the welfare impacts of housing temperature and different handling methods.

1.1. Olfactory system and OR37 subsystem

1.1.1. Olfactory system

The olfactory system is used by animals to perceive chemical stimulation. Across all mammals and rodents, mice in particular, have become one of the most prominent animal models for studying vertebrate chemical communication (Chamero, Leinders-Zufall, & Zufall, 2012). Chemical signals from the environment are sensed by the olfactory system and elicit diverse behavioural responses, including communication, navigation, foraging, and avoiding predators (Brechbuhl et al., 2013; Brennan & Keverne, 2004; Wyatt, 2014; Yang & Crawley, 2009).

The olfactory system senses chemicals in the environment by their stimulation of receptors in the membrane of sensory neurons and are then transduced into electrical signals conveyed by axons to the olfactory bulb in the brain. The signals will then be relayed to the appropriate brain region for the related physiological or behavioural

responses (Martini & Nath, 2010). The mouse olfactory system consists of different systems, which are: the main olfactory system (MOS), the accessory olfactory system (AOS), the Grueneberg ganglion (GG), and the septal organ, (Wyatt, 2014). These olfactory systems have quite similar, yet distinctive roles in behavioural outputs. The information from different olfactory systems will then be integrated in the higher brain area to produce appropriate responses to the stimulation from the odorant molecules or the chemical mixtures in different contexts.

1.1.1.1. Mouse main olfactory system (MOS)

The olfactory system in general consists of olfactory sensory neurons (OSNs) in the main olfactory epithelium (MOE), which are located in the upper nasal cavity. The OSNs express a single olfactory receptor type, which has a broad response range overlapping with other receptors. The OSNs work in combination with different receptor types to convey the information about the identity of the odorants to the olfactory bulb, which is located in the anterior part of the brain cortex (figure 1.1).

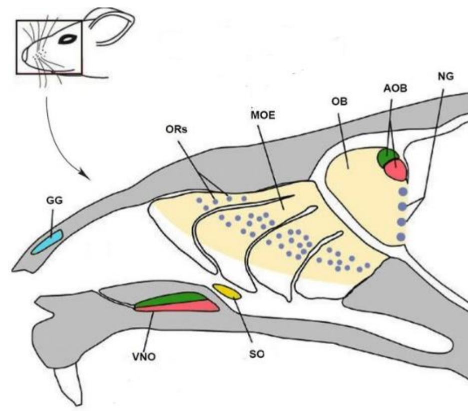

Figure 1.1 : Mouse olfactory system. Grueneberg Ganglion (GG), Vomeronasal organ (VNO), septal organ (SO), olfactory receptors (ORs), main olfactory epithelium (MOE), olfactory bulb (OB), accessory olfactory bulb (AOB), necklace glomeruli (NG). Image adapted from Hussain (2011). Copyright © 2011, CC BY 3.0.

The OSN is a bipolar neuron with cilia on the apical side, which is covered with a mucus layer produced by the bowman gland, and axon on the other side projecting to the olfactory bulb. The mucus layer facilitates odorant binding proteins to bind on dissolved odorant and assist the odorant receptors in the membrane of the cilia (Martini & Nath, 2010). Surrounding the OSNs are the supporting cells that make up the structure of the upper nasal cavity with basal cells attached to the lamina propria,

which regenerate, replacing the worn-out olfactory sensory neurons (Martini & Nath, 2010). The receptors on the cilia are G-protein coupled receptors, which recognise different shapes of odorant molecules and when activated, lead to the activation of adenylyl cyclase (Martini & Nath, 2010). The cascade of the adenylyl cyclase converts adenosine triphosphate (ATP) into adenosine 3'-5'-cyclic monophosphate (cAMP), which targets the cyclic-nucleotide gated (CNG) non-selective cation channel on the plasma membrane, allowing the entry of sodium ions, and depolarising receptor potential and increases action potential firing rate at cell soma (Martini & Nath, 2010). The OSN axon projects through the cribriform plate of the skull to synapse in the glomeruli of the main olfactory bulb (MOB).

The olfactory bulb can be divided into the main olfactory bulb and the accessory bulb. Both sections have similar functions in encoding the odorant information, however, they have different sources of input. The main olfactory bulb consists of different layers, which are the glomerular layer, the plexiform layer, and the granular layer. The glomerular layer contains a bundle of synaptic connections called glomeruli, which is the first level of synaptic processing of the main olfactory system (Hamilton et al., 2005). Individual glomeruli receive information from a single olfactory receptor type creating a distinct odour map, which produces different patterns of glomerular activity in response to different odorants. Information from the OSNs arriving at the olfactory glomeruli will then be relayed to the mitral cells, which are located on the plexiform layer or to tufted cells, which are located in the external plexiform layer (Scott et al., 1993). Together with the periglomerular cells, the granule cells, which are located on the granular layer, will contribute to a process called lateral inhibition of the mitral cells, refining the pattern of activity in the mitral cells within the main olfactory bulb into other processing centres, such as piriform cortex (Schröder et al., 2020; Scott et al., 1993). The cells in piriform cortex are sensitive to patterns of activity across the population of mitral cells, which they associate to form representation of odour objects, which will then be processed on other brain regions to associate odour objects with meaning and context. It is to be noted that not all MOB outputs project to this general odour analysing system, such as the odorant receptor (OR) 37 family that projects to the hypothalamus (Klein et al., 2015). The mitral cells also responded to input from social odorants and projects to subcortical regions such as amygdala and basal nuclei

of stria terminalis (BNST) for social recognition and pheromonal responses (Ferguson et al., 2001).

The classical olfactory transduction pathway is dependent upon the G-protein coupled receptors, with the G-protein called G_{olf}, in the OSNs that utilize the cAMP pathway cascade. However, more recent findings show that there are a small population of sensory neurons on the MOE that lack the G_{olf} protein (Liberles & Buck, 2006). These receptor neurons, expressing *Taar* genes instead, are classified into a new type of olfactory receptor, the trace amine-associated receptors (TAARs) that recognize volatile amines (Liberles & Buck, 2006). It was considered as an important finding due to these receptors responding to specific amines found in predator urine, such as phenylethylamine (PEA), or to amines that have been proposed to have pheromonal effects (Dewan et al., 2013; Liberles & Buck, 2006). This suggests that MOE also plays a role in social chemo signal communications.

1.1.1.2. Mouse accessory olfactory system (AOS)

Another olfactory system found within mice is the AOS, also known as the vomeronasal system. As shown in figure 1.1, the AOS vomeronasal sensory neurons are located in a different area to the OSNs within the MOS. The primary sensory neurons of the AOS are located within the vomeronasal organ (VNO). The VNO, also known as Jacobson's organ, is located in the nasal septum of the nasal cavity and above the oral palate (D'Aniello et al., 2017; Schröder et al., 2020). Within the VNO, there are two types of primary sensory neurons, apical and basal, which are distinguished by their position relative to the surface and the receptor types they express (figure 1.2). The apical sensory neurons express the $G\alpha_{i2}$ protein and are classified as V1R receptor subtype. The basal sensory neurons express the $G\alpha_{0}$ protein and are classified as V2R receptor subtype (Knoll et al., 2001; Leinders-Zufall et al., 2004; Schröder et al., 2020; Zufall & Munger, 2001). When these primary sensory neurons are activated by molecules, the information will be relayed to the accessory olfactory bulb (AOB), which is located in the dorsoposterior region of the MOB (Schröder et al., 2020). Stimulation of the vomeronasal sensory neurons (VSNs) will be relayed to the AOB, bypassing the MOB, with the apical V1r-expressing VSNs to the anterior part of the AOB and the basal primary sensory neurons to the posterior part of the AOB. Similar to the processing pathway of the MOB, the first level of the synaptic processing happens in the olfactory glomeruli within the glomerular layer of the AOB. Signals

from the VSN terminals in the olfactory glomeruli will then be relayed to the mitral/tufted cells, which is then projected to specific nuclei of the medial amygdala (MeA), posteromedial cortical amygdala (PMCoA), bed nucleus of the stria terminalis (BNST), and bed nucleus of accessory tract (BAOT) (Brennan, 2010; Knoll et al., 2001).

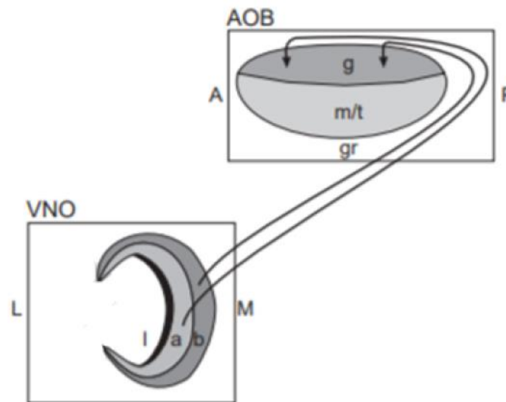

Figure 1.2 : Vomeronasal sensory neuron divisions and their projections to accessory olfactory bulb. Vomeronasal Organ (VNO), Lateral (L), Medial (M), lumen (l), apical (a), basal (b), Accessory Olfactory Bulb (AOB), Anterior (A), Posterior (P), glomerular layer (g), mitral/tufted cells (m/t), granular cell layer (gr). Image adapted from Knoll et al. (2001) with modification and permission. Copyright © John Wiley and Sons, 2005.

In early studies, the projection from the medial amygdala nuclei to hypothalamus was thought to be through the subcortical region of the stria terminalis, bypassing the cortical region, and shaped the conception of exclusively separate pathways for the MOS and the AOS (Mucignat et al., 2012; Raisman, 1972). This misconception of exclusivity between MOS and AOS was also reinforced with studies and hypotheses regarding the position of the MOE and the VNO and the nature of the odorant molecular weight and volatility. It was hypothesised that the location of the MOE facilitates the reception of airborne odorant with low molecular weight while the VNO is more likely to be receptive to odorant molecules with heavier molecular weight and low volatility due to mechanical pumping mechanism and the location of the VNO that sits in a liquid-filled lumen area (Meredith & O'Connell, 1979; Mucignat et al., 2012). Although they are specialised to respond to airborne stimuli sensed at a distance for MOE versus fluid borne stimuli that require direct contact with scent source for VNO, there is an overlap in the stimuli that they respond to. More recent studies show the exclusivity between MOS and AOS was proven to be incorrect, since it was found that

the MOE also reacted to similar chemo signals that commonly processed by the AOS and vice-versa that volatile odorants molecule can be detected by both the MOS and AOS such as the TAARs system (Liberles & Buck, 2006; Lin et al., 2004; Mucignat et al., 2012; Pro-Sistiaga et al., 2007; Trinh & Storm, 2004; Wang et al., 2006). The neural projections are also not as separate as the dual pathway hypothesis would suggest. The projection of both AOB and MOB are both converging to the same neurons in the MeA and extensive interconnectivity between main olfactory and vomeronasal regions of amygdala (Baum & Kelliher, 2009).

1.1.1.3. Mouse grueneberg ganglion and mouse septal organ

In recent years, it was known that the olfactory system cannot be simply divided into two exclusively separate pathways of MOS and AOS (Mucignat et al., 2012; Raisman, 1972). Recent studies also show that there is a small population of olfactory receptor neurons that expressed *Taar* genes instead of G_{olf} on the MOE (Liberles & Buck, 2006). There are another small population of olfactory sensory neurons that lacked the G_{olf} and the cAMP pathway, and in its place having guanylyl cyclase-D (GC-D) and cyclic guanosine monophosphate (cGMP) specific subunits that are independent to G_{olf} and cAMP pathway (Juilfs et al., 1997; Leinders-Zufall et al., 2007; Meyer et al., 2000). Another distinct characteristic of the GC-D neurons is that they project to the necklace glomeruli, which are located in the caudal region of the main olfactory bulb (Cockerham, Puche, & Munger, 2009; Meyer et al., 2000). It was later found that GC-D neurons are stimulated by the CO_2 that permeates the membranes and are changed into bicarbonate (Sun et al., 2009).

It was found that there were other receptors that share similar cGMP signalling pathways to GC-D neurons outside the MOE, which was identified as a cluster of sensory neurons on the rostral tip of the nose (see figure 1.1) and are called Grueneberg ganglions (GG) (Grüneberg, 1973; Liu et al., 2009; Meyer et al., 2000). Despite its unusual location outside the MOS and the AOS, GG expresses olfactory marker proteins (OMP), a protein that is specifically expressed by mature chemosensory systems (Fleischer et al., 2006). GG neurons were found to be projecting their axons to the caudal area of the main olfactory bulb, which is the same area where the necklace glomeruli are located (Liu et al., 2009). However, there were distinct differences between the necklace glomeruli that received input from the MOE and the one with input from the GG (Liu et al., 2009). Due to the receptors being activated by CO_2, it

was first suggested that the GG function was to detect CO_2 (Liu et al., 2009). However, a further study by Brechbühl, Klaey, and Broillet (2008) showed that exposure to an alarm chemo signal from another individual within the same species evoked the calcium channel stimulated a response in the GG.

Finally, another system within the olfactory system is the septal organ. There were not many studies regarding the septal organ, other than the known location and anatomical structure. A study done by Kaluza et al. (2004) showed that the septal organ expressed a similar olfactory receptor type that is also expressed on MOE. There was no evidence suggesting the septal organ expressed VNO receptor families. Additionally, a study done by Ma et al. (2003) shows that signal transduction properties of septal organs mainly use similar majority cAMP pathway and small amount of cGMP pathway as seen on the MOE. It was also seen that the septal organ projects into the main olfactory bulb, and the only difference, other than the position just slightly rostral from the VNO (figure 1.1), was the shape of the receptor neurons morphology, which were flattened somata and shorter dendrites than the OSNs. There were not many studies regarding the function of the septal organ. However, due to its location and similarities with the MOE, it was suggested that septal organ assists the detection system of MOE during quiet respiration and to low volatility compounds (Ma et al., 2003; Wysocki, Wellington, & Beauchamp, 1980).

1.1.2. Olfaction to physiology and behaviour

The olfactory system conveys information about odours and chemosignals that elicit behavioural and/or physiological responses. Wyatt (2014) categorised the chemosignals by the benefits to sender and receiver. Chemosignals that involve members of different species are called allelochemicals with further distinction into allomones, kairomones and synomones, according to which species benefits from the communication. On the other hand, chemosignals that involve different members of the same species are classified as pheromones. The chemosignals categorisation is described as shown on figure 1.3.

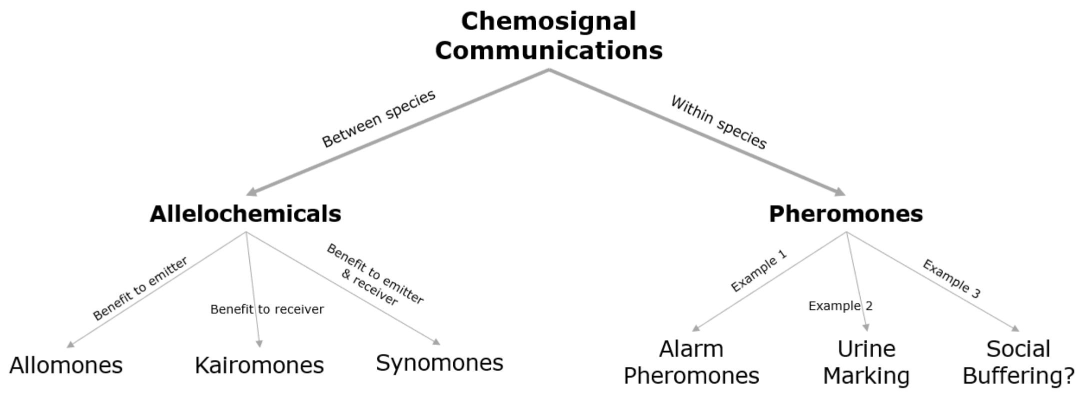

Figure 1.3 : Different categories of chemosignals according to the involved subjects. Image adapted from Wyatt (2014)

Examples of allelochemicals in nature can be observed during inter-species encounters, such as during prey and predator encounters. An example of allomone in mammals is the emission of the anal sac secretions as a defensive spray by striped skunks to repel predators (Wood, 1999). While the example of kairomone in mammals is the induction of fear and freezing behaviour in mice due to the exposure to fox faeces, which is identified to contain 2,4,5-trimethylthiazoline (TMT) (Wallace & Rosen, 2000). It was then identified that the GG system was responding the highest to the TMT, despite the MOE and the VNO can also detected the TMT and inducing freezing responses in mice (Brechbuhl et al., 2013). Lastly, an example of synomones is in relationships between plants, herbivores, and carnivores. When plants are being eaten by herbivores, some species of plants produced more extrafloral nectar which is a signal for predators regarding the presence of herbivores as their prey (Heil & Kost, 2006).

Similarly, such an example of pheromones in nature can be observed during intra-species communications. The first example of pheromonal interaction is alarm pheromones, which are volatile compounds that are secreted by prey animals (such as mice) to alert their conspecifics regarding the presence of a predator. It was later discovered that the mouse alarm pheromone was 2-*sec*-butyl-4,5-dihydrothiazole (SBT) which has a similar structure and triggered a similar pathway as the active compounds that is available in a predator's urine or faeces (Brechbuhl et al., 2013). Another example of a more common pheromonal interaction function is during the territorial marking of mice interactions. It was found that the constituent of the urine, notably termed as major urinary proteins (MUPs), a family of lipocalin ligand binding proteins, plays important role in conveying individuality (more typical MUPs) and

male pheromonal attraction to females (atypical MUPs, such as Darcin) (Roberts et al., 2010). The MUPs prevented the male mice from marking the territory that had been marked with their own MUPs and promoted counter marking behaviour in a territory with MUPs from other mice (Kaur et al., 2014). Although urine marks left by male could be considered as a competitive signal to other males, a study done by Klein et al. (2015) shows that the secretions of conspecifics could be used to reduce the stress response level during novel environment stress. The stress reduction effect by having a conspecific presence is known as social buffering. It was previously thought that the presence of individuals during a stressor was the one that induced it, however, study by Klein et al. (2015) showed how the secretions were sufficient to reduce a stress response parameter.

1.1.3. Main Olfactory System's Subsystem – The Odorant Receptor 37 Family

A study done by Strotmann et al. (1992) discovered an unusual odorant receptor (OR) family with extension of the extracellular three (EC3) loop, which is the OR37 family. The OR37 family are highly conserved across mammals. According to the Hoppe, Breer, and Strotmann (2003) study, the mouse OR37 (mOR37) family could be categorised into cluster I (consisting of 5 genes) and cluster II (consisting of 3 genes) based on loci position in the mouse chromosomes. 1 out of 5 genes within the cluster I and the 3 genes within the cluster II were pseudogenes. Similar genes were also found in humans, however, contrary to mOR37, the human OR37 (hOR37) family were mostly pseudogenes on cluster I and were functional on cluster II. Nucleotide sequence identity percentages between the mOR37 to and the hOR37 are between the range of 60% to 85%. It was also known from other studies that the sequence identity of mOR37 families is also present in other mammalian species such as: rats, dogs, pigs, horses, and elephants (Davies et al., 2018).

The OR37 subsystem is also considered to be unusual due to its projection pathway that does not follow the common projection pathway of the main olfactory system, which directly projects to the paraventricular nuclei (PVN) of the hypothalamus and to the posterior dorsal part of the medial (MePD) region of the amygdala (Bader, Breer, & Strotmann, 2012; Bader et al., 2012). Such pathways are more commonly found in the accessory olfactory system's type of pathway, however, the OR37 family is found

within the main olfactory system. It was also known from the Bader, Klein, et al. (2012) study that this direct projection to the PVN of the hypothalamus was more targeted on the vasopressinergic neuron types from a trans synaptic labelling using a Dil crystal injection into the MOB.

The OR37 subsystem activation was primarily driven by long-chain fatty aldehydes compounds. Bautze et al. (2012) showed that different long-chain fatty aldehydes, such as pentadecanal (15 carbon long), hexadecanal (16 carbon long), and heptadecanal (17 carbon long), activated different subtypes of the OR37 projection glomeruli in the olfactory bulb, as shown in figure 1.4. It can be seen that the OR37 glomeruli A reacted the highest to pentadecanal (C15al), the OR37 glomeruli B reacted the highest to hexadecanal (C16al), and OR37 glomeruli C reacted the highest to heptadecanal (C17al). However, it can also be seen that each glomerulus is also being activated by other long-chain fatty aldehydes, despite having smaller responses to the ligands with the highest activation level. Interestingly, the hexadecanal (C16al) activated all three OR37 glomeruli unlike pentadecanal (C15al) and heptadecanal (C17al).

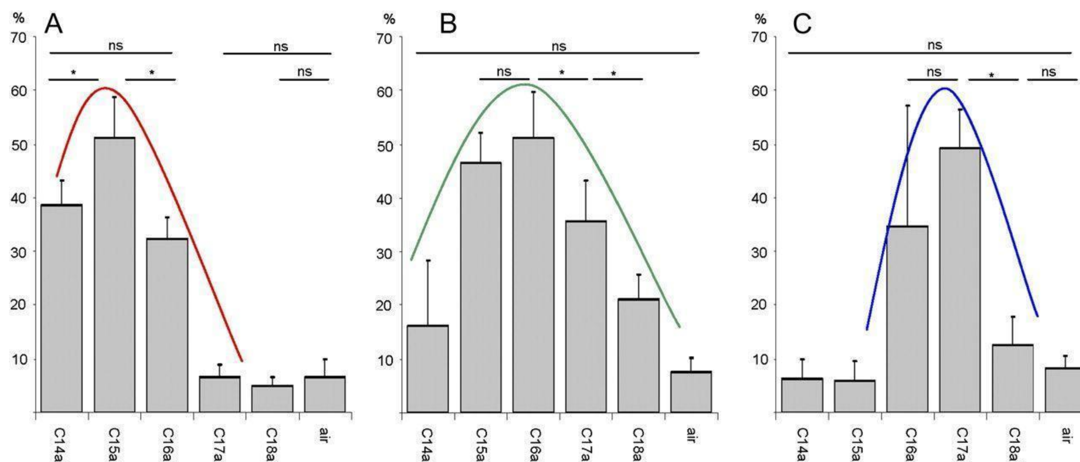

Figure 1.4: Different activation level of the OR37A, -B, and -C glomeruli after exposure of mice to long-chain aldehydes or air. Percentage of c-Fos positive Juxtaglomerular Cells (JCs) at the (A) OR37A glomerulus, (B) OR37B glomerulus, and (C) OR37 glomerulus, the curve illustrates the response profile of the OR37A, -B, and -C glomeruli toward C14–C18 aldehydes. *, statistically significant; ns, statistically not significant. Reused with permission from Bautze et al. (2012). Copyright (2012) Oxford University Press.

A more recent study by the same group has shown that the activation of the OR37 glomeruli by their respective ligands, a combination of synthetic C15-C16-C17, had a similar effect on c-Fos in the PVN as conspecific secretions during a novel

environment testing (Klein et al., 2015). This finding suggested the OR37 ligands as a candidate for artificial pheromones in stress response reduction via the social buffering effect. It was also found that the C16 natural source was identified on mouse faeces' surface, which most likely to be produced by anal gland secretion. Despite that the tracer study by Bader, Klein, et al. (2012) showed that the OR37 subsystem projects to vasopressinergic neurons and the novel environment stress study by Klein et al. (2015) showed that the PVN activation on novel environment stress was attributed to the Corticotropin Releasing Hormone (CRH) neurons, there is a gap in the knowledge regarding the changes in activated neurons during stress as an effect of the OR37 subsystem activation.

1.2. Stress and stress responses

1.2.1. Sources of stress in laboratory environment

Stress can be defined as a state where homeostasis is being threatened by a perceived intrinsic or extrinsic agent (termed as stressor), resulting in physiological and behavioural adaptive responses (Goldstein, 1995; Pacak & Palkovits, 2001; Tsigos et al., 2016). Although stress is commonly associated with negative impacts, stress is necessary in promoting behavioural adaptations within species to ensure survival (Smith, 1982). When stress is maintained for long periods, the stress response becomes maladaptive and detrimental and the stress will change into a state called distress (Russell & Burch, 1959). Based on their duration of exposure, stressors can be grouped as (1) acute stress, which is a single-time, or short period stress and (2) chronic stress, which is a continuous, repetitive exposure or over a prolonged time (Pacak & Palkovits, 2001).

Before addressing the source of stressors, it is important to understand the differences between the natural environment of the house mice and the captive environment of the laboratory mice. In their natural environment, wild house mice commonly have more complex social organisation and social behaviours compared to laboratory mice. The complex social organisation promotes survival by avoiding relatedness within mating choice, promoting cooperative behaviours, and prevents aggression between closely related siblings (Barbara, 1994; Ruch et al., 2009; Kurvers et al., 2013). In addition to

maintaining genetic diversity, the complex social organisation and behaviours in wild house mice are also influenced by seasonal changes (Evans, Lindholm & König, 2021). However, the opportunities for social behaviours are very limited when it comes to laboratory mice. Inbred strains are commonly used within laboratory mice to minimise genetic variability, which makes the inbred strains more genetically homogenous when compared to wild house mice (Casellas, 2011). Consequently, by having genetic diversity removed from consideration, the inbred strains lose the means to manifest the complex social organisation and behaviours, unlike wild house mice. Therefore, the captive environment itself does impact how laboratory mice manifest their behaviours. It is important to understand the reasoning and factors that influence the appearance of the behaviours, such as social behaviours and aggressions within the laboratory animal context, to understand the source of the stressors for mice in captivity.

There are substantial literatures on organising stressors into different categories, however, this work will focus on the sources of stressors for captive animals. According to Morgan and Tromborg (2007), the sources of stress for animals in captivity can be categorised into 2 groups which are: (1) the abiotic environmental stressors and (2) the confinement-specific stressors. In non-captive animals, stress drives adaptive behaviours which then control the stress, thus limiting its chronicity, whereas in the laboratory environment, the animals may not be able to achieve this and hence, the stress becomes uncontrollable and detrimental (Mason, 2006).

1.2.1.1. Abiotic environmental stressors in captivity

One major setback for animals in captivity is that the environment that they are being held in commonly does not reflect their natural environment. To address this issue, the concept of refinement was first introduced by Markowitz (1978) in order to improve behavioural developments and to promote the behavioural opportunities of zoo animals. In line with the principle of humane experimental techniques by Russell and Burch (1959), enriching the artificial environment as a refinement to achieve good animal welfare for captive animals is necessary. The enrichment of the artificial environment is important for the captive animals to not restrict their natural behaviours, which could potentially become another source of stress. According to Morgan and Tromborg (2007), some aspects that should be considered as potential stressors for

captive animals include sound, light levels, odours, temperature, and substrates due to their lack of ability in controlling these aspects.

Sound

It is obvious that the sound level and the noise profile within a controlled laboratory environment will be different to the sound available in the captive animals' natural habitat. A study done by Waser and Brown (1986) showed that an ambient noise level recording of a rainforest habitat ranges between 27 dB and 40 dB (time recording between 06:00 and 15:00). Other biomes such as riverine habitats and savannah habitats also show similar noise levels which are between 20 dB to 37 dB. As a comparison, the noise level of a laboratory environment during a similar time period was recorded as exceeding 90 dB, and over 110 dB when ventilation equipment was used (Pfaff & Stecker, 1976; Sales, Milligan, & Khirnykh, 1999). A more recent study by Lauer et al. (2009) recorded that the background sound levels were around 80 dB and increased by 30-40 dB during working hours.

Other than the sound level, there is also another problem regarding the ultrasonic sound. The human hearing range cannot perceive frequencies above 20 kHz, which is the ultrasonic range. However, the ultrasonic range is commonly used in animal communications. Study by Sales et al. (1988) described how different common laboratory and/or husbandry equipment (such as TV cameras, oscilloscopes, cage washers, etc) do produce noises within the ultrasonic range. The problem with ultrasonic noise itself is the limited number of studies done in rodents and their stress response as an effect of the ambient ultrasonic noises.

Higher noise levels in a laboratory environment (both sonic and ultrasonic) compared to the natural habitat of the animal are examples of sound as a stressor in a captive environment. Acute noise stress has been shown to be detrimental to the stress axis and cognitive performance in mice (Jafari et al., 2017). While during chronic exposure to constant traffic noise, a stress axis hyperactivity and impairments in memory followed by anxiety-like behaviours were observed in mice (Jafari, Kolb, & Mohajerani, 2018).

Light Levels

The artificial lighting that is used in a laboratory environment has been proposed to be a stressor to laboratory animals. The main focus in artificial lighting is the aspect of the duration of lighting (photoperiodicity), the intensity of lighting, and the light quality. While the 12:12 hours of dark and light cycle are commonly adopted for the laboratory environment, it is also quite common that the dark phase is not completely dark. A study by Studholme, Gompf, and Morin (2013) showed that brief light stimulation (5 minutes of stimulation) during the dark phase could induce the body temperature to drop, which is followed by a change in the electroencephalogram (EEG) reading, and subsequently induced sleep in mice. Another study by Bedrosian et al. (2013) also showed the detrimental effect of light pollution during the dark phase, which showed that a dim light exposure during the dark phase affected this active period by eliciting responses akin to the resting period.

While the light intensity could also become a source of stress. The light intensity could vary between laboratories. However, compared to a natural lighting source, artificial lighting in laboratories could be considered to be a lower light intensity than the natural lighting source. While the light intensity in the natural habitat could vary, the animals do have an option to avoid or to approach such a light source as a behavioural response, which in a captive animals' case, is not an option. Review by González (2018) summarised that a constant low light intensity during the dark phase had both short and long term effects on the molecular clock, physiology, metabolism, behaviour, mood, and cognition of animals.

Thermal

The temperature within the laboratory is commonly maintained within a narrow range around 23°C. However, this temperature range is set to facilitate the experimenter rather than the laboratory animals. More recent studies show that the mouse thermoneutral zone is around 30°C, which suggests that mice are exposed to constant cold temperatures within the laboratory environment (Maher et al., 2015; McKie et al., 2019). This poses a problem itself since being in constant cold stress could affect a mouse's physiological balance, immune response, and other measurements (Cichoń et al., 2002; Karp, 2012).

Substrate

While providing a substrate in the cage is now a common laboratory practice for laboratory animals, it is another thing to consider in relation to the effect of different substrates for the mice. A study by Gordon (2004) showed how different bedding materials (beta chips vs wood shavings) induced an increase to metabolic rates and consequently body temperature. The thickness of the bedding is also another factor to be considered since it will allow the mice to burrow to adjust their body temperature. The problem with poor bedding materials and their heat conductance property is that it will then become another source of stress, which is similar to the thermal stressors.

1.2.1.2. Confinement-specific stressors

Other than the abiotic environment stressors, being held in captivity creates a whole new set of stressors which are unique to the captive animals. The main difference with the environmental stressors, which are also found in the animals' natural habitat, is the animal's ability to control the impact of physical factors by adjusting its behaviour. Being in captivity is most likely to result in the absence of the animal's options to control the stressors' effect. Most of the stressors that are unique to the captive environment are the result of the restricted living space, the forced proximity to humans, the routine husbandry procedures, the restriction of food/foraging opportunities, and the social group situation, which does not reflect the natural group size.

Restricted Space

Animals are allocated a designated cage within a laboratory. Despite the regulations and recommended occupiable surface area of the cage, it is nowhere near the size of the animal's natural habitat. Studies such as the one done by Van Loo et al. (2001) found that the cumulative fighting between male mice was observed more often in smaller cage sizes compared to the bigger cage sizes. It is also known from Poon et al. (1997) that a bigger cage size promotes higher activity over a 24 hour activity recording. A study by Roemers et al. (2019) shows that the smaller cage size negatively affects the physical fitness of the mice. Although too small a cage size is shown to be a problem for animals in captivity, increasing the cage size does not necessarily mean

removing the source of the stressors. Prey animals such as mice, innately avoid open spaces, which are commonly used as a fundamental of open field arena testing.

Forced Proximity to Human

It is uncommon for most animals to constantly live in proximity with humans in their natural habitat. Despite some form of symbiosis between humans and some animals, animals in their natural habitat have a measurable flight initiation distance, which differs between species (Tarlow & Blumstein, 2007). Although the flight initiation distance for laboratory mice is quite difficult to be known due to the nature of animals in captivity, the higher incidence of flight initiation distance for spiny mice (*Acomys cahirinus*) is between 0-2 meters followed by 4-6 meters, with the distance in between initiate a freezing response (Ilany & Eilam, 2008). Although laboratory animals will be habituated to the presence of humans, it does not necessarily mean that the stress caused by being in proximity to humans will be diminished. Instead, this situation will force animals to adapt to the condition, therefore, changing their behaviour into one that would not be observed in nature.

Routine Husbandry

On top of being forced into close proximity with humans, husbandry procedures are common practice to ensure the hygiene and the health of captive animals. Part of the husbandry procedure for the laboratory animals is handling. Handling habituates the animals to the handler. A study by Hurst and West (2010) showed how repeated handling produced a taming effect, where the anxiety-like response in the mice was reduced. However, another problem with handling is the familiarity of the animals to the handler. In some experimental husbandry situations, different people will perform the handling, which is another unfamiliar stimulus, despite the animals being used to being handled. The study by Hurst and West (2010) also showed the high variability between 9 different handlers to particular handling methods.

Restricted Feeding and Foraging Opportunities

Predictability is something that is not commonly available in an animal's natural habitat. Contrary to the predictability of negative stimulus, the predictability of positive stimulus is not necessarily beneficial to animals. One example of positive predictability for laboratory animals is the regular food source. A study by Luby et al.

(2012) showed that during the 24 hour activity recording, an anticipatory activity was recorded in mice prior to their regular feeding time. This showed how mice behaviour was conditioned to feeding time, which changed the behaviour of the mice. The problem with anticipatory behaviour is that when the predictable became unpredictable, the changes could potentially become a source of stress. The change of feeding behaviour itself also becomes a source of stress since the mice could not express the normal foraging behaviour and possibly compensate for such behaviour in some other way.

Abnormal Social Group

Following the problem with the space, laboratory animals are commonly being held in a caging system where animals are forced to live in groups or to live on their own for extended periods. It is quite uncommon for male mice to live in a group in their natural habitat. Aggression between male mice is not uncommon in a laboratory condition. The problem with male aggression in group housing is that, after the fighting bout, both winner and loser males normally move away from each other. However, due to the limited space, both males could not move away from each other and force the fight instances continue to occur. This becomes a source of stress for both the winner and the loser. This continuous fight will put the loser into a chronic social defeat paradigm, which affects the HPA axis function and stress responses (Keeney et al., 2006; Keeney, Hogg, & Marsden, 2001).

Some experimental procedures require animals to be individually housed for extended periods. A number of studies have shown the detrimental effect of individual housing for extended periods to stress responses such as anxiety-like behaviours, social aggression, and hypothalamic-pituitary-adrenal (HPA) axis stress responses (Francès et al., 2000; Heck et al., 2020; Liu et al., 2019; Võikar et al., 2005). The extent of detrimental effects of the individual housing will be further explored in the social isolation chapter (see Chapter 4).

Odours

Odours are something that are commonly underappreciated for a captive animal’s environment. Besides less enriched odours in captivity compared to the natural environment, there is also a problem concerning the constant exposure to other species'

smell within a close vicinity. It is known that rats' odours elicited fear-like or aversive behaviour in mice (Papes, Logan, & Stowers, 2010). It is quite common for a laboratory to have different species being held in the same animal unit and within the same air circulation system. This is another potential stressor for captive animals, especially mice.

Other than the smell of other species, there is another potential source of stress regarding the mice's own smell as mice use their urine for territorial marking. Cage cleaning, as a part of the husbandry procedure, regularly removes the mice's smell, which is equal to disturbing the mice's territory. A study by Gray and Hurst (1995) showed that cage cleaning disturbed the odour cue for territory and increased the aggressions between males in a group house situation. On the other hand, a study by Lopez-Salesansky et al., (2015) showed how the use of the same gloves for handling between mice were considered as a common practice. The main problem with using the same gloves for handling different mice, especially different sex mice, is that it could potentially cross-contaminate the olfactory cues between mice, which might potentially become another source of stressors.

Another potential stressor within a captive environment in relation to odours are the presence of abiotic odorants that commonly present within the laboratory settings, such as the odour of cleaning products. The study done by Lopez-Salesansky et al., (2015) also showed how the use of cleaning products were varied between institutions when cleaning the euthanasia chamber. Consequently, a study done by Hershey et al. (2018) also showed how the use of different cleaning products for a test arena affects the outcome of anxiety-like behavioural tests in mice when compared to the control group using water. Although it is possible that the odour of the cleaning products is not necessarily as detrimental as a stressor, the fact that it does change behavioural output suggests that it is necessary to be aware of the abiotic odour within the captive environment.

1.2.2. Stress Responses

As a response to the stressors, the animals will then produce different stress responses to mitigate the detrimental effects of the stressor. There are numerous parameters commonly used to measure the stress responses of animals. However, this work limits the measurement of stress responses to two categories, which includes physiological stress responses, and behavioural stress responses.

1.2.2.1. Physiological

Physiological responses to stressors are commonly closely linked to each other as a system. However, this section will focus on physiological stress responses such as the body weight changes to stress, the stress-induced thermal changes, and the endocrine responses related to stress. The endocrine responses will be discussed later in the chapter altogether with the HPA axis.

Body weight

Body weight has been used as a simple measurement of health for laboratory animals. Although it may not be reliable or robust enough to determine the immediate and specific problem with the animals, body weight reduction normally correlates with the deterioration of clinical signs (Wu et al., 2018). A study by Allen et al. (2010) showed how stress could induce weight loss in mice via muscle-mass reduction, which, if the direction of the logic were reversed, poor health and growth could be an indicator of a stressed animal.

Body temperature

Body temperature has also been proposed as a measure of the stress response, which is more rapid than the body weight. Body temperature can be measured from various areas, such as the core or peripheral body temperature using an implanted chip, rectal temperature using a probe, ear temperature, or more recently, a non-invasive eye temperature using a thermal camera (Gjendal et al., 2018). Oka (2018) described how the temperature changes according to exposure to stressors. Although body temperature is closely linked to the metabolism rate, which is a by-product of the stress response release of glucocorticosteroids, it does not necessarily mean that the body temperature will increase as a response to the stressor. Oka (2018) also mentioned how

stressed animals also experience a stress-induced temperature decrease, which is suggested to be a stress response to a long-lasting and inescapable stress.

1.2.2.2. Behavioural

As physiological responses are an animal's defence mechanism in maintaining homeostasis from a stressor's disturbance, an animal's behaviour also facilitates the physiological response in responding to stressors. Behavioural measurements could be a more direct and simple measure than physiological stress responses, which commonly involves longer procedures in sample processing. Common behavioural measurements for assessing stress responses include overt behaviour measurements, stereotypic behaviours, anxiety-like behaviours, defensive behaviours to external threats, and changes in affective biases. The affective biases function as both a factor that modulates the behavioural responses and a measure of stress responses. The affective biases are related to an animal welfare state that is affected by stressors, which will be discussed later in this chapter.

Overt behaviours

Overt behavioural observations are the simplest form of behaviour observation in animals that do not require specific equipment to be observed. Despite the limited interpretation due to a variety of possible factors that could affect behavioural outcome, overt behaviours can be used as a measure of a response to specific types of stressors. A study by Machida et al. (2019) measured freezing behaviour and its association with changes to EEG activity in a fear conditioning study. A study by Ruiz-Miyazawa et al. (2015) showed that the mice's paw response to nociception was related to cytokine response in an analgesic drug study. A study by Denmark et al. (2010) showed that the grooming pattern of subordinate mice became disorganised after a chronic social stress, which suggests the grooming pattern is associated with novelty-induced anxiety-like behaviours. These studies showed how the overt behaviour could be used alongside the measurement of physiological parameters. Interpretation of overt behaviours needs to be performed with care on their own, however, a change in the observed overt behaviour can potentially be linked with other physiological parameters.

Stereotypic behaviours

One specific form of overt behaviour that is commonly observed specifically in laboratory animals is stereotypic behaviour. Stereotypic behaviour is an excessive production of motoric behaviour that results in repetition (Ridley, 1994). Stereotypic behaviour is observed in rodents as a repetition of running in a circle, pacing up and down in one part of the cage, bar gnawing, and backward somersaulting (Nevison, Hurst, & Barnard, 1999; Würbel, Stauffacher, & von Holst, 1996). This behaviour is thought to be a result of being in a confined space and a normal response to an abnormal environment. A study by Gross et al. (2012) showed how enriching the cage can reduce the occurrence of stereotypic behaviours, which is possibly caused by the opportunity of performing species-specific behaviour. The problem with stereotypic behaviour is that once this behaviour manifests, stereotypy can restrict the animal's behavioural repertoire and make the animals less responsive and less likely to exhibit other behaviours in different behavioural observations (Pogorelov et al., 2005).

Anxiety-like behaviours

Other than overt behavioural measurements, behavioural output will depend on the context of the testing being performed. One of the common measurements of stress response-related behaviour is the behaviour measurement during an anxiogenic environment context. In mice, the anxiety-like behaviours commonly observed by exposing the mice to an anxiogenic environment using various testing batteries, such as: the open field arena test, which assess exploratory activity in an unfamiliar area; the two chambered light and dark transition test, which assess the conflict between the exploratory drive and the anxiogenic property of bright area; the hole board "head dip", which assess exploratory behaviour; and the novelty suppressed feeding test, which assess the conflict between hunger drive and the anxiogenic environment (Bailey & Crawley, 2009). Although there are various tests that could be done to assess anxiety-like behaviours, these tests were developed as a response to anti-anxiety drugs (Bailey & Crawley, 2009). Anxiety itself is an anthropomorphic term, which is arguable in relation to whether the animals are actually experiencing the anxiety itself. Although anxiety itself has its own clinical definition, a range of tests should be performed to assess anxiety-like behaviours before attributing an anxiety-like stress response to an anxiogenic environment. A study by Hurst and West (2010) showed the use of anxiety-

like behaviour measurement to show how different ways of handling mice could affect their anxiety-like state.

Anxiety-like behavioural outputs will also differ between different strains of mice. The evidence base has repeatedly shown how different strains of mice have different anxiety-like behavioural baseline levels (Sartori, Landgraf & Singewald, 2011; van Gaalen & Steckler, 2000). Parmigiani et al. (1999) suggested that the difference in strains could possibly affect the gene that encodes the receptors involved in anxiety-like phenotypes. Sartori, Landgraf & Singewald (2011) also mentioned that the inbreed C57BL/6 strain has been known to be the most common strain to be used for anxiety-like behavioural tests.

Aggressive behaviour

Aggression is another behavioural measure that is affected by stress. Based on the aggression characteristic in mice, aggressive mice are divided into Short Attack Latency (SAL) and Long Attack Latency (LAL) mice (Veenema & Neumann, 2007). The difference between these aggressive characteristics in mice were attributed to the difference in the HPA response to stress (Veenema et al., 2003). The study by Veenema et al. (2003) also showed how the basal HPA axis response on both SAL and LAL did not differ, however, the responses started to change during stressful events, where the LAL mice showed a prolonged response to stress. Although there were differences in the attack latency, both types of mice did not experience social defeats during a standard opponent test on a neutral cage.

Other than the aggression characteristics, similar to the anxiety-like behaviours, different levels of aggression persist between different strains of laboratory mice. A survey done by Lidster et al. (2019) supported the existing evidence base regarding the CD/1 strain as having higher aggression when compared to the lower aggression strains such as the C57BL/6. Interestingly, the study also discussed how the preconception regarding the C57BL/6 as the "highly aggressive strain" was possibly due to the higher number of this strain being used for studies involving mice. In addition, Parmigiani et al. (1999) highlighted the differences between strains and their aggressive behaviour could also be related to the artificial selection process, possibly a selective breeding process, occurring within inbred mice strains, such as the

C57BL/6 strain. Further details into aggressive behaviours will be discussed within Chapter 4.

1.2.3. The HPA Axis

One of the common threads between all the stress responses in animals, specifically in mice, is that the stress response involves the (HPA) axis, which is one component of the stress response. The stress input could possibly come from different regions of the brain depending on the source of the stressor, however, the signal for the stress response is commonly processed first by the hypothalamus (Herman et al., 2016). The hypothalamus will then relay the signal to the pituitary and subsequently to the adrenal gland. The adrenal gland will then release the corticosterone hormones to the blood system to facilitate energy mobilisation as a stress response (Simpson & Waterman, 1988). It is also known that the signal also works as a feedback loop to the HPA axis in regulating the stress response (figure 1.5)

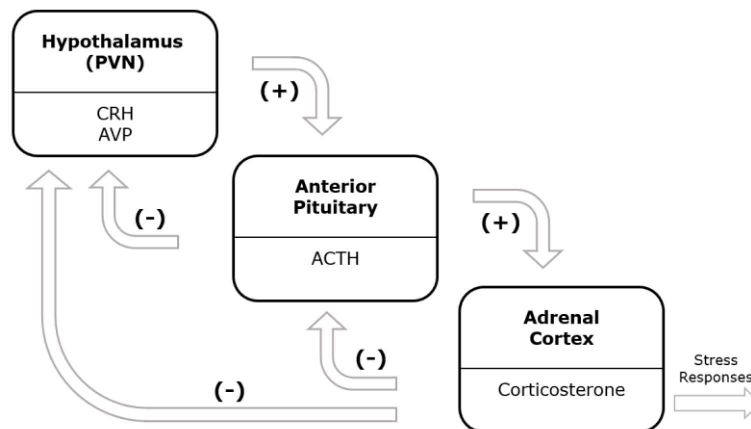

Figure 1.5: The Hypothalamic-Pituitary-Adrenal (HPA) Axis and the feedback loop. Adapted from Papadimitriou and Priftis (2009) and Martini and Nath (2010).

1.2.3.1. Hypothalamus

The hypothalamus is a part of the HPA axis, part of the limbic system, and located between the optic chiasm and the mammillary body (Martini & Nath, 2010). Components of the hypothalamus that play an important part of the HPA axis are the paraventricular nuclei (Martini & Nath, 2010). The hypothalamus has diverse functions, which includes feeding reflexes, regulation of heart rate and blood pressure,

regulating water retention in kidneys, regulation of body temperature, regulation of sleep-wake cycle, and the regulation of the stress axis (Martini & Nath, 2010). The main nuclei in the hypothalamus that plays the role in the stress axis is the PVN of the hypothalamus. The neuroendocrine cells in the PVN are further divided into magnocellular neurons, which includes oxytonergic and vasopressinergic neurons, and parvocellular neurons which includes vasopressinergic neurons, CRH neurons, and the more recently discovered population of oxytocinergic neurons (Aguilera & Liu, 2012; Eliava et al., 2016).

The magnocellular neurons, which are located on the lateral part of the PVN, receive various inputs both excitatory and inhibitory from various areas of the brain. Notable inputs to the magnocellular area are the dopaminergic input, from the interneurons of the perinuclear zone of the PVN, and the glutamatergic input, from the olfactory bulb (Brown et al., 2013). The oxytocinergic neurons in the magnocellular area also specifically received an input from the parvocellular oxytocinergic neurons in relation to pain processing (Eliava et al., 2016). For the output, magnocellular cells extended their axons into the posterior pituitary area to release the oxytocin and vasopressin into the bloodstream (Aguilera & Liu, 2012; Eliava et al., 2016).

Similarly, the parvocellular cells, which are located in the medial part of the PVN, also receive a range of inputs from hypothalamic areas. Notable inputs to the parvocellular area are from other hypothalamic areas (the medial preoptic area, the posterior hypothalamus, and the dorsomedial nucleus), the nucleus of the solitary tract, and the interneurons of the perinuclear zone of the PVN (Herman et al., 2016). The dorsomedial part of the PVN, which consists of mostly CRH neurons, also specifically receives the input of glucocorticoids as a negative feedback response of the HPA axis (Evanson et al., 2010). During glucocorticoids stimulation, the CRH neurons rapidly produces and releases endocannabinoids, which will bind to CB1 receptors on the presynaptic areas and inhibiting glutamate release, inhibiting the excitatory input of the CRH neurons (Di et al., 2003). Contrary to the magnocellular neurons, the parvocellular neurons release their peptides into the pituitary portal and therefore to the anterior pituitary (Aguilera & Liu, 2012).

The expressions of both CRH and arginine-vasopressin (AVP) play an important role in determining the appropriate response to the duration of the stress. During the basal

condition, ~50% of the dorsomedial parvocellular CRH neurons co-express AVP (Whitnall, 1993). During the acute stress paradigm, both CRH and AVP are rapidly released into the pituitary to produce adrenocorticotropic hormone (ACTH) (Plotsky, 1991). However, during chronic stress, the CRH release decreases after repeated exposure to the same stressors. On the other hand, high levels of AVP release are maintained, although the full mechanism itself is still not fully understood (Lightman, 2008).

Neurohormones, also known as neuropeptides, such as the CRH and the ACTH are difficult to measure due to the low concentration in biological samples and the speed of the degradation (Sandberg & Weber, 2003). A common measurement that is a more reliable and relatively cheaper option to assess the neuropeptide is the radioimmunoassay measurement and another measurement that assess the mRNA that can be done is by using an in-situ hybridization (Sandberg & Weber, 2003; Wong et al., 1994). Some studies such as Klein et al. (2015) used a proxy to measure the activity of neural cells within the hypothalamus that produced the CRH. Such a proxy could be done using the c-Fos protein measurement, which is a protein expressed from the c-fos Immediate Early Gene (IEG) during an extracellular stimulation event and one of the markers of activated neurons (Herrera & Robertson, 1996). During stressful events that stimulate the hypothalamus, the CRH neuron cells will be stimulated to be activated and the CRH. Although the limitation of the c-Fos measurement is that it is not clear which neural cells were being activated, and needs to be validated with colocalization approach, c-Fos measurement provided an insight to the neural activity during exposure to stressors. This neural activity, coupled with proper double labelling, could provide an insight to a functional approach to the stress response axis to particular stressors.

1.2.3.2. Pituitary Gland

The pituitary gland is the second major part of the HPA axis. The pituitary gland is located within the sphenoid, inferior to the hypothalamus, and connected to hypothalamus by the infundibulum. The pituitary gland is divided into two regions, the anterior and the posterior, which were classified into different subregions due to their developmental origin and their functions (Martini & Nath, 2010). The anterior pituitary originates from the epithelial ectoderm, resulting in the structure of the

anterior pituitary consisting of a modification of epithelial cells (Larkin & Ansorge, 2017). On the other hand, the posterior pituitary is originated from the neural ectoderm, resulting in the structure of the posterior pituitary to be primarily neuron cells, which are also connected with an axon from the hypothalamus (Aguilera & Liu, 2012; Larkin & Ansorge, 2017).

The main region of the pituitary that plays a role in the HPA axis is the anterior pituitary. The anterior pituitary consists of gonadotroph cells, thyrotroph cells, somatotroph cells, lactotroph cells, and corticotroph cells (Larkin & Ansorge, 2017). The CRH signal from the parvocellular region of the hypothalamus will bind to the CRH-R1 receptors in the corticotroph cells in the anterior pituitary (Abou-Samra et al., 1987). Simultaneously, the vasopressin signal from the parvocellular cells of the hypothalamus will bind to the vasopressin receptor (AVP1B) to activate the Protein Kinase C (Aguilera & Rabadan-Diehl, 2000). The CRH, complemented with the Protein Kinase C signals will then activate the pro-opiomelanocortin (POMC) to produce the ACTH. The resulting ACTH will then be transported, via blood stream, to the adrenal cortex to produce glucocorticoids (Aguilera, 1994). The POMC activation process is also downregulated by the presence of the glucocorticoid, which is another part of the negative feedback of the HPA axis (Gagner & Drouin, 1985).

1.2.3.3. Adrenal Gland

Finally, the last part of the HPA axis is the adrenal gland. The adrenal gland is located on the superior border of each kidney and divided into the subregions of the cortex and the medulla. The boundaries between the cortex and the medulla are irregular, however, both areas have distinct cellular characteristics, with the cortex cells storing lipids. The cells in the medulla produce the epinephrine and the norepinephrine hormones (also called adrenaline and noradrenaline), while the cells in the cortex produce the glucocorticoid hormone (Martini & Nath, 2010).

The cortex region of the adrenal gland is the main area that is involved in HPA axis regulation. The ACTH from the pituitary gland will bind to the melanocortin-2-receptors (MC2R) in the zona fasciculata and produces adenyl cyclase-dependent responses, which increases intracellular cAMP levels (Hadley & Haskell-Luevano, 1999). The increase in intracellular cAMP levels will then increase the cholesterol

biosynthesis and result in glucocorticoid production (Simpson & Waterman, 1988). The glucocorticoids, which are predominantly cortisol in humans and corticosterone in rodents, are common measures of stress in animals. The glucocorticoids will then be transported, via the bloodstream, to other regions for the activation of the stress response, and at the same time, relay the signal for negative feedback to both the hypothalamus and the pituitary (Evanson et al., 2010; Gagner & Drouin, 1985). When the glucocorticoids are released into the bloodstream during stressful events, the glucocorticoids attach to the glucocorticoid receptors within the cytosol of the target cells. The attachment to the receptors will then either start the transactivation process by attaching to the DNA to increase the expression of certain genes or start the transrepression process by attaching to the DNA where the transcription factors are supposed to attach and prevent the transcription of other genes (Newton & Holden, 2007; Revollo & Cidlowski, 2009). In general, the glucocorticoids are known to be mainly involved in metabolic function to synthesise glucose, which is an important substrate for energy production (Martini & Nath, 2010). Other than energy metabolism, glucocorticoids are also known to affect anti-inflammatory responses (Martini & Nath, 2010).

Timing wise, the glucocorticoid is the longer response for stress, while the epinephrine and norepinephrine are considered to be the more immediate responses for stress, which are the regulator of the "fight-or-flight" response as a part of the sympathetic nervous system (McCorry, 2007; Ziegler, 2004). During the stressful events, both the epinephrine and the norepinephrine, together with acetylcholine that is produced by the preganglionic neurons, regulate both the sympathetic nervous system and the parasympathetic nervous system, which results in either the fight response or flight response (McCorry, 2007). As a part of the "fight-or-flight" response, the hormones also affect vasoconstriction, vasodilation, muscle contraction, and muscle relaxation in different parts of the body including heart, lungs, liver, stomach, intestine, pancreas, sweat glands, urinary bladder, kidney, and immune systems (McCorry, 2007; Won & Kim, 2016). As the stressor persists and the epinephrine and the norepinephrine responses subside, the HPA axis is then activated to produce glucocorticoids.

1.3. Laboratory Animal Welfare

1.3.1. Definition of Laboratory Animal Welfare

Laboratory animal welfare, which subsequently will be termed as animal welfare, is one branch in applied science with the main objective being to promote humane methods in laboratory animals. Animal welfare in the United Kingdom is regulated following the Animals (Scientific Procedures) Act 1986 (ASPA) that covers all vertebrates and cephalopods undergoing a procedure which might cause the animals a level of pain, suffering, distress, or lasting harm equivalent to, or higher than the introduction of a needle in accordance with good veterinary practice (Animals (Scientific Procedures) Act 1986, 2012). According to the report written by the select committee of the House of Lords (2002), the ASPA 1986 is the tightest system of regulation in the world compared to the regulatory system in Japan, The United States, and France. Current guidelines for good animal welfare practice in the UK is detailed in Home Office's Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes (2014). The code of practice encompassed all animals that are protected under the ASPA 1986 which are vertebrates and cephalopods.

Supplementary to ASPA 1986 and the Home Office's Code of Practice, Russell and Burch (1959) defined the principles in humane experimental techniques which will be subsequently referred as The Principle. The main aim of animal welfare, according to the principle, is to promote humane behaviour to reduce the total sum of pain and fear. Both pain and fear, if prolonged, can induce distress which will lead to detrimental physiological stress responses. Hypothetically, if a rank can be assigned to the total sum of pain and fear and when the treatment drives the animals to the detrimental end, the treatments will be considered as inhumane. Then following that logic, the reduction of pain and fear will move the rank to the opposite end and be considered as humane. It is important not to confuse the humane method with human psychological description in experimental procedure and ethical criticism.

Objective measurement on pain and fear is important in animal welfare. Pain is a complex phenomenon where, when being broken down, pain involves the perception of the stimulus, or also called as nociception, processed on the central nervous system, and being reported to the observer. This paradigm itself is complex enough due to the

concept of consciousness and the ability to report the pain to the observer, however, the nociception itself is quantifiable and the term nociception will be used instead to focus the topic on animal welfare. While fear, described as an arousal of the central nervous state to avoid stress, might, but not always, cause distress in animals. Fear has become an important concept itself in animal welfare. The conflict of fear will override behavioural outcome and affecting the measurements.

1.3.2. Animal welfare measurement

According to Russell and Burch (1959), the principle in measurement of distress parameters in animals can be determined by the presence of distress in terms of rank on a scale. Any treatment that induces a lower rank in mood than before the treatment can be considered as imposing a measurable amount of distress. When the principle is expanded from just frustrations of needs, such as hunger and bodily discomfort, the observation of behaviours of the animals become important. The Principles separated the behaviours into three separate distinctions, which are: the general behaviours, the autonomic behaviours, and the sexual behaviours in order to reproduce efficiently. There were other writings that tried to categorised animal welfare measurement concepts such as works by Broom (1991) that categorised the measurements into life expectancy, responsiveness to stimulus, stereotypes, and the value of preference tests, and works by Hemsworth et al. (2015) that categorised the measurements into biological functioning, affective state, and natural living. However, most recent review by Mellor et al. (2020), after multiple series of updates spanning 25-years, categorised the assessment of animal welfare which encompassed other works quite well into the five domains models: Nutrition, Environment, Health, Behaviour, and subsequently Mental Domain as a result of the other 4 domains as on figure 1.6. The first three domains: nutrition, environmental, and health are focused on internal imbalances of the animals while the behaviour domain is focused on external restriction and/or interaction of the animals in presence of other animals or humans as an output in form of behavioural responses.

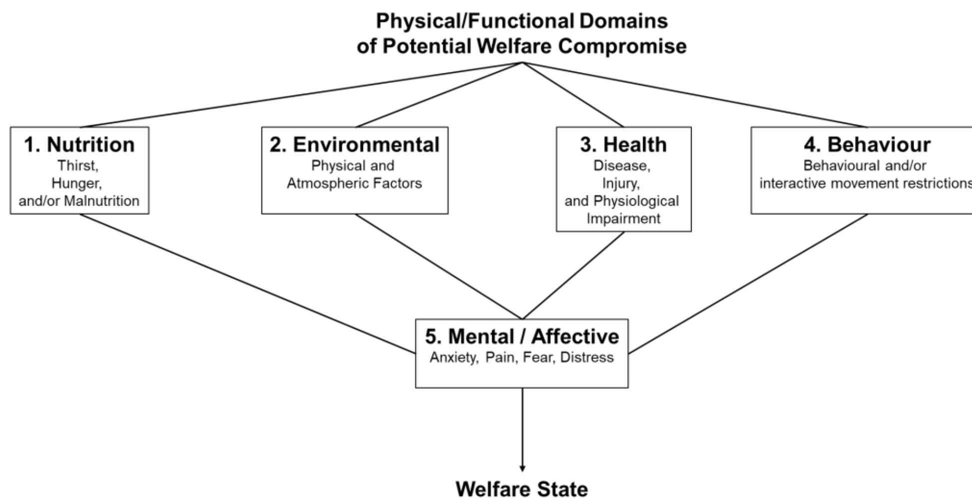

Figure 1.6: 5 domains models for animal welfare measurements. Adapted from Mellor and Reid (1994) and Mellor et al. (2020).

The first nutrition domain refers to the availability of water and food to animals. Imbalances or deprivation of the first domain will lead to a negative state of mental domain such as thirst and hunger which in turn compromise the welfare state. On the other hand, such conditions can be reversed by giving the animals opportunities to remedy the imbalances, which in turn will induce positive affective states. The second environmental domain refers to the physical and atmospheric condition that the animals are exposed directly. If the environment becomes unfavourable and cannot be avoided, this will induce a negative affective state. Removing this unavoidable factor is then categorised as an enhancement of physical factor, which will induce a positive affective state. The third health domain is straightforward in terms of negative health conditions will negatively affect the affective state and vice versa with the positive health condition. Lastly, the fourth behavioural domain focused on how the animals interacts with their environment, other animals, or humans. When certain behavioural outputs are restricted due to captivity, particular negative affective states are anticipated. However, when the behavioural outputs are no longer restricted or the animals are provided with opportunities to express the behaviours, the condition will allow situational negative affective state to be replaced by positive affective state.

In addition to these domains, Mellor and Reid (1994) also propose a grading system to assess the severity of the compromises within each domain. The grading system consists of five grades from null and increasing to severely compromised. They also specified that with a combined procedure, the severity grade must represent the state

of the animal at the time of maximum impact. More recent work by Mellor, Patterson-Kane, and Stafford (2009) further explained the details of the five graded scale into grade A,B,C,D, and E according to the intensity, presence, and duration of the negative affect affliction on the animals. Grade A and B represents the no and tolerably low level of severity while the Grade E represents the most severe negative impacts. Grade C and D refers to an intermediate-level of compromise to animal welfare.

Apart from the 5 domains model introduced by Mellor et al. (2020), a review by Mason and Mendl (1993) explained the challenges in measuring animal welfare that need to be considered when observing certain parameters. One of the biggest challenges in animal welfare measurement is that there are no one simple parameters that can be measured as a good animal welfare condition. Most of the measurements of animal welfare states are not exclusive measurements of the state of animal welfare (take corticosterone or CORT for example, which is also a measure of ultradian rhythm and arousal), which makes any measurements difficult to interpret. It is quite common for a treatment to have different interpretations (such as moving to a new cage could be either beneficial or detrimental) and for a parameter of animal welfare not being able to be replicated with the same interpretation in different settings.

The problem with replicating welfare measurement results suggests the presence of underlying factors that are difficult to account for. Mason and Mendl (1993) described underlying factors that could potentially affect the welfare measurements: the type of aversive stimulus, the timing and duration of the aversive stimulus, the time point of the response measurement after the stimulus, species difference, age and sex difference, individual animal variations, and animal psychological state. It is necessary to consider such factors when assessing animal welfare, since a change in the parameters needs to be tied in with the animal's basic needs and bodily function in order not to confuse the parameters as poor welfare status.

Further to the welfare status, there is another problem regarding the definition of welfare itself. Although most works agree that good animal welfare means that an animal's biological function is not impaired, however, when two of the measurements of the biological function contradict each other, it is not clear what conclusion can be drawn (Barnett & Hemsworth, 1990; Broom, Johnson, & Broom, 1993). Another hypothesis to assess animal welfare is by measuring animal subjective experience

(Dawkins, 1990). The work by Mendl and Paul (2008) used the term affect as a measure of an animal's mood and how the affective state is influenced by past experiences, as a form of episodic learning. Furthermore, Mendl et al. (2009) explored how this affective state influenced an animal's motivation to perform a task. Mendl et al. also showed that the affective state, in the form of cognitive bias, can be manipulated to predict the outcome during a presentation of an ambiguous cue. Based on this, such measurements that assess the animal's cognitive bias can be used to assess the animal's affective state. The assessment of an animal's affective state is more reliable as a measure of welfare due to the general principle of the measurements to measure the emotional valence rather than arousal (as commonly measured by other physiological parameters).

1.3.3. Animal welfare and the 3Rs concept

Based on The Principle, the fundamental of improving laboratory animal welfare can be grouped into 3 different concepts: Replacement, Reduction, and Refinement, which are also referred to as the 3Rs. Before discussing the 3Rs concept any further, it is important to establish the concept of the source of inhumanity. According to the principle, the source of inhumanity can be separated as direct inhumanity, which results from direct treatment, and contingent inhumanity, which results from other factors apart from the direct treatment such as husbandry. The objective of the 3Rs is the removal of inhumanity, be it direct or contingent, by mitigating the hypothetical scale to move towards more humane treatment (Russell & Burch, 1959). It is to be noted that it is almost impossible to fully remove the inhumanity and the application of the 3Rs component might overlap with each other in order to promote animal welfare.

1.3.3.1. Replacement

The first concept in the 3Rs is replacement, which according to The Principles is defined as "the substitution of conscious living animals or insentient material". It is important to note that the main keyword of the definition is the word "Insentient". Replacement does not simply means replacing with other animals, be it considered as "lower" by other works as extensively described by Tannenbaum and Bennett (2015). According to their work, it is quite a common misconception in the replacement concept of using the terms "less-sentient" rather than "insentient". It may sound

semantic; however, the misconception potentially drifts the concept of replacement into something entirely different and could not be justified from the animals' point of view. As previously mentioned, Russell and Burch's (1959) 3Rs objective is to minimise and/or to remove inhumanity in animal experimentation and the concept of inhumanity according to The Principles was established surrounding the conscious animals. However, the use of the term less-sentient itself is problematic due to the arbitrary exclusion of free-living invertebrates from the consciousness paradigm in The Principles. The replacement objective also needs to be upheld strongly in regard to the inhumanity aspect since it is very easily entangled with the anti-vivisectionist perspective on animals' experimentation. The concept of replacement should not be used as a justification for not using animals in research. The replacement concept in The Principles was divided into two categories which were relative replacement and absolute replacement.

The concept of relative replacement from The Principles refers to the usage of sentient animals but treated in order to be insentient. Such an example could be achieved by having the animals under deep anaesthesia during non-recovery experiments and all the treatments and data collection were completed while the animals were unconscious. While on the other hand, the concept of absolute replacement according to The Principles is fully substituting the animals with insentient material. As mentioned by Tannenbaum and Bennett (2015) that the drifting definition of the relative replacement into less-sentient animals instead of insentient animals which explain the importance of the semantic point previously. It is also mentioned that the more recent target of replacement is around the absolute replacement without the capabilities to define the removal of inhumanity. It is important to stick to the concept of the replacement from The Principles to promote humane treatment in animal experiments.

It is important to stick to the concept of removal of inhumanity from sentient animals, however, it does not mean that the experiment has to be compromised due to the replacement. The concept of replacement according to The Principles is still work in progress and until this date, there has not been other works describing the general theory of the replacement. However, other works such as Balls (1994) and Balls (2013) still refer to the same common problem on The Principles' replacement section: the high-fidelity fallacy. The high-fidelity fallacy refers to a bias towards using one group of animals simply because of the preconception that animals from the same taxonomy

level would have similar responses to the original target animal. For example, the preconception of using other mammals as replacement to human studies rather than using invertebrates with similar target system's function. From the concept of high-fidelity fallacy, it is then important to find a suitable replacement with similar function rather than being attached to the preconception.

1.3.3.2. Reduction

When it is not possible to replace the animals with other insentient material, the second concept in the 3Rs, reduction, plays an important role in promoting animal welfare. The reduction objective is straightforward: to reduce the numbers of animals employed in the studies. It needs to be ensured that the reduction, according to The Principles, does not equate to minimising the number of animals used for an experiment. It is another question regarding the separating line between 3Rs reduction and minimising, which brings the importance of experimental design and employing appropriate strategies in research.

Regarding the nature of animal study itself, it is important to acknowledge variability within the population of animals. The variability of the data of a study is one important measurement of statistical analysis. However, it is important that the variability in the study can be accounted for and properly controlled in order to come up with the sufficient sample size needed. One of the good practices in determining the sample size for population data and producing strong evidence to answer the research question, is by using a power analysis to determine the appropriate sample size for the study (Field, 2013). The work by Clark and Bate (2014) elaborated the importance of reducing variability in animal research in order to increase the signal-to-noise ratio.

The work also mentioned various experimental design types, notably, regarding the aspects that need to be considered for power analysis (Clark & Bate, 2014). It is important to hypothesize the effect size of the treatment for the study either from literature study or expected effect level on the observed parameter. Given that the widely accepted power is between 70% and 90%, it is also important to know the statistical tests and assumptions that will be performed on the data in order to avoid bias and balance the probability of type-I and the type-II statistical errors.

1.3.3.3. Refinement

Finally, after considering replacement and reduction, the last part of the 3Rs is refinement. As mentioned on The Principles that it is almost impossible to remove inhumanity completely, however, the refinement process is essential in mitigating the total sum of pain and/or distress in animals. This can be achieved by modifying the currently available or commonly used practice in order to reduce the total sum of pain and/or distress. The source of inhumanity becomes very relevant in this aspect of 3Rs because this will help to clarify the objective of the refinement. The refinements of both direct and contingent inhumanities are similarly important.

An example of the refinement of direct inhumanity can be seen in the works done by Hinchcliffe et al. (2017). In their study, Hinchcliffe et al. used a modified oral dosing method. Commonly, oral dosing was performed using a gavage needle. The problem with using gavage needles is that a proper training is necessary in order to successfully introduce the treatments into the stomach of the animal. Arantes-Rodrigues et al. (2012) mentioned how this method is stressful to animals and does possess a mortality rate, however, the mortality rate can be minimised by having an experienced technician carry out the procedure. The modified oral dosing method used a milkshake solution to deliver the treatments and letting the animals drink the treatments by themselves due to the nature of the palatable solution. This method does pose limitations when the used drugs will react with the palatable solution, however, this method does minimise the risk of accidental injury.

Refinement on the direct inhumanity can also be done by changing a more stressful method to a less stressful method to achieve the same conclusion. Such work was done by Hinchcliffe et al. (2017) by using an affective bias test, which is considered to be a mild stress procedure according to ASPA 1986, based on a hypodermic needle injection, instead of the forced swimming test, which is a moderate stress procedure, according to ASPA 198 based on the swimming stress, to test for a drug study that affect the negative affective state. The use of the affective bias test is another method in recognising the negative affective state to assess the animal welfare state in general. The Affective Bias Test is in line with Mendl et al. (2009) Judgement Bias Task in answering the recognition of pain and suffering to measure an animal's welfare state.

An example of the refinement of the indirect inhumanity, in regard to the husbandry procedure, can be seen in the UK Code of Practice (2014) and the recommendations on the RSPCA resources (2020). The UK code of practice regulates the minimum surface area for mice housing. A study done in the mid-20th century explored the effect of group size by putting 16 mice in a ~980cm2 cage (Southwick & Bland, 1959). A study by Dörner et al. (2006) also showed how overcrowding can have detrimental effects to mice. Other than the surface area, the recommendation of the RSPCA also mentioned cage cleaning. Cage cleaning has been known to affect animals in different ways, especially mice, as a consequence of disturbing the odour information in the home cage (Gray & Hurst, 1995). Although cage cleaning is not detailed within the UK code of practice, it is commonly done every week by moving the animals into a new clean cage. However, this has been proven to be detrimental and the recommendation by the RSPCA suggests to perform spot cleaning of the soiled bedding every fortnight to minimise disturbance. Although group size and cage cleaning might not be applicable to all experimental procedures, by addressing what the animals need and trying to fulfil those needs is a step towards the refinement of animal welfare.

Another example of the refinement of the indirect inhumanity can be seen in the handling refinement study done by Hurst and West (2010). It is common practice to handle mice by picking them up by their tail. It is still not fully understood why it is aversive, but various studies have shown negative effects associated with the tail pick up. Hurst and West (2010) showed an alternative, non-aversive, method of picking up mice, either through the cupping method, or by using their enrichment tube. The study also showed how the alternative method did not induce anxiety-like behavioural parameters, unlike the tail handling method, however, it is within the animal's interest to assess other parameters to show whether the animal's welfare state was compromised by tail handling or not.

The whole 3Rs perspective in minimising and addressing animal suffering and pain has developed since the time the principles were written by Russel and Burch. Although it is still a work in progress, plenty of advancement has been made in the interest of animal welfare and recognising their pain and suffering. In the bigger picture, the improvement of animal welfare is important for the replicability of science

because the improvement of animal welfare also reduces variability between experiments.

1.3.4. Animal welfare and experimental replicability

After all the considerations of animal welfare theory, it is also important to understand why animal welfare is important in science. It was mentioned briefly how animal welfare might affect the experimental outcome. According to Pritt and Hammer (2017), experimental replicability is one of the big problems in life science. The main cause of the lack of reproducibility is primarily caused by underpowered studies. Apart from other causes such as incorrect data interpretation, the absence of control groups, selective data reporting, blatant fraud, self-correction that supports fundamental experimental flaws and publication bias towards positive results, the animal welfare aspect is commonly less emphasised.

Granted, that there are various factors playing into this replicability issue, but according to Pritt and Hammer, animal welfare itself might play a bigger role than previously thought and not enough attention has been given. Poole (1997) elaborated how good science is governed by factors such as: important problems for which an answer is being sought, the answer to the problem that is not an ambiguous result, and controlled variables which are not part of the problem. The variable controlling aspect becomes important when it comes to animal welfare since inhumanities could directly affect the variability of the physiological and behavioural responses in the animals. Poole described the term of a “happy animal” as busy, confident, behaves normally, not in pain, resists disease, and successfully reproduces. This concept is now more commonly known as the wellbeing of the animals which is commonly sought as the goal of the refinement part of the 3Rs (Tannenbaum & Bennett, 2015).

1.3.5 Animal welfare and olfaction

Olfactory cues have been known to play a major role in modulating behavioural development and expression in animals. Previous studies have tried to use olfactory cues to enrich the environment of captive animals, including zoo and farm animals. A study by Wells and Egli (2004) shows that olfactory enrichment of catnip for black-footed cats could increase the amount of time spent in active behaviours. While a study by Blackie and de Sousa (2019) shows that the addition of garlic oil significantly

improves activity in weaned pigs. In addition, a study by Van Loo, Van Zutphen, & Baumans (2003) shows that the addition of soiled beddings, which contain natural olfactory cues, from the previous cage to the fresh cage during cage cleaning regime could decrease aggression within group housed laboratory mice. However, when it comes to laboratory mice, there were limited literatures where the studies added synthetic odorants to improve animal welfare.

The way of enriching the environment of the captive animal's environment falls within the refinement aspect of the 3Rs. As previously mentioned, the aim of the refinement is to minimise the distress of the animals. The application of the OR37 ligands in mice as previously shown by Klein et al. (2015) suggested that these olfactory cues affected the component of the stress axis by reducing the activation of the PVN of the hypothalamus. Although further investigation is necessary for the effects to be replicated within different contexts, it is a possibility to take advantage of this mechanism as a form of olfactory enrichment in animal welfare refinement context.

1.4 Thesis Aims and Objectives

In summary of the information presented in this chapter, it is known that exposure to OR37 ligands in mice reduces the activation of the PVN, which is a part of the stress axis, within a novel environment testing box. However, it is currently unknown whether this effect can be replicated in different contexts and whether this effect can be taken advantage of to improve animal welfare. It is also known that odour is only one of the components of stressor source within captive animals and there are numerous other possibilities for refinement within the source of stress for captive animals. The overarching aims of this thesis are to elucidate the wider application of the OR37 ligands, including within an animal welfare context. Additionally, this thesis will also further investigate other refinement possibilities within laboratory mice without the use of the OR37 ligands.

Specific hypotheses being tested:

- Acute OR37 ligands application in mice affects the PVN of the hypothalamus within an anxiety-like behavioural context.
- Chronic OR37 ligands application in mice as an olfactory enrichment to improve animal welfare.
- Refinement of animal welfare without the use of the OR37 ligands can be done within the context of potential stressors such as from thermal stress and routine husbandry procedure stress.

Chapter 2: General Methods

This chapter covers all the methodological aspects that were not exclusive to once chapter. All general details regarding animal husbandry procedure and repeating experimental procedures will be covered within this chapter while the small changes in each protocol/procedure will be described on each individual chapters.

2.1. Animals and Housing

Mice used in the studies were either C57BL/6J (Harlan), CD/1 (Harlan) or CRH-cre/tdTomato (inhouse breeding). The CRH-cre/tdTomato is a line of transgenic animal with C57BL/6J background that expresses the tdTomato reporter gene in CRH-expressing neurons. The mice arrived at the animal service unit at 7 weeks old and acclimatised for 1 week before any experimental treatment or habituation to the experimenter. The mice were always handled using their cage cardboard tube or a fresh cardboard tube which subsequently will become the replacement tube in their cage. The mice came into the unit as group housed and were subsequently separated into individual housing 1 week prior to the beginning of experimentation, unless they were used in the experiment as a group housed condition. The mice were randomly assigned to a number and were randomly allocated to a cage to minimise the bias while allocating the mice into a cage. All cages of mice were randomly assigned to their respective experimental treatments using a random number generator specific to their experimental procedure.

All mice were housed in a conventional mouse cage (365 x 207 x 140 mm, 1284L Eurostandard Type II L, Techniplast, UK) with an open top lid. The mice were kept on a 12:12h conventional lighting regime (white lights on at 7 AM and off at 7PM), or a reversed lighting regime (white lights off at 8 AM and on at 8 PM, with motion sensor-activated red lights during the dark condition), with housing temperature 23±1 ^{o}C. Mice were provided with standard enrichments of cardboard tube(s), bedding material (sawdust), nesting materials (paper shavings, unless otherwise stated in other chapter methods) and wooden blocks. All mice were fed with standard laboratory rodents chow (Purina, UK) on ad libitum feeding regime and had free access to water,

except during testing. The cages were cleaned at the start of every week (unless stated in other chapter methods) by moving the mice using their cardboard tube into a clean cage with their previous cage's lid (including the food pellets and the water bottle), cardboard tube(s), and wood block(s). No behavioural tests were performed within the same day of the cage cleaning unless mentioned otherwise. All experimental procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, Home Office licenses, and were approved by the local ethical review group (University of Bristol).

2.2. OR37 ligands preparation

The preparation of the OR37 ligands mixture for experiments was based on Klein et al.'s (2015) paper. 1 mg of each: pentadecanal (C15, Abcr, Germany), hexadecanal (C16, Cayman chemical, USA), and heptadecanal (C17, Abcr, Germany) were weighed and added into 1 L of a 1,3-propandiol (Sigma) solvent, heated to 37 ^{o}C and stirred until dissolved to make the concentration of 10 ng/ 10 µl. The ligands mixture was decanted into smaller aliquots for single use and stored in temperatures of -20 ^{o}C. The ligand mixture was always heated to 37 ^{o}C in a water bath before being used. A similar protocol was followed when making pentadecanal-heptadecanal mixture and hexadecanal-only mixture.

2.3. Open Field Test

Mice were observed in an open field arena (60 cm x 60 cm x 30 cm) under normal lighting conditions (~120 lux on the centre of the arena, unless specified otherwise in detailed chapter method). During the test day, the mice were moved from their home cage to the centre of the open field arena using their cardboard tube. Behavioural activities were recorded with a video camera for 30 minutes and then analysed offline using Ethovision XT (Noldus Information Technology, inc). The observed behaviours included rearing (mice standing with two forelimbs raised from the ground, either leaning against the wall or not against the wall), defecating (leaving a pellet/faecal count), and locomotion. Behaviours other than locomotion were observed and scored using the Observer 9 software (Noldus Information Technology, inc) while the analysis of locomotion was automated using the Ethovision software. Within the

Ethovision software, the arena was divided into 16 equal squares in a 4x4 configuration. The squares were defined as corners (4 corner squares), centre (4 squares in the middle), and borders (all squares except centre) as shown on figure 2.1. The locomotion behaviours were further specified to time spent in zone (in seconds) and to total distance travelled (in cm). A mouse showing higher anxiety-like behaviours were expected to have lower time spent in centre, higher locomotive activity, and more faecal pellets found within the centre of the arena (Kraeuter, Guest & Sarnyai, 2019).

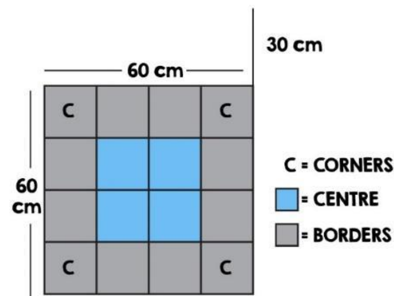

Figure 2.1: Open field maze used in experiment and the area zonation within Ethovision

2.4. Elevated Zero Maze Test

Mice were observed in an elevated zero maze arena (diameter 60 cm, track width 5 cm, height 30 cm, 2 closed arms and 2 open arms), as shown on figure 2.2, under normal lighting conditions (~120 lux on the open arm). During the test day, the mice were moved from their home cage to one closed arm (consistent across all experiments) of the elevated zero maze arena using their cardboard tube. Behavioural activities were recorded with a camera for 10 minutes and analysed offline manually, either using The Observer XT9 software or BORIS v. 7.9.7 Software (Friard & Gamba, 2016). Measured behavioural activities were time spent in the open arm, latency to the open arm, and frequency of transition between open arms. The mice were defined as entering the other arm by the means of all four limbs entering the other arm. The increase in open arm activity reflects an anti-anxiety behaviour (Walf & Frye, 2007).

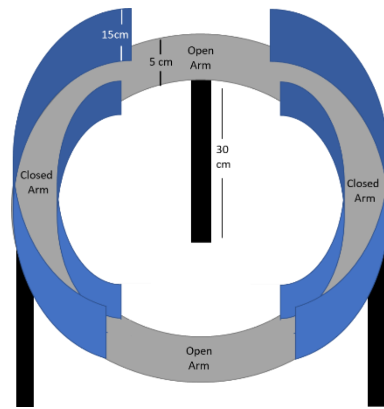

Figure 2.2: Diagrams of the zero maze arena

2.5. Tissue Collection, Preparation, and Storage

Animals were sacrificed at the time point of 90 minutes after the beginning of any test or treatment (i.e., at 60 minutes after 30 minutes of open field test). The animals were killed with either an overdose of Euthanal followed by perfusion or by a schedule 1 killing of cervical dislocation. Perfusion was done with 5 ml of phosphate-buffered saline (PBS) followed by 20 ml of a 4% formaldehyde solution. The brains were collected after perfusion or cervical dislocation and post-fixed in a formaldehyde solution for 4-5 hours at 4°C and changed to 30% sucrose solution at 4°C overnight. After the brain had sunk within the sucrose solution to the bottom of the container (± overnight), the brain was trimmed coronally in a flat skull position at bregma 0. After trimming, the brain was positioned and either cut straight away or frozen and stored at -20°C. The freezing was done either using isopentane together with liquid nitrogen while being covered in OCT solution or using a metal plate on top of dry ice while covering the brains with OCT solution.

The brain was cut using freezing microtome into 40 µm thickness and stored in cryoprotectant at -20 °C for free floating immunohistochemistry protocol or was cut using a cryostat into 15 µm thickness on subbed slides and stored in -20 °C for a slide mounted immunohistochemistry protocol. Sections were collected starting from the end of the anterior commissure until the ventral hippocampus was visible, or after collecting 60 sections for 40 µm sections, or 160 sections for 15 µm sections. The sections were either collected into a full set of serial sections, or two sets of alternating sections.

2.6. Immunohistochemistry

2.6.1. Free-floating method

3,3-Diaminobenzidine (DAB) Protocol

Two sets of alternate sections were used for this protocol. One set of the sections was washed with PBS, tris-buffered saline (TBS) and incubated in 0.6% hydrogen peroxidase in TBS for 30 minutes to block endogenous peroxidase. The sections were washed with TBS and incubated for 30 minutes in TBST (0.1% triton-x in TBS) with a 3% normal goat serum (30 µl in 1 ml TBST) at room temperature. The sections were incubated overnight in primary antibody (Rabbit polyclonal anti-c-fos, Millipore ABE457, 1:5000) with a 3% normal goat serum at 4ºC. On the next day, the sections were washed using TBST with a 3% normal goat serum and incubated in a secondary antibody (goat anti rabbit biotin, ABCAM, Inc., 1:400) in TBST with a 3% normal goat serum at room temperature for 1 hour. After being incubated with the secondary antibody, the sections were washed with TBST and incubated on a shaker with avidin-biotin complex (ABC) reagent (Vectastain ABC kit, Vector, Inc.) for 1 hour at room temperature. The sections were then washed using 0.05 M tris buffer and visualised by a DAB reaction (DAB kit, Vector, Inc.). Cold PBS was used to stop the reaction and the sections then washed in TBST solution and mounted on the slides and cover slipped. The other set of the sections were stained using cresyl violet to stain for cell nuclei and used as a marker to locate the PVN.

Immunofluorescent protocol

For the first day, the sections were washed with TBS four times with 10 minutes wash time in between washes in order to wash out the cryoprotectant. Afterwards, the sections were incubated with a TBST + 3% normal goat serum for 30 minutes followed with overnight incubation of primary antibody in a TBST + 3% normal goat serum at 4 ºC. On the second day, the sections were washed three times with a TBST + 3% normal goat serum with 10 minutes in between and incubated with secondary antibody in a TBST + 3% normal goat serum for 3 hours in the dark. After secondary antibody incubation, the sections were washed with TBS 3 times with 10 minutes in between and mounted in minimal light conditions. The sections were mounted on PBS solution for the free-floating setup onto subbed slides and cover slipped with antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI).

2.6.2. Slide mounted immunofluorescent protocol.

Prior to the staining, the slides were selected for the sections containing the PVN and were drawn around the sections using immunohistochemistry hydrophobic pap pen (Dako, Sweden) to prevent the solutions from spilling during the immunohistochemistry protocols. After the slides reached room temperature, the sections were washed using a TBS solution pH 7.4 for 4 times with 10 minutes sitting time for each wash. After the TBS wash, the sections were incubated with TBST solution with a 3% normal goat serum for 30 minutes. After the incubation, the sections were incubated with a primary antibody solution (rabbit anti c-Fos ABE457 in 1:500 dilutions to TBST with a 3% normal goat serum, Millipore) in a 4°C humidifying chamber overnight.

After the overnight incubation, the sections were washed 3 times with 10 minutes sitting time for each wash with a TBST solution with a 3% normal goat serum. The sections were incubated with a secondary antibody (Alexa fluor 488 goat anti rabbit, Invitrogen, diluted to 1:500 in TBST with a 3% normal goat serum solution) for 2 hours under dark conditions and everything that was done after this step was done under dim lighting. After the incubation, the sections were washed using TBS solution 3 times with 10 minutes sitting time for each wash. After the final wash, 2 drops of a mounting medium with DAPI (Vector) were added to the slides and cover slipped. The slides were air-dried for 10 minutes before nail polish was applied to the sides of the coverslips to prevent the coverslips from slipping during the image acquisition process.

2.7. Image Capturing and Cell Counting

Images were captured using either a bright field microscope connected to a camera, or widefield inverted microscope (Leica DMI6000 inverted epifluorescence microscope, Wolfson Bioimaging Facility, University of Bristol, UK) connected to camera (Leica DFC365FX monochrome digital camera). 3 different filters were used: DAPI (cell nuclei labelling), GFP for alexa 488 green fluorescent (c-Fos labelling), and CY3 for alexa 568 for red fluorescent (OXY or AVP labelling). Leica Application Software X (Leica microsystem) was used for capturing the images.

Images were captured under 5 times or 10 times objective magnification depending on the observed brain region. Exposure duration was adjusted on a red-black colour

palette to make sure there was not any under/over exposure for each filter and was kept consistent for all image acquisition. Files were saved as a .lif format for cell counting analysis and/or z-stack colocalization analysis.

Captured .lif images were then analysed manually in Fiji (Schindelin et al., 2012). Prior to analysis, all image files were blinded using code to minimise observational bias. Analysis was performed by cropping the area of the region of interest for measuring the area size and using multiple selection tools as a cell counter. The area measurement and the cell counting were repeated and averaged for 3 images on 40 µm sections and 5 images on 15 µm sections. PVN c-Fos activation was calculated by converting the PVN area of the brain sections into mm2 and using the said value to divide the number of visible cells labelled by the c-Fos staining that overlap with the stained nuclei on the DAPI channel. The value of cells per mm2 was then averaged for each brain to produce PVN c-Fos activation value for each animal.

2.8. Radioimmunoassay for CORT and ACTH

After cervical dislocation, the trunk blood was collected into a small petri dish with 50 µl of ethylenediaminetetraacetic acid (EDTA) and 50 µl of aprotinin (bovine lung serum, Sigma-Aldrich), to prevent coagulation and to preserve the hormones. The aprotinin was not used when the blood only collected for CORT analysis. The small petri dish was weighed before and after adding the blood to calculate the dilution factor for each blood sample. The blood was then transferred to a microtube and centrifuged in 8000g for 10 minutes to separate the serum plasma and the red blood cells. The plasma was then transferred to a new microtube and stored in a box with dry ice before being stored at -80°C until analysis.

RIA analysis was performed by Mr Zidong Zhao and/or Ms Megan Jackson at the Dorothy Hodgkin Building, University of Bristol, UK, following the protocol described before by Spiga et al., 2007. Briefly, 10 µl of blood plasma sample was diluted in 490 µl of citrate buffer solution. 100 µl triplicates from the diluted samples were then added with 50 µl of the radio-labelled corticosterone antibody or the radio-labelled ACTH antibody and incubated overnight at 4°C. On the next day, samples were precipitated with 500 µl of a charcoal/dextran T70 solution and centrifuged at

3256g for 15 min at 4°C. Supernatant from the samples were then aspired and the pellets were loaded into the gamma counter for corticosterone detection.

After the value was obtained and adjusted according to the standard curve and 1:50 dilution factor, the value was corrected following the sample collection dilution factor, which follows the formula:

$$Corrected\ Value = \frac{Gamma\ Counter\ Value}{\left(\frac{Blood\ plasma\ weight}{Blood\ plasma\ weight +\ EDTA\ weight}\right)}$$

2.9. Eye Thermal Recording

The eye temperature data was recorded using the FLIR C2 thermal camera (FLIR System, Inc.). For the recording, the FLIR camera was connected to a laptop with the FLIR software and positioned approximately 30 cm above the cage of the animals. Image sequences were captured for 5 seconds with 2-3 frames per second. Analysis was done blinded and 3 consecutive images were selected with criteria of showing the same side of the eye of the mice. Using the selection tool, a 3x3 pixel was deployed and the maximum temperature value was recorded for each image. The 3 values were then averaged to denote the eye temperature of a mouse from one sampling.

2.10. Cotton Bud Biting Test

The cotton bud biting test (also called as cotton swab biting test) was used as a measure of non-social aggressive behaviour. This method was adapted from Park et al. (2015). The mouse was scruffed and held facing the experimenter. A cotton bud (wooden stick with cotton on both ends) was then presented in front of the mouse's mouth. The cotton bud was presented 10 times with a 5 second gap between each presentation. The total number of trials in which biting occurred and the duration of each bite with a maximum of 5 seconds were recorded. Different cotton buds were always used between animals to prevent contamination from the previous mouse.

2.11. Data Analysis and Statistics

Power analysis was performed using the software GPower (Universitat Dusseldorf) to calculate sample size needed for groups in PVN c-Fos activation experiments. The effect size (d) was calculated based on Klein et al (2015) data for control and 10 ng/10 µl OR37 ligands mixtures (means and standard deviation) and resulted in a value of d=1.8. From a power calculation based on α=0.05 and power=0.80, the minimum required sample size for each group was 6.

All data was analysed using IBM SPSS Statistics (version 25) and all the graphs were produced using the software GraphPad Prism 6.0 (GraphPad Software, USA). The standardised residuals and Cook's distances from the ANOVA were checked to identify potential influential data points. The standardised residuals were checked for the normality using Saphiro-Wilk test and the appearance of residuals histogram and normality plot were manually observed. A data point was considered as an influential data point when the z-value was higher than 3 times SD and/or the Cook's distance value was greater than 1 (Field, 2013). Normally distributed data with more than 2 groups were analysed using one-way ANOVA, to see the difference between groups or two-way ANOVA, to see the difference between groups, sex, and the interaction between group and sex. When there was a significant difference between groups from the ANOVA, either planned contrast analysis was performed appropriately following the a-priory planned comparisons, or post-hoc analysis was performed with Gabriel correction when the group sizes were unequal or Dunnett correction when the group sizes were equal to check the specific difference between groups. When the data was not normally distributed, the non-parametric Kruskal-Wallis test was used instead when there were more than two groups to be compared, or the Mann-Whitney test when there were two groups. When the group sizes were unequal, the Levene test was also considered and when the data did not pass the Levene test, the Welch correction was applied on the ANOVA. For preference data, a one-sample t-test was used to check whether the preference value significantly differed from 0. For multiple recordings from the same animals over a period of time data, a Repeated Measures ANOVA was used with WEEKS as a within-subject factor and GROUPS as a between-subjects factor. The sphericity of the ANOVA was checked and if the sphericity assumption was violated, a correction factor was applied. The epsilon value was used as a cut-off point whether to use the Huynh-Feldt correction (epsilon > 0.75) or the Greenhouse-Geisser correction (epsilon < 0.75)(Field, 2013). When the data

type was not a continuous variable, the non-parametric equivalent of the Friedmann test was used in place of the Repeated Measures ANOVA for the within-subjects factor and using the Kruskal-Wallis or Mann-Whitney test for the between-subjects factor.

Chapter 3: OR37 Ligands Properties on Acute Stress Responses

3.1. Chapter Aim and Objectives

The aim of this chapter is to investigate the hypothesis of whether acute application of the OR37 ligands in mice affects the PVN of the hypothalamus within an anxiety-like behavioural context. Specific experimental objectives within this chapter include:

- To investigate mice natural preference to the solvent of the OR37 ligands.
- To confirm the reduction effect of exposure to OR37 ligands on PVN c-Fos activation in anxiogenic tests using an open field arena.
- To determine the effects of acute OR37 ligands on anxiety-like behaviours.
- To determine the effects of acute OR37 ligands on blood plasma CORT and ACTH in anxiogenic tests.
- To determine the effectiveness of different mixtures of OR37 ligands in reducing the PVN c-Fos activation.
- To determine the concentration-response effects of the OR37 ligands.
- To determine whether oxytocinergic and vasopressinergic neurons are being activated by exposure to the OR37 ligands.

3.2. Introduction

The OR37 pathways are known to have unique direct projection to the PVN of the hypothalamus and medial amygdala (Bader, Breer, & Strotmann, 2012; Bader, Klein, Breer, & Strotmann, 2012). The study done by Klein et al. (2015) showed that the activation of the OR37 subsystem using a mixture of the OR37 ligands during a novel environment stressor significantly reduced the PVN c-Fos activation. In a separate experiment, Klein et al. (2015) also showed that the PVN c-Fos activation during the novel environment stressor colocalized with CRH neurons instead of oxytocinergic or vasopressinergic neurons. This finding raised a question whether it is possible to apply the OR37 ligands in a broader context other than the novel environment stressor. Therefore, we tried to replicate the PVN c-Fos activation reduction effect on anxiety-like behavioural measures, which in this case, we used the open field test and elevated zero maze test. Respectively, we are also interested to see whether this significant drop of PVN c-Fos activation will also change the anxiety-like behavioural outputs.

The PVN of hypothalamus is known to regulate the HPA axis by producing CRH as a response to stress, which in turn will affect the pituitary to produce ACTH. The ACTH will then be transported to the adrenal glands to signal the CORT release in the bloodstream and facilitate energy mobilisation. The ACTH and the CORT play an important role in stress response signalling in order to mobilise the energy reserve and ultimately modify the animal behaviours as another layer of response to the stressor. An example of the relationship between CORT and anxiety-like behaviours in mice was shown in a study by Rasmussen et al. (2011) where cage cleaning increased both the time spent in the centre of the open field arena and the serum corticosterone level. In addition, a study by Murray, Smith & Hutson (2008) showed that injection of chronic low dose corticosterone in mice decreased the time spent in the light area of the light/dark box test. A significant drop in neural activity in the PVN due to the OR37 ligands exposure might suggest a change of everything under the same HPA axis pathway, including neuroendocrine and behavioural responses. Following the anxiogenic tests, it is also an interesting question to see the relationship between the anxiety-like behaviours and the neuroendocrine responses within the context of the OR37 ligands exposure. Addressing this question, we will also attempt to validate the OR37 ligands exposure on anxiogenic tests and check the neuroendocrine responses, which are the blood plasma CORT and ACTH level.

Regarding the OR37 ligands themselves, the study done by Bautze et al. (2012) showed that the OR37A, -B, and -C glomeruli did not activate exclusively only to one ligand. It is interesting how one ligand could also activate different glomerulus, hence showing some degree of overlap in activating different glomerulus with other ligands. Despite the study by Klein et al. (2015) using the mixture of the pentadecanal (C15), hexadecanal (C16), and heptadecanal (C17), the Bautze et al. (2012) study suggested that the hexadecanal activated the three OR37A, -B, and -C glomeruli. Meanwhile, pentadecanal only activated the OR37A, and -B glomeruli and heptadecanal only activated the OR37B, and -C. It is still not known whether individual ligands or different mixture compositions will affect the PVN activation differently, even further, to other stress response parameters. To address this, we will have a treatment group of hexadecanal solution due to its capability of activating all three OR37 glomeruli and see whether it also decreases the PVN c-Fos activation. In addition, we are also going to test another group of combined pentadecanal and heptadecanal mixture, due to the combined two's supposed ability to stimulate all three OR37 glomeruli as well. The groups of the hexadecanal on its own and the combined pentadecanal and heptadecanal are expected to have a similar reduction effect of PVN activation to when all three components of the OR37 ligands are present.

Other than the composition of the OR37 ligands mixture, it is also important to understand the concentration-response relationship of the OR37 ligands. The concentration-response relationship of the OR37 ligands is complicated due to the limitation on measuring the airborne concentrations and the effect of different modes of exposure. The exposure to the OR37 ligands mixture on Klein et al. (2015) was done with the concentration of 10ng/ 10µl which resulted in the significant PVN activation reduction and 1ng / 10µl which did not affect the PVN activation similar to the solvent control group. There have not been other studies done on the OR37 ligands which shows a gap in knowledge regarding the effects of different concentrations of the OR37 ligands to be unknown. It is interesting to see whether direct exposure to the maximum concentration will increase effectiveness and whether the concentration in between 10ng/10µl and 1ng/10µl will show different effectiveness levels. To address this question, we will investigate a concentration-response relationship between the 10ng/10µl and 1ng/10µl to the PVN activation. We will also investigate the effectiveness of higher exposure concentration in the PVN activation reduction .

Lastly, the PVN activation during the novel environment stressor was shown to colocalize with the CRH neurons instead of vasopressinergic or oxytocinergic neurons (Klein et al., 2015). However, a study by Bader, Klein, et al. (2012) showed that the OR37 glomeruli have direct projection to vasopressinergic neurons in the PVN. Interestingly, the study done by Klein et al. (2015) was done separately between the PVN activation colocalization and the PVN activation reduction to OR37 ligands exposure. This leaves the gap in knowledge of the direct effect of OR37 ligands exposure to PVN neuronal type during stress conditions. To address this, we will investigate the colocalization using double labelling immunohistochemistry method to the samples of stressed animals with and without OR37 ligands exposure.

3.3. Methods

3.3.1. Animals and Housing

The mice used in the studies were either the C57BL/6J strain or the CRH-cre/tdTomato strain. The mice were cared for by following the husbandry procedure described in chapter 2, section 2.1 Animals and Housing. Animals were kept either at reverse lighting or conventional lighting. Further details regarding the strain, number of animals used, group size, and the lighting regime are specified in table 3.1. The experiment was planned to be done on the CRH-cre/tdTomato strain, however, due to a breeding issue, the CRH-cre/tdTomato mice did not produce c-Fos protein. The C57BL/6J strain was used instead starting from experiment 2, due to the C57BL/6J being the background strain of the CRH-cre/tdTomato. Both male and female mice were used from the experiment 1 up to experiment 5. However, there was no sufficient evidence from the 5 experiments that there were differences between sexes. In order to minimise variability, only male mice were used in experiment 6 and 7. The experiments were initially done on a reversed lighting cycle. However, in order to increase the anxiety-like behavioural responses, the experiment was done within the light phase of the conventional lighting cycle. Both handling and cages were as described in Chapter 2, section 1, Animals and Housing.

Table 3.1: Strain, number of animals used, group size, and lighting regime.

Experiments	Strain Used	Animal Numbers		Group Size	Lighting
		Male	Female		
Experiment 1 : Unconditioned place preference	CRH-cre/tdTomato	12	12	Group (3 per cage)	Reverse
Experiment 2 : Pilot project on open field	C57BL/6J	9	9	Group (3 per cage)	Reverse
Experiment 3 : Group housed and stress hormone on open field	C57BL/6J	27	15	Group (3 per cage)	Reverse
Experiment 4 : Singly housed on brighter open field	C57BL/6J	15	15	Individual	Conventional
Experiment 5 : Singly housed and stress hormone on open field	C57BL/6J	21	21	Individual	Conventional
Experiment 6 : Concentration response study on zero maze	C57BL/6J	30	0	Individual	Conventional
Experiment 7 : Colocalization study on zero maze test	C57BL/6J	16	0	Individual	Conventional

3.3.2. OR37 Ligands Exposure Method

The Klein et al. (2015) study used an airflow system that was integrated in the novel box arena to deliver the OR37 ligands. Although it was possible to scale the novel box arena into an open field arena, the integrated airflow system was not practical to be used in other types of behavioural measurement with an open region such as the elevated zero maze. To address this issue, all anxiety-like behavioural tests within this chapter were performed inside an exposure chamber (100 cm x 100 cm x 50 cm) with a lid to contain the OR37 ligands treatments. Despite the exposure chamber being a closed environment with a lid, the exposure chamber was not airtight, which was similar to the novel box arena that was used by Klein et al. (2015).

The volume of solution used was calculated based on the volume used on an unfamiliar closed plastic box (11.5 cm x 14 cm x 9 cm) described by Klein et al. (2015). Based on the 10 µl OR37 ligands mixture used within the volume of the unfamiliar closed

plastic box (1,449 cm^3) and need to be scaled to the volume of the exposure chamber (500,000 cm^3), 1.5 ml of OR37 ligands mixture was applied in the exposure chamber to equate the concentration of 10 ng/ 10 µl in the novel box arena used by Klein et al. (2015). Similar calculation was also performed when exposing in the mouse home cage (Techniplast 1284L) with an open top lid.

Inside the enclosure, an assembly made of a computer fan with a metal wire cage was placed on each corner. The OR37 ligand mixtures or solvent were applied on a 5 x 7 cm filter paper and placed on top of the assembly and blown by the computer fan facing upwards to saturate the exposure chamber with the testing solution. Total volume of the solutions for the treatment was divided equally for each corner (for example 750 µl for each corner of 10 µg/10 µl in the 3 ml treatment group). Solutions were let to saturate the air inside the chamber for 15 minutes before any animal behavioural testing.

3.3.3. Unconditioned Place Preference (UPP)

The UPP was done in order to check whether there was any innate avoidance to the solvent (propanediol) of the OR37 ligands mixture based on personal communication with the investigator on the Klein et al. (2015) study. Another candidate for solvent (diethyl phthalate) was also tested from the personal communication. The UPP test was done with 1,2-propanediol against blank and diethyl phthalate against blank. The test was performed in a rectangular three-chamber plexiglass box divided into a middle chamber (28 x 14 x 30 cm) and two side-chambers (15 x 14 x 30 cm each). Each chamber was divided by a clear plexiglass divider with a small hole (5 x 5 cm) on the middle-bottom part that allowed mice to traverse between chambers. Petri dishes with holes on the lid with 2.5 cm x 2.5 cm filter paper were placed in each side of the chambers. The chambers were filled with a thin layer of clean bedding (sawdusts) and changed for every test. An individual mouse was placed in the middle chamber and left for 5 minutes to habituate to the testing arena and the behaviour of the mice during the habituation phase was recorded for offline analysis. After 5 minutes of habituation, the petri dishes were changed with a clean one. 100 µl of test solution was spotted onto the filter paper in either the right or left petri dish that has been counterbalanced while the other side's petri dish was left with only dry filter paper in it. The behaviour of the mouse was recorded again for another 5 minutes. After each test, the box was cleaned

with detergent and wiped dry before being used for the next test. The amount of time spent in a chamber was defined starting from when the entire head of the mouse entered the chamber until the entire head entered another chamber. Preference for the solutions were calculated with the formula:

$$preference\ to\ solution: \frac{duration\ in\ solution\ chamber}{(duration\ in\ solution\ chamber + duration\ in\ blank\ chamber)}$$

From the results of the calculation, a value of 1 translates into a total preference and value of 0 as total aversion.

3.3.4. Open Field Test

The open field test was done following the protocol described on Chapter 2, section 2.3 Open Field Test and was done within the enclosure chamber mentioned previously in this chapter.

3.3.5. Elevated Zero Maze Test

The elevated zero maze test was done following the protocol described on Chapter 2, section 2.4 Elevated Zero Maze Test and was done within the enclosure chamber mentioned previously in this chapter.

3.3.6. Tissue Collection, Preparation, and Storage

The tissue collection, preparation, and storage were done following the protocol described on Chapter 2, section 2.5 Tissue Collection, Preparation, and Storage.

3.3.7. Immunohistochemistry

The immunohistochemistry was done following the protocol described in Chapter 2, section 2.6 Immunohistochemistry.

3.3.8. Image Capturing and Cell Counting

The image capturing and cell counting were done following the protocol described on Chapter 2, section 2.7 Image Capturing and Cell Counting.

3.3.9. Radioimmunoassay for (RIA) CORT and ACTH

The radioimmunoassay was done following the protocol described on Chapter 2, section 2.8 Radioimmunoassay.

3.3.10. Animals and Experimental Setup

3.3.10.1. Experiment 1: Determination of mice innate preference to propanediol and diethyl phthalate using Unconditioned Place Preference Test

This experiment was conducted to investigate the innate preference of the mice to the solvent used to dissolve the OR37 ligands, which could potentially affect future behavioural measurements. The experiment was done within one day and the order of animals were counterbalanced for sex. The arena was positioned so that both chambers were at a similar distance to the experimental room's door to avoid bias towards one side. During the behavioural recording, the observer was standing away from the arena and care was taken to not cause disturbance to the mice.

3.3.10.2. Experiment 2: Confirmation of OR37 ligands' PVN Activation Reduction Effect during Anxiogenic Test using Open Field Test

This experiment was done to replicate the reduction effect of the PVN c-Fos activation within an open field arena. Prior to the study, a power analysis was performed following the procedure described in Chapter 2, section 2.11 to determine the sample size. Groups used for this experiment were solvent group (propanediol), 1.5 ml of 10 ng/10 µl OR37 ligands (pentadecanal, hexadecanal, and heptadecanal) mixtures (low volume), and 3 ml of 10 ng/10 µl OR37 ligands mixtures (high volume).The volume added to the arena was changed to increase the volume available for dispersion within the closed enclosure. 3 ml was chosen to see the effect of the mixtures if the concentration was doubled in given chamber's volume. Total animals used in this experiment were 18 mice (9 male and 9 female) that randomly assigned to 3 groups

with computer generated random numbers and counterbalanced for sex on each block. The order of treatments for each block was also counterbalanced.

18 animals were group housed in a reverse lighting room with a cage size of 3 and were transported to the testing room to be tested in an OF arena over 3 days of experiment. The animals were moved to the arena using the cardboard tube from their cage and 90 minutes after the start of the OF test, the animals were sacrificed. The animals were perfused and the brains were processed immediately for the sectioning using a freezing microtome to get 2 sets of 40 μm thickness sections. Immunohistochemistry was done following the free floating - DAB protocol.

3.3.10.3. Experiment 3: Different combinations of OR37 ligands mixture and neuroendocrine responses

Following the successful pilot in confirming the OR37 ligands' PVN activation reduction effect on the Open Field Test, the aim of this experiment was to see whether the mix of all three components required. Another aim of this experiment was to see the neuroendocrine responses to the OR37 ligands exposure. Following the same experimental setup as the previous experiment, the animals were group housed with at least 2 animals and maximum 4 animals in 1 cage with male and female separated. 30 mice (15 male and 15 female) were allocated into 5 groups and counterbalanced for sex. The 5 groups were cage control (taken straight from home cage without experimental procedure and used as a baseline control for c-Fos expression), solvent control group, the 10 ng/ 10 μl OR37 ligands mixture group, 10 ng/ 10ul hexadecanal (C16) group, and 10 ng/ 10 μl pentadecanal with heptadecanal mixture (C15C17). The C16 alone was used as there is an overlap of OR37 responses to the aldehydes which has activity over the three receptor types. Similarly, the combination of the C15 and the C17 meant to have activity over the three receptor types. The volume used was 1.5 ml for all groups following the result from a previous experiment that showed significant effects but also on the same concentration as the previous study had done.

One day prior to the experimental day, the animals were transported from their holding room to the experimental room. On the experimental day, the animals were moved from the home cage to the open field arena using their cage's tube and the time spent on the centre was recorded using an overhead video camera. After 30 minutes, the

animals were returned to their home cage and 60 minutes later, the animals were killed with cervical dislocation for brain and blood collection. The brains were fixed and cut into 40 µm sections and stained using the immunofluorescent procedure for c-Fos. The blood sample was processed for the RIA.

A day after, additional 12 male mice were used for determining the time course of the CORT and ACTH. Due to the nature of the CORT release should come after the ACTH release, a time course was needed to show the optimum time point for CORT and ACTH collection. The time points chosen were 0 minutes, 15 minutes, 30 minutes, and 90 minutes. 3 animals were used for each CORT 15 minutes, ACTH 15 minutes, CORT 30 minutes, and ACTH 30 minutes. To make up the 0 minutes and the 90 minutes time point, the CORT and ACTH data from the cage control and the solvent control were used. The mice for the CORT and ACTH time points were placed on the open field arena following their respective time duration and culled for the blood collection. The experiment was conducted with the experimenter being blinded to the treatment groups.

3.3.10.4. Experiment 4: Reconfirmation of the different OR37 ligand mixtures with increased level of stressors

Following the result from the previous experiment 3 that show the low level of endocrine responses on the stressed animals, few adjustments were made to increase the stress level such running the experiment during the light phase, increasing the illumination on the open field arena, and individually housing the mice to control for the mice interaction during the waiting time before the animals were killed. Followed the same open field testing procedure as previous experiment, 30 animals were used (15 male and 15 female) and randomly divided into 5 groups and counterbalanced for sex. The 5 groups were cage control (taken straight from home cage), solvent control, the OR37 ligands mixture, hexadecanal (C16), and pentadecanal with heptadecanal (C15C17). The experiment was conducted following similar design to the previous experiment with the experimenter being blinded to the treatment groups.

The animals were group housed during the handling time. 3 – 5 days prior to experimental day, the animals were moved from the holding room to the experimental room and individually housed. On the experimental day, the animals were moved from

the home cage to the open field arena using their cage's tube and tested using additional lighting to reach ~250 lux. The animals were left in the open field arena for 30 minutes and their behaviours were recorded using a camera. The observed behaviours were moving duration, time spent in the centre, and faecal pellets count. After 30 minutes, the animals were returned to their home cage and 60 minutes later, the animals were killed with cervical dislocation for brain collection. Brains were then fixed and cut into 40 μm sections and stained for c-Fos using the immunofluorescent protocols.

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3.3.10.5. Experiment 5: Reconfirmation of the OR37 ligands' effect on the PVN and the neuroendocrine response with increased level of stressors

Following the previous experiment 4, a meta-analysis was done and showed a significant reduction on the time spent in the centre of the open arms due to the effect of the OR37 ligands (figure not shown). This experiment was done in order to replicate the effect of the OR37 ligands to mice stress responses on Open Field Test. The stress responses used were the PVN c-Fos activation level, anxiety-like behaviours, and the CORT level. Animals were used following the details on section 3.3.1, performed similarly with experiment 4 with an addition of plasma CORT measurement, and divided into groups following Table 3.2. The orders of animals for solution testing were blocked into days and were counterbalanced for treatment and sex.

Table 3.2: Animal group allocation for individually housed mice in open field test with PVN c-Fos activation and plasma CORT measurements

Group	Animal Number			
	Male (Brain)	Male (Blood)	Female (Brain)	Female (Blood)
Cage baseline	3		3	
Solvent	3	3	3	3
OR37 Ligands	3	3	3	3
Hexadecanal (C16)	3	3	3	3

The animals were run with similar setup as previous experiment (experiment 5) to maximise the stress response. The mice from the brain group were returned to the cage

following the open field and killed 60 minutes after by cervical dislocation. The brains were then taken, fixed, and cut into 40 µm sections for c-Fos immunohistochemistry using the free-floating immunofluorescence protocol. The mice from the blood group were killed straight away following the end of the open field which corresponds to the 30 minutes time point for the CORT analysis. The mice from the cage baseline were killed straight from the home cage as a cage baseline control for both CORT and PVN c-Fos, and both the brain and the blood were collected after. The brains were then fixed and processed for c-Fos immunohistochemistry and the bloods were then processed for the RIA.

3.3.10.6. Experiment 6: Different concentrations of the OR37 ligands mixture

Another aim in this chapter is to investigate the concentration-response effect of the OR37 ligands. Following the previous experiments that the open field tests were considered not stressful enough and another member of the lab used a more robust anxiogenic test, the elevated zero maze test was used for this experiment. Animals were used following the details on section 3.3.1. This experiment was done on male mice to minimize variability by removing the factor of sex as the previous experiments not shown any sex difference. The animals came to the animal facility as a group housed (group size of 4), which was 1 week prior to the experimental week, and were habituated to handling during this period. For the experiment, at the end of the handling period, the animals were divided into 5 ligands mixture concentration groups which were 1 ng/10µl, 2 ng/10µl, 4.5 ng/10µl, 10 ng/10µl and the ligand crystals. No solvent control group was used due to the constraint by the need to do the experiments in the morning which limits the number of groups tested in a block. The 1 ng and 10 ng were chosen because the response was known from Klein et al (2015) study. There was a consideration regarding whether to use the 1ng or the solvent control, however, the 1ng/10µl was chosen to complete the set of the concentrations and the 1 ng was supposed to have the same level of effects as the solvent control. The 2 ng/10µl and 4.5 ng/10µl were chosen because both, as the middle concentrations, fit the log 0.3 and log 0.6 point of the response graph. As the final group, the crystal was used to see whether by reaching towards the saturated concentration on the air will have a lower PVN c-Fos activation level than the 10ng/10µl. All of the lower concentrations were made by diluting down the 10 ng / 10 µl stock with the solvent to achieve the

concentrations. The ligands crystal mixture was made by combining ~30 ng for each crystal (pentadecanal, hexadecanal, and heptadecanal) to separate aluminium foil boats on the left and right side of the enclosure. The aluminium foil boat was placed on top of a heating unit (3 cm x 7cm, ~40°C) from 15 minutes before the experiment started.

3 – 5 days prior to experimental day, the animals were moved from the holding room to experimental room and individually housed. On the experimental day, the animals were moved from the home cage to the elevated zero maze using their cage's tube inside the enclosure and conditioned using additional lighting to reach ~250 lux. The animals were left on the elevated zero maze for 30 minutes and recorded using a camera on top of the enclosure. After 30 minutes, the animals were returned to their home cage and 60 minutes later, the animals were killed by cervical dislocation for brain collection. Collected brains were then fixed and frozen with the isopentane-liquid nitrogen method for storage. The brains were then cut into 15 µm thickness with half of the brains were cut using the University of Bristol Biomedical Science Histology Facility service. Immunohistochemistry was done using the slide mounted protocol for c-Fos immunofluorescence (see section 2.6.2.).

3.3.10.7. Experiment 7: OR37 ligands and Colocalization of PVN c-Fos activation with oxytonergic and vasopressinergic during anxiogenic test

The final aim of this chapter was to investigate whether the PVN reduction effect was colocalized with the oxytocinergic neurons or the vasopressinergic neurons. Following the robust result using the zero maze from the previous experiment, the zero maze was used. Animals were used following the details on section 2.3.1 and were divided into 2 groups: solvent control and the OR37 ligands mixture. The experiment setup was done following the protocol on the previous experiment (experiment 6). However, due to a change in the project license, the testing time was changed from 30 minutes to 10 minutes. The brains were fixed and frozen using the metal plate method (see section 2.3.5.) and were cut into 40 µm thickness using freezing microtome. The brains were cut into 2 sets of alternate sections for the free floating immunofluorescent staining. 1 set of the sections were stained using c-Fos and oxytocin staining with both primaries run together in one solution then followed by both secondaries together. The other set of the sections were stained using c-Fos and vasopressin staining. The details of the

primary and the secondary antibodies used are described on Table 3.3 Image capturing was performed as a z-stack with ± 200 µm thickness from the focal point.

Table 3.3: Antibody combinations for colocalization experiment

Target	Primary Antibody		Secondary Antibody		Colour
	Name and Supplier	Dilution	Name and Supplier	Dilution	
c-Fos	Anti c-Fos Polyclonal Rabbit Antibody ABE457 (Millipore, UK)	1:3000	Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen)	1:200	Green
Oxytocin	Anti-Oxytocin Polyclonal Guinea Pig Antibody 408 004 (Synaptic System, Germany)	1:2000	Alexa Fluor 594 goat anti-guinea pig IgG (H+L) (Invitrogen)	1:200	Red
Vasopressin	Anti-Vasopressin Polyclonal Guinea Pig Antibody 403 004 (Synaptic System, Germany)	1:1500	Alexa Fluor 594 goat anti-guinea pig IgG (H+L) (Invitrogen)	1:200	Red

3.3.11. Data Analysis

All data were analysed following the Chapter 2, section 2.11. Data Analysis. PVN c-Fos activation were calculated by converting the PVN area of the brain sections into mm^2 and using the said value to divide the number of visible cells labelled by the c-Fos staining that overlap with the stained nuclei on the DAPI channel. The value of cells per mm^2 were then averaged for each brain to produce PVN c-Fos activation value for each animal. The number of oxytocin labelled cells and vasopressin labelled cells were calculated similarly with c-Fos labelled cells. Colocalization percentage was quantified by dividing the number of c-Fos positive cells divided by the number of oxytocin positive cells or vasopressin positive cells.

All data were checked whether the data were normally distributed or not and whether there was any significant outlier or influential data point to be excluded following the procedure described in Chapter 2, section 11 Data Analysis. On experiment 1, The preference data was analysed using one sample t-test to see whether there was significant avoidance toward the tested solutions.

On experiment 2, the PVN c-Fos data was analysed using a Two-Way ANOVA with GROUPS and SEX as between-subject factors. A non-orthogonal planned contrast analysis was done between the solvent group vs the pooled OR37 ligands groups (low and high volume) and between the OR37 ligand groups (low vs high concentration)

and the p value was corrected using the Holm-Bonferroni correction. The anxiety-like behavioural data (faecal counts, duration in centre, and time spent moving) were analysed using a Two-Way ANOVA with GROUPS and SEX as between-subject factors.

On experiment 3, the plasma CORT level and the anxiety-like behaviour (duration in centre) were analysed using a Two-Way ANOVA with GROUPS and SEX as between-subject factors. The plasma ACTH level and the PVN c-Fos activation were analysed using a Kruskal-Wallis test for the group differences and the Mann-Whitney U test for the sex difference.

On experiment 4, the anxiety-like behaviour data (faecal counts, duration in centre, and time spent moving) and the PVN c-Fos data were analysed using the Two-Way ANOVA with GROUPS and SEX as between-subject factors. The comparison of time spent in centre between experiment 3 and experiment 4 was analysed using the Two-Way ANOVA with GROUPS and EXPERIMENTS as between-subject factors.

On experiment 5, the PVN c-Fos data and the plasma CORT data were analysed using a Two-Way ANOVA with GROUPS and SEX as between-subject factors. A post-hoc analysis using Gabriel correction was performed. The anxiety-like behaviour data (duration in centre and time spent moving) were analysed using a Two-Way ANOVA with GROUPS and SEX as between-subject factors. Meta-analysis of time spent in the centre of the open field arena was performed using the ESCI tools which is a companion software from The New Statistics book (Cumming & Calin-Jageman, 2016). Data of time spent in the centre of the open field were obtained between the solvent group and the OR37 ligands group from four experiments (experiment 2, 3, 4, and 5). Data were inputted accordingly as mean duration value in seconds, standard deviation, and group size. Output of the data were in form of forest plot with the square indicating the mean difference of the particular study, the size of the square indicating the weight of the particular study to the overall meta-analysis, and the whisker extended from the square indicating the 95% confidence interval (CI) of the mean. The diamond shape at the bottom of the forest plot indicates the summary of the studies included in the meta-analysis. The p-value of each studies indicate whether the difference between groups were significant and the p-value of the diamond signify whether there was significant conclusion to be drawn from the meta-analysis or not.

On experiment 6, the PVN c-Fos data and the anxiety-like behaviour data (transitions between arms, latency for first transition to the open arm, and duration in the open arms) were analysed using a One-Way ANOVA. A post-hoc analysis was done using the Gabriel correction.

On experiment 7, the PVN c-Fos data, the oxytocin colocalization, the vasopressin colocalization, and the anxiety-like behaviour data (transitions between arms, latency for first transition to the open arm, and duration in the open arms) were analysed using an independent t-test.

3.4. Results

3.4.1. Experiment 1: Determination of mice innate avoidance to propanediol and Diethyl phthalate using Unconditioned Place Preference Test

There was no evidence for either of the tested solutions being aversive in the UPP. Habituation phase of the unconditioned place preference did not show a preference to either chambers (one sample t-test, p=0.532, figure 3.1). During the test phase, there was also no preference either to the chamber that had propanediol in it or the chamber without anything in it (one sample t-test, p=0.245). Another candidate for solvent of the OR37 ligands, Diethyl phthalate, was also tested against an empty chamber and there was no preference to either chamber (p=0.997). There were no preference changes between the habituation and the test phase of the propanediol ($t_{(22)}$=-0.222, p=0.826) which strengthened the evidence that there were no changes to the preference index when the propanediol was introduced to the arena.

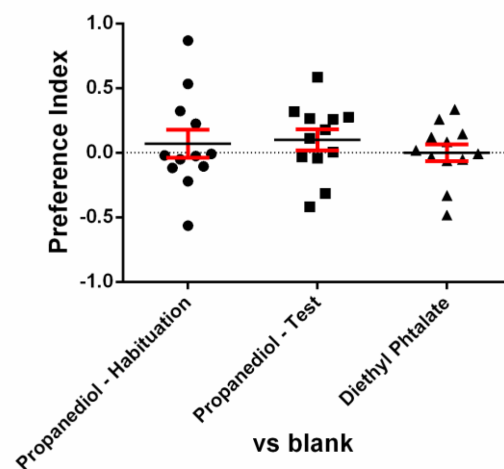

Figure 3.1: Unconditioned place preference shows that mice do not have innate preference or avoidance of propanediol and Diethyl phthalate. Data shown as mean ± SEM.

3.4.2. Experiment 2: Confirmation of the OR37 ligands' Reduction Effect on the PVN Activation during Anxiogenic Test using Open Field Test

The experiment successfully confirmed the OR37 ligands' PVN activation reduction effect during the open field test both on low and high volume of the OR37 ligands without any differences in anxiety-like behavioural measurements. 4 data points (2 from solvent and 2 from the 3 ml group) were missing from the analysis due to technical difficulties both on the cutting procedures and on the staining process. One data point on the OR37 ligands (6ml) group was excluded from c-Fos analysis due to being an influential data point based on Cook's distance value of 0.948 (red dot on figure 3.2). PVN c-Fos activated cell count showed significant difference between groups ($F_{(2,7)}$=5.437,p=0.038, figure 3.2), with no difference between male and female ($F_{(1,7)}$=0.296,p=0.603), and no interaction between sex and groups ($F_{(2,7)}$=0.771,p=0.498). Planned contrast analysis with equal variances assumed and sex factor omitted between solvent to OR37 Ligands group (3ml and 6ml combined) showed a significant reduction of PVN c-Fos activation ($t_{(10)}$=3.380, p=0.007). Individual contrast showed significant reduction of PVN c-Fos activation between solvent vs OR37 ligands (3ml) (p=0.027) and between solvent vs OR37 ligands (6ml) (p=0.008).

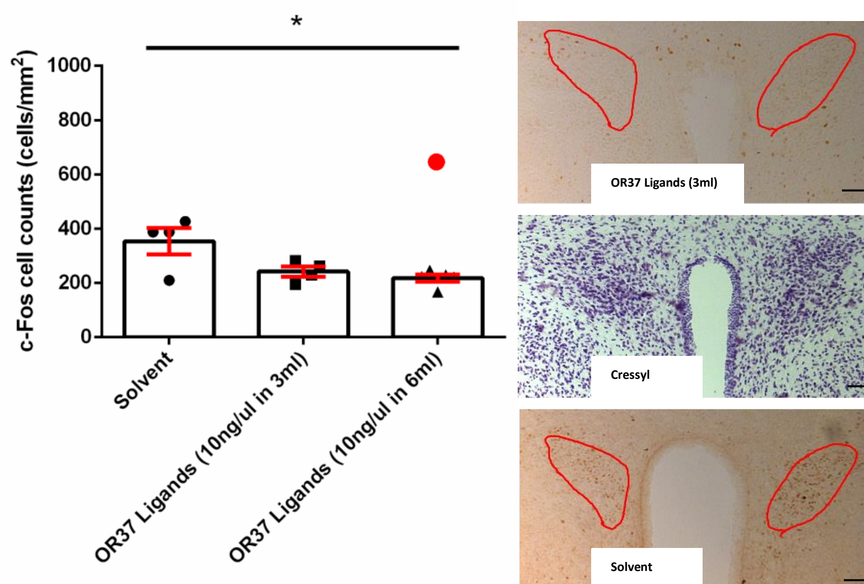

Figure 3.2: OR37 ligands significantly reduced PVN c-Fos activation in an open field test. Data shown as mean ± SEM, *p<0.05.

There were no differences among groups in either time spent in centre of the open field arena ($F_{(2,12)}$=1.929, p=0.180, figure 3.3A), duration of moving on the open field arena ($F_{(2,12)}$=0.16, p=0.985, figure 3.3B), and faecal pellet measurements ($F_{(2,12)}$=0.120, p=0.887, figure 3.3C). There were also no main effects of sex on time spent in centre of the open field arena ($F_{(1,12)}$=0.107, p=0.749),), duration of moving on the open field arena ($F_{(1,12)}$=0.008, p=0.930), and faecal pellet counts ($F_{(1,12)}$=1.036, p=0.329).

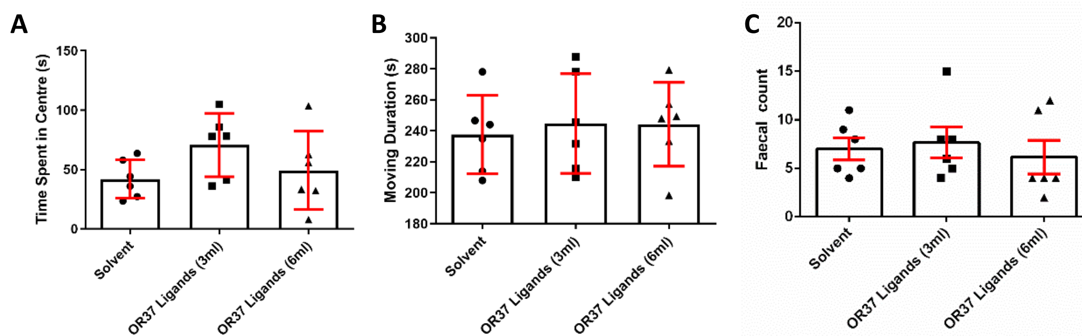

Figure 3.3: OR37 ligands did not affect behaviours on the open field test. (A) time spent in the centre of the open field, (B) total moving time, (C) total faecal counts. Data shown as mean ± SEM.

3.4.3. Experiment 3: Different combinations of OR37 ligands mixture and neuroendocrine responses

Following the previous significant reduction of PVN c-Fos activation, this experiment was performed to measure the effect of the OR37 ligands exposure to the plasma CORT and plasma ACTH measurements. This experiment also tried to investigate the different combinations of the OR37 ligands component (the C16 group and the C15-C17 group). Due to problems with the exposure level during image acquisition, the c-Fos fluorescent could not be distinguished with the noise. Therefore, no c-Fos data could be shown from this experiment.

From the blood sample that was collected at the same time point as the brain collection for c-Fos (90 minutes from the start of the experiment), there were no differences between treatment groups on CORT plasma level ($F_{(4,20)}$=0.627, p=0.649, figure 3.4A) and ACTH plasma level ($\chi^2_{(4)}$=1.351, p=0.853, figure 3.4B). There was also no difference between groups on time spent in the centre of the open field ($F_{(3,16)}$=0.548,

p=0.655, figure 3.4C). There was no main effect of sex found either on CORT plasma level ($F_{(1,20)}$=0.530, p=0.475), ACTH plasma level (U=70,p=0.078), and behaviour on time spent in the centre of open field ($F_{(1,16)}$=4.185, p=0.058).

The time course graph of the plasma CORT (figure 3.4D) showed that the peak concentration with the smallest variability on the captured time frame was at 30 minutes. On the other hand, the plasma ACTH (figure 3.4E) did not show a similar pattern to the plasma CORT and had a higher variability.

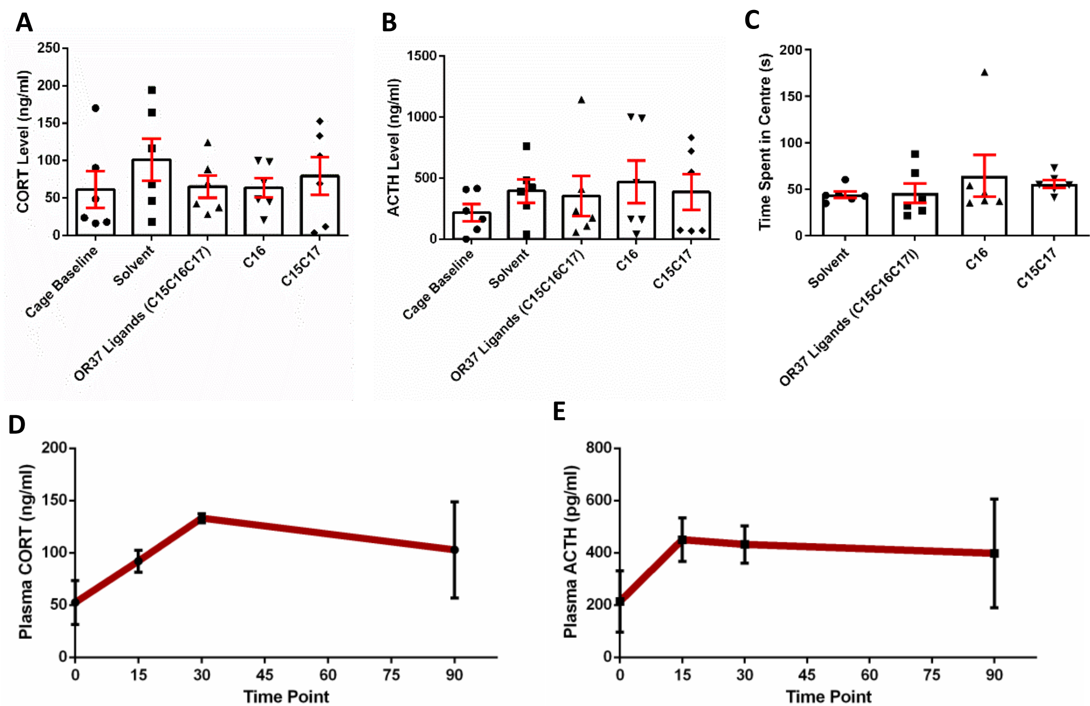

Figure 3.4: no significant differences between groups on CORT and ACTH at 90 minutes or time spent in centre with the no differences in the endocrine responses could be attributed to the incorrect time point for sampling as shown on the time course graph. (A) plasma CORT level at 90 minutes, (B) plasma ACTH level at 90 minutes, (C) time spent in the centre of the open field arena, (D) time course of plasma CORT which shows 30 minutes as ideal collection time, and (E) time course of plasma ACTH. Datasets for the 0 and the 90 minutes of the time course were taken from the male mice of the cage baseline and the solvent groups (CORT from figure A and ACTH from figure B). Data shown as mean ± SEM.

3.4.4. Experiment 4: Reconfirmation of the different OR37 ligand mixtures with increased level of stressors

Following the non-significant CORT level difference between the cage baseline and the stressed animals from experiment 3, this experiment was performed with increased level of stressors by singly housing the animals and increasing the illumination within the open field arena. Although this experiment was performed to confirm the c-Fos measurement for the combination of the different ligand mixtures, due to the issue with the c-Fos staining, no c-Fos data can be shown from this experiment.

Even with an increase of the stressor levels, there were no significant differences between groups on time spent in the centre of the arena ($F_{(3,14)}$=0.655, p=0.590, figure 3.5A), moving duration on the open field arena ($F_{(3,14)}$=0.130, p=0.940, figure 3.5B), or faecal pellet counts ($F_{(3,14)}$=2.704, p=0.089, figure 3.5C). Consistent with the previous experiment, there was no difference between male and female on time spent in the centre of the arena ($F_{(1,14)}$=0.204, p=0.659), moving duration on the open field arena ($F_{(1,14)}$=3.625, p=0.078), or faecal pellet counts ($F_{(1,14)}$=1.055, p=0.323). Comparison of the time spent in centre within 30 minutes observation time between experiment 3 and experiment 4 (figure 3.5D) showed a significant reduction of time spent in centre on experiment 4 ($F_{(1,38)}$=21.35, p<0.0001).

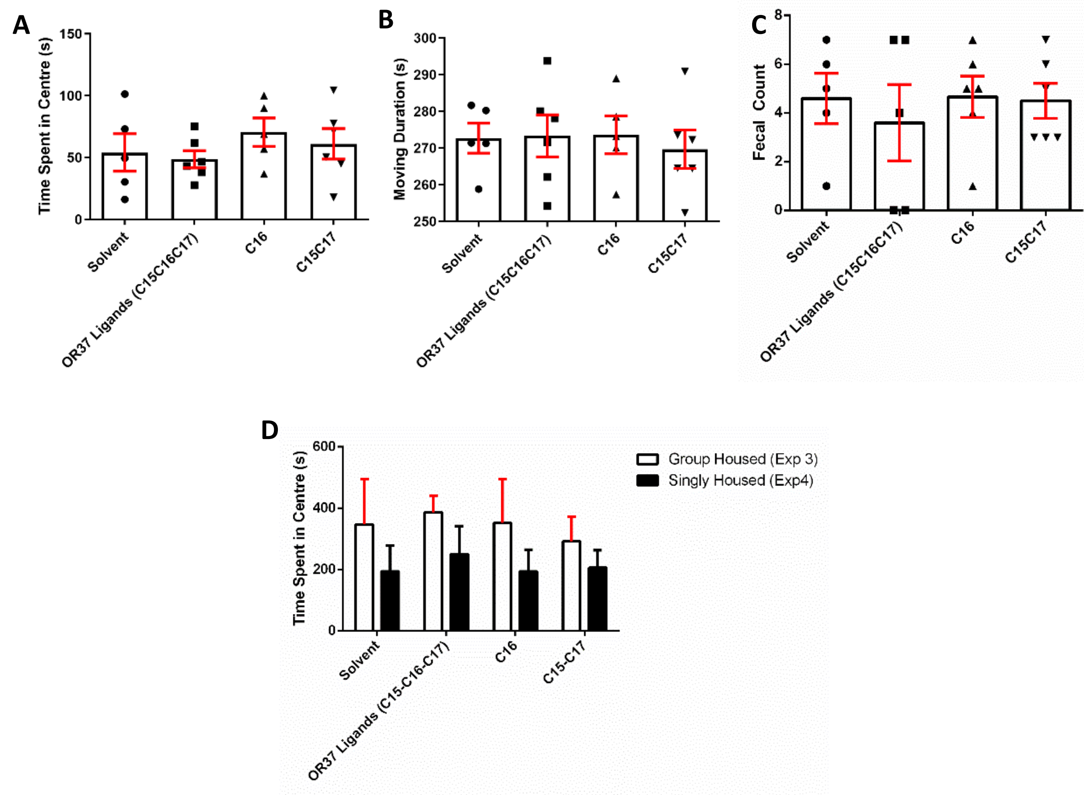

Figure 3.5: No significant differences between OR37 ligands treatment, hexadecanal (C16), or pentadecanal-heptadecanal mixtures (C15+C17) to solvent in measurements using the open field arena but with a different behavioural response level between experiment 3 and 4. (A) time spent in the centre of the open field arena, (B) total moving time, (C) faecal count, (D) comparison between experiment 3 and 4 showed difference between time spent in centre from total 30 minutes of observation time. Data shown as mean ± SEM.

3.4.5. Experiment 5: Reconfirmation of the OR37 ligands' effect on the PVN and the neuroendocrine response with increased level of stressors

Following figure 3.5D in experiment 4 that showed that animals from experiment 4 spent less time in the centre, it could be inferred that having the animals from experiment 4 have increased the baseline of their anxiety level. Although it is possible that the lower time spent might be due to the animals being tested during their light phase, however, the setup from experiment 4 was more anxiety inducing compared to that of experiment 3. Therefore, experiment 5 was performed to confirm the OR37 ligands effect to the reduction of c-Fos activation and to plasma CORT with the stress response meant to be distinguishable from the baseline animals. A meta-analysis was performed after experiment 4 to analyse the time spent in centre from experiment 2, 3

and 4 (figure not shown), however, the data included in (figure 3.6E) suggested a weak effect, therefore this experiment was meant to confirm this effect. Following the meta-analysis, the time spent in the centre measurement within this experiment was focused on the first 5 minutes since the beginning of the experiment.

Similar to experiment 2, there was a significant difference between groups on PVN c-Fos activated cells ($F_{(3,13)}$=4.311, p=0.026, figure 3.6A) without a sex difference ($F_{(1,13)}$=0.589, p=0.457) and no interaction between groups and sex ($F_{(3,13)}$=0.717, p=0.559). Post-hoc analysis with the Gabriel correction showed that the cage baseline group had significantly lower PVN c-Fos activated cells to the OR37 ligands group (p=0.030), but not to the solvent group (p=0.788), or to the C16 hexadecanal group (p=0.169). The mean of the OR37 ligands group was not significantly different from the solvent group (p=0.158) or to the C16 hexadecanal group (p=0.807).

Analysis on the plasma CORT level (figure 3.6B) showed significant difference between groups ($F_{(3,16)}$=45.070, p<0.0001) without a main effect of sex ($F_{(1,16)}$=2.977, p=0.104) and no interaction between groups and sex ($F_{(3,16)}$=0.738, p=0.545). Post-hoc analysis with the Gabriel correction showed that the cage baseline plasma CORT level was significantly lower than the solvent group (p<0.0001), the OR37 Ligands group (p<0.0001), and the C16 hexadecanal group (p<0.0001).

This experiment was consistent with the findings of the previous experiments that the time spent in the centre of the open field (figure 3.6C) was not affected by different treatment groups ($F_{(2,30)}$=1.302, p=0.287) or sex ($F_{(1,30)}$=1.255, p=0.271). There was also no interaction between groups and sex ($F_{(2,30)}$=0.655, p=0.526). A similar trend was found on locomotion activity in the open field arena (figure 3.6D) with no differences between groups ($F_{(2,30)}$=0.022, p=0.978) or sex ($F_{(1,30)}$=1.501, p=0.230) and there was no interaction between group and sex ($F_{(2,30)}$=0.433, p=0.653). The meta-analysis of the time spent in the centre between solvent and OR37 ligands groups (figure 3.6E) did not show a difference between groups (p=0.366).

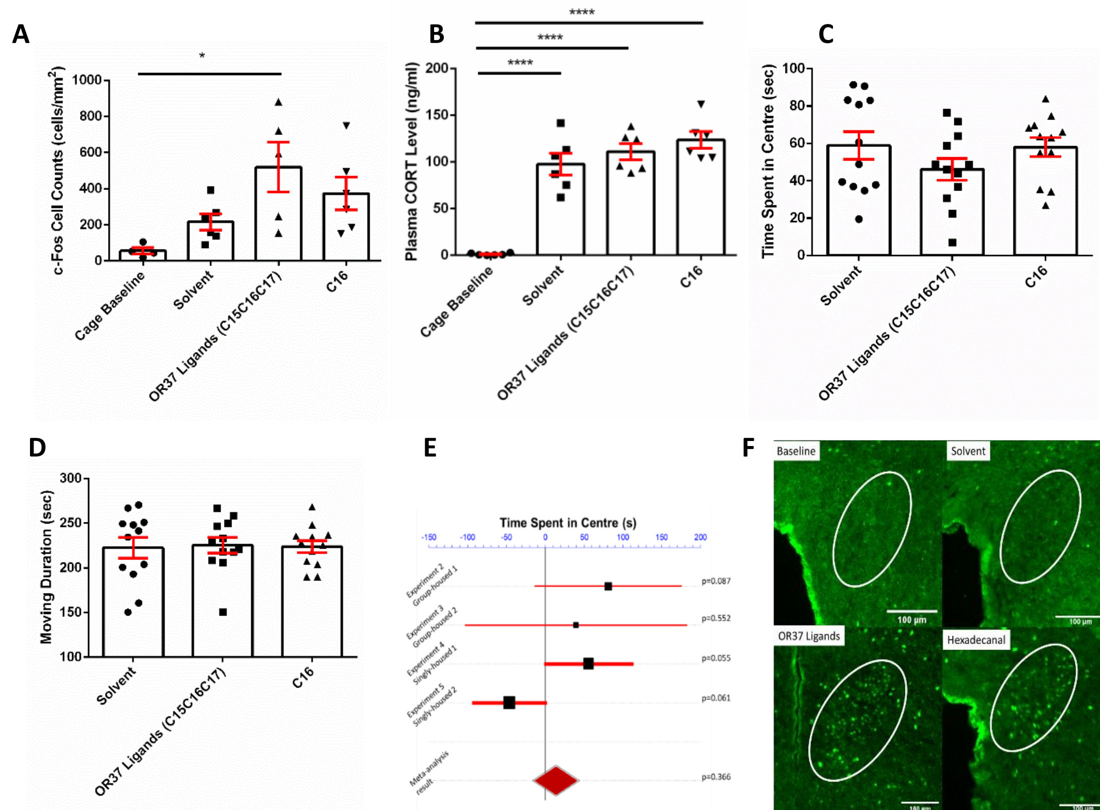

Figure 3.6: Significant differences between solvent and OR37 ligands groups were found in the PVN c-Fos but not in plasma CORT level and behavioural results. (A) PVN c-Fos level, (B) plasma corticosterone level, (C) time spent in the centre, (D) locomotion duration, (E) meta-analysis of all the open fields results across all experiments using time spent in the centre parameter between solvent and OR37 ligands group, and (F) PVN c-Fos images taken randomly from each cage baseline group, solvent group, OR37 ligands group, and hexadecanal group. Data shown as mean ± SEM on bar chart. The black square in the forest plot represents the mean difference between groups (OR37 ligands group subtracted by solvent group) and the whiskers represent the 95% Confidence Interval, the diamond represents the fixed effect within 95% Confidence Interval.

3.4.6. Experiment 6: Different concentrations of the OR37 ligands mixture

This experiment was performed to investigate the different concentration's effects of the OR37 ligands using an elevated zero maze test. The zero maze test was chosen as another form of anxiogenic test. 3 data points were missing (1 from the 2ng/10µl and 2 from the crystal group) due to no c-Fos fluorescent. There was a significant difference of PVN c-Fos activation between groups on the zero-maze test ($F_{(4,22)}$=4.665, p=0.007, figure 3.7A). Post-hoc test with Gabriel correction showed that the 1ng group was significantly higher to the 2ng group (p=0.013), the 10ng group (p=0.035), and the crystal group (p=0.034) but not to the 4.5ng group (p=0.425). However, the 4.5ng was also not significantly higher to the 2ng group (p=0.571) nor to the higher doses.

As for behavioural results, there were no significant differences between groups on the frequency of transition between arms ($F_{(4,35)}$=0.232, p=0.918, figure 3.7C), the latency to the open arm ($F_{(4,35)}$=0.246, p=0.910, figure 3.7D), or the amount of time spent in the open arm ($F_{(4,35)}$=0.346, p=0.845, figure 3.7E).

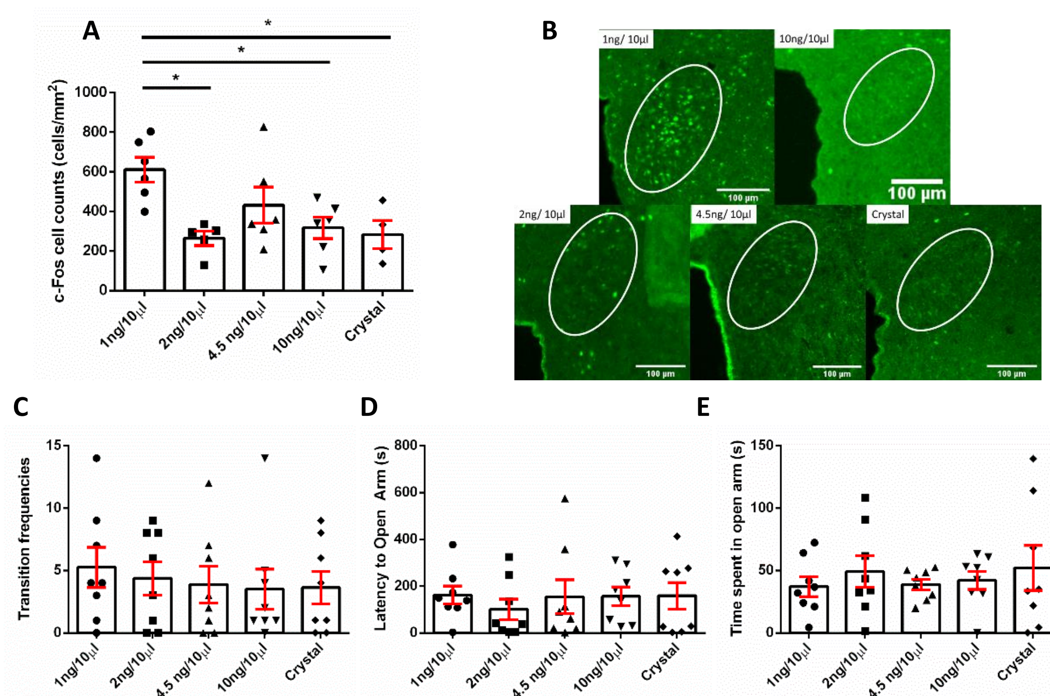

Figure 3.7: Significant reduction of PVN c-Fos expression to various concentrations and different forms of OR37 ligands without any obvious behavioural changes. (A) PVN c-Fos activation level, (B) randomly selected images from each group, (C) total transition between closed arms, (D) time for first entry to open arm, (E) total time spent in the open arm. Data shown as mean ± SEM, *p<0.05.

3.4.7. Experiment 7: OR37 ligands and Colocalization of PVN c-Fos activation with oxytocinergic and vasopressinergic during anxiogenic test

This experiment was conducted to investigate more details on neuronal type that was affected by the OR37 ligands exposure. 6 data points were missing from the oxytocin samples (3 from the solvent group and 3 from the OR37 ligands group) and 5 data points were missing from the vasopressin samples (3 from the solvent group and 2 from the OR37 ligands group) due to issues with the immunohistochemistry staining.

Contrary to the previous experiment, the c-Fos activation (figure 3.8A) did not differ between the solvent and the OR37 ligands groups both on the oxytocin samples ($t_{(8)}$=0.223, p=0.828) and the vasopressin samples ($t_{(9)}$=0.160, p=0.876). There was also no change to colocalization (figure3.8B) between the c-Fos – OXY ($t_{(8)}$=0.438, p=0.672) and the c-Fos AVP ($t_{(9)}$=0.306, p=0.766). Similar to the previous experiment, there were no differences between the solvent group and the OR37 ligands group on the latency to the open arm ($t_{(14)}$=1.018, p=0.326, figure 3.8C) and the time spent in the open arm ($t_{(14)}$=1.721, p=0.107, figure 3.8D).

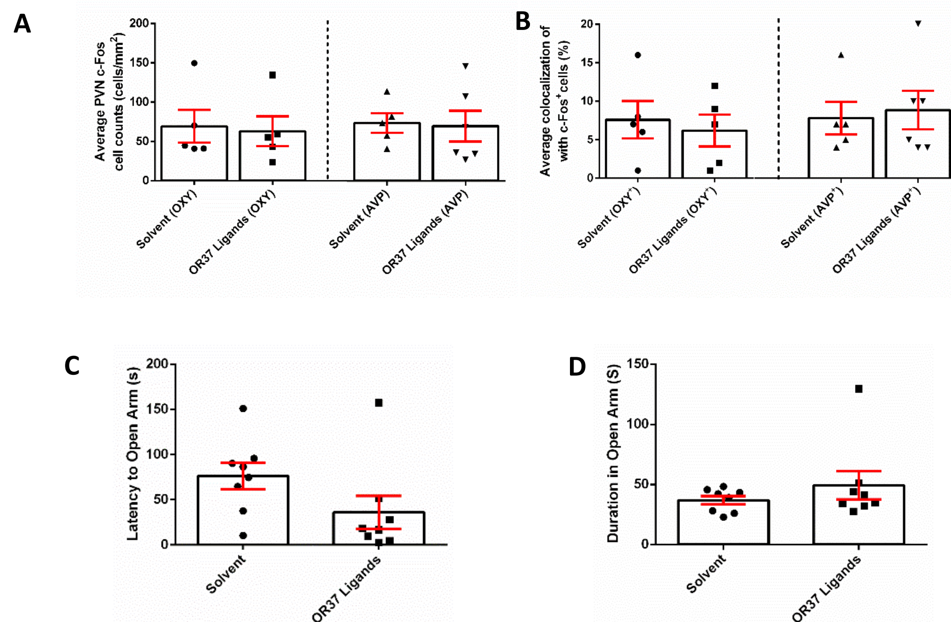

Figure 3.8: c-Fos activation, colocalization between c-Fos - OXY and c-Fos – AVP, and anxiety-like behavioural measures in zero maze did not show significant difference between the solvent group and the OR37 ligands group. (A) PVN c-Fos counts, (B) percentage of oxytocinergic/vasopressinergic cells that colocalized with the c-Fos cells, (C) representative images from solvent and OR37 ligands group for both OXY and AVP staining(D) time spent in the open arm, and (E) latency for first transition to open arm, Data shown as mean ± SEM

3.5. Discussion

This chapter provided insight on how the OR37 ligands mixture affects mice in anxiety-like behavioural tests. The focuses of the OR37 ligands effects were divided into 3 separate components of the stress responses: the PVN c-Fos activation, the endocrine and neuroendocrine responses assessed by the plasma CORT and ACTH, and the behavioural output from the anxiogenic environments.

OR37 ligands and Mice PVN c-Fos responses in anxiogenic environments

To begin with, it was necessary to show that the PVN c-Fos activation reduction on novel environment stress paradigms was replicable in an anxiety-like stressor paradigm. Results of the PVN c-Fos activation from experiment 2 showed that the reduction of c-Fos expression in the PVN was indeed replicable in novel anxiety-like behavioural tests. There was also no evidence of sex difference which is consistent with the Klein et al. (2015) study. The results also suggested that the amount of OR37 ligands mixture volume did not produce a bigger reduction of PVN c-Fos activation, therefore, the lower volume was chosen to prevent unnecessary contamination, and also was the equivalent volume as what Klein et al. (2015) did. There are still issues about unknown volatility and airborne concentration of OR37 ligands in air, however, this result validated exposure methodology for use with the open field.

An attempt was made to replicate these results within the open field tests, however, the experiment within the group housed mice with more parameters (experiment 3), and the experiment within singly housed mice (experiment 4) were met with technical problems. Due to over exposure in experiment 3, which blurred the separation between artefacts and c-Fos signals, and due to no/very low c-Fos fluorescent on experiment 4, which meant that no c-Fos results could be quantified, there were no c-Fos results for both experiments. One final experiment within the open field was conducted to confirm the PVN c-Fos reduction effect (experiment 5), however, there was a concern regarding the blinding of the solutions due to the c-Fos results showing the opposite direction to the previous experiments. The suspicion regarding the blinding was that the labels for the OR37 ligands and the solvent groups were mixed up. Unfortunately, there is no way to retrace back the solution's blinding, which puts the results of the solvent and the OR37 ligands group to be unreliable. Although the PVN c-Fos

reduction in the open field from experiment 2 could not be replicated in other experiments due to technical issues, it still shows that the OR37 ligands exposure reduced PVN c-Fos activation in an open field test.

Another test to measure anxiety-like behaviour is the elevated zero maze. Experiment 6 was done using the zero maze arena to see whether the PVN c-Fos activation reduction of OR37 ligands exposure was specific in the open field arena or can be generalised in another anxiety-like context. Although there was no solvent control in this experiment, the 1ng/10µl concentration was meant to have a similar effect as the solvent control group. The comparison between the 1ng/10µl and the 10ng/10µl groups showed that the PVN c-Fos reduction can be replicated within the zero maze test.

Another question regarding the properties of the OR37 ligands is whether the 10ng/10µl concentration is already the maximum possible effect on PVN c-Fos activation reduction and the PVN c-Fos expression response with concentration in between 1ng/10µl and 10ng/10µl. To answer these questions, one group on this experiment included a crystalline form of the OR37 ligands and heated them on a heating plate with the expectation that this would be the most saturated concentration of OR37 ligands in the arena. Other groups included were 2ng/10µl and 4.5ng/10µl with the expectation if the OR37 receptors behaving similar to a dose-response curve (however, due to the method of exposure, concentration-response curve terminology is more appropriate), the 2ng/10µl and 4.5ng/10µl (respectively 0.3 and 0.6 points on log curves) will capture the data point that resemble the log curve. Based on the Klein et al. (2015) study, the 1ng/10µl and the solvent control had the same level of effects. In order to ensure that consistency in making the curve, 1ng was chosen and treated as a control. There is also another approach to minimize the factor of sex in this experiment to control the variability despite there were not any significant differences between sex on previous experiments.

As for the results from experiment 6, the PVN c-Fos activation reduction was already observed from the 2ng/10µl concentration. Despite the 4.5ng/10µl not significantly different to the 1ng/10µl group, the 4.5 ng/10µl also not significantly different with the 2ng/10µl group which might suggest the one particular animal with the very high PVN c-Fos activation was driving the means of the population to be higher. On the other hand, the crystal group had a similar PVN c-Fos activation reduction level as the

10 ng/10µl group. This result shows that: the method of heating up the OR37 ligands crystal was proven to be successful as a method of delivery without using a solvent and the 10ng/10µl concentration could be concluded to be the highest PVN c-Fos activation reduction to be expected from the OR37 ligands.

There were quite few studies done previously on mice using anxiety-like behavioural tests looking at PVN c-Fos activation. Despite that the anxiety-like behavioural tests such as open field test and elevated maze were developed to test anxiolytic effect of benzodiazepine family drugs, study done by Sartori et al. (2012) did not show significant changes in PVN c-Fos activation which might be due to methodological concerns with the lack of reduction of anxiety-like behaviour on control group. Interestingly, study by Linden et al. (2004) shows that the injection of anxiolytic drugs (LY354740, metabotropic glutamate agonist) increased the PVN c-Fos activity instead. There were few studies that includes complex stress such as restraint stress (Choi et al., 2013) and chronic variable stress (Borrow et al., 2019) in anxiety-like paradigm and using c-Fos, however, will be omitted to maintain focus on direct measurement of anxiety-like behaviours and PVN c-Fos activation. Unfortunately, there were limited studies investigating benzodiazepine drugs on C57BL/6 mice PVN c-Fos activation during anxiety-like tests. However, similar studies done on rats were more available such as Salminen, Lahtinen, and Ahtee (1996) that shows acute benzodiazepine treatment increased PVN c-Fos activation instead which is interestingly in line with Singewald, Salchner, and Sharp (2003) study that used different anxiogenic drugs that increased PVN c-Fos activation. This suggests that anxiety-like behaviour is not as simple as it is and the relationship between anxiety-like behaviours, drug treatments, and PVN c-Fos activation are complex. PVN of the hypothalamus itself contains many different types of neurons and sends signals to other parts of the brain for diverse physiological function. It is important to characterise a direct comparison on anxiety-like behaviour context, OR37 ligands treatment, and PVN c-Fos activation neuron type.

The PVN neuron type that was affected by the OR37 ligands exposure study unfortunately could not be concluded from the colocalization study (experiment 7). Although experiment 7 was performed similarly to experiment 6, the change in the duration of the zero maze test did reduce the stress level observed in the mice on the zero maze. This reduction of the stress level of the zero maze was shown by the similar level of the c-Fos expression between the solvent and the OR37 ligands groups, which

is consistent between the set of sections that were used for oxytocin staining and vasopressin staining. Following the no change in c-Fos expression between groups, there were also no differences observed to the colocalization percentage on both sections for oxytocin and vasopressin staining.

Lastly, another question regarding the OR37 ligands was whether the combination of the three ligands components (pentadecanal, hexadecanal, and heptadecanal) was necessary to produce the PVN c-Fos activation reduction effect. Based on Bautze et al. (2012) study, hexadecanal (C16) was found, despite not having the highest response on OR37A and OR37C glomeruli, also activated these two other OR37 receptor types. The response was different when compared to pentadecanal (C15) or heptadecanal (C17) that only have the highest response on one OR37 glomeruli and are not as effective in activating all three OR37 glomeruli. Based on that, the groups of C16 and C15-C17 were added to the experiment 3 and 4. However, due to the unreliable PVN c-Fos results, the question could not be answered. In addition, the C16 group was added to the final open field experiment (experiment 5). Although the result showed a response higher than the cage baseline, it was difficult to interpret due to problems with the blinding on the solvent control and the OR37 ligands mixture groups.

OR37 ligands and mice Plasma CORT and ACTH responses in anxiogenic environments

ACTH and CORT are part of the stress response pathway mediated by the PVN activity (Kinlein et al., 2019). Due to the nature of the expression, ACTH and CORT were expected not to be on the same time point for collection with the PVN c-Fos expression. To begin with, a time course study to determine the concentration of ACTH and CORT on blood plasma was done on experiment 3. From the results on figure 2.6, the CORT response graph shows that the optimum time point from the experiment is the t30. It suggests that at the end of the 30 minutes open field arena testing that all animals perceived a similar level of stress. It might be possible that the t30 is not the actual maximum peak of CORT stress response. However, after being taken out from the testing arena and being checked at t90, it appears that the CORT response became highly variable. This suggests that the CORT response is less reliable at T90, thus, not suitable to be done at the same animal as the c-Fos study. The ACTH response was fairly similar with the CORT in terms of time course. The obvious difference was at t30 where it is still fairly similar to the previous time point (t15).

This could suggest that there might be a peak between them or reflect the more rapid time course of ACTH response. One way to address this issue was by having a data collection on higher time axis resolution. There was also another factor to be considered regarding ACTH stability that it is less stable than CORT (Evans et al., 2001). Even though there was aprotinin added onto the samples, it could be possible that the ACTH was degraded on the processing during the blood collection protocol. This suggests that unless there is a more reliable method of processing the sample for ACTH, it is better to use CORT results for the meantime.

A study done by Malisch et al. (2007) found that mice (*Mus domesticus* strain Hsd:ICR) that were treated with restraint stress for 40 minutes have significantly higher corticosterone levels when compared to control for both male and female. A study by Coleman et al. (1998) also mentioned that corticosterone increased gradually over the first 30 minutes before reaching its peak at approximately 40 minutes for the same breed of mice in a forced running test. Even though the N number for the corticosterone assay was small, the corticosterone result from this experiment is consistent with the findings from both studies. A review by Bornstein et al. (2008) mentioned a dissociation between ACTH and glucocorticoids. This suggests that the time point for ACTH and corticosterone might be different. Subsequently, a study done by Veenema et al. (2003) found that ACTH levels after 15 minutes were not significantly different to the basal group while corticosterone has become significantly different to the basal group. However, the ACTH and corticosterone were different across mice of the same species with different aggressive phenotypes. This suggests that further study regarding the endocrine profile for the mice population being used for this experiment needs to be analysed.

The CORT and ACTH results between groups on experiment 3 did not show significant differences. This might be a source of concern with the methods considering that the CORT level was higher than expected in baseline controls. Given that the animals were in their light phase at a time when their CORT and ACTH levels were supposed to be low, these results might an indication of few scenarios: another factor that influence the CORT and ACTH variations such as the ultradian variability, the cage baseline animals were stressed out, the open field were not stressful enough hence did not resulting on higher stress responses, or the concern for the methodological for the measurement itself. The mice were transported to the

experiment room a day prior and were individually housed to minimise the stress from sources other than the experimental treatments. The stress level in the open field can be enhanced by increasing the illumination on the arena.

It would have been interesting to determine if there was a correlation between the endocrine measurements and the PVN c-Fos, but the technical difficulties made c-Fos measurement to be unreliable. Similarly, in experiment 5, although the baseline animals' CORT levels were low, the technical issues made the c-Fos results to be unreliable to be made into a correlation with the CORT level. However, despite the differences between the solvent and the OR37 ligands groups on the PVN c-Fos, there was no difference between the solvent and the OR37 ligand groups on the plasma CORT levels. Although this might suggest a dissociation between the PVN c-Fos and the plasma CORT, further investigation is necessary to draw conclusive evidence.

OR37 ligands and mice behavioural responses in anxiogenic environments

Regarding the mice capabilities of discriminating the OR37 ligands and the solvent, a habituation-dishabituation test has been done by a member of the lab and presented on Davies et al. (2018). From the habituation-dishabituation test, the mice showed no ability to detect or discriminate OR37 ligands in a habituation-dishabituation test, which suggests that they are either not perceived or a lack salience in this context. The results from the conditioned place preference experiment were straightforward: no innate preference or aversion to the solvent, and without side bias on the habituation phase. This result has been controlled to the unbiased design, hence, there should not be any biases towards the chamber positions and the placement of the tested solvent. Since there were no changes between the habituation and the test phase, it was sufficient to say that the solvent itself did not drive preference or aversion. The diethyl phthalate mentioned on experiment 1 was due to personal communication (2017) with Jorg Strotmann, of the Klein et al. (2015) study. The diethyl phthalate was considered to be another solvent for the OR37 ligands due to solubility issues with OR37 ligands mixture that could not be dissolved in higher concentration than 10ng / 10µl in propanediol.

Despite the significant drop of PVN c-Fos activation, there was no evidence of changes in anxiety-like behaviours on experiment 1. According to Seibenhener and Wooten

(2015), common measurement of anxiety-like behaviours of open field including locomotion activity, time spent in zones that signify thigmotaxis behaviours, and faecal boli left on the arena after observation. The study was done by using a knockout p62 gene which heightened anxiety-like behaviours on mice and based on the study, the mice that exhibited more anxiety-like behaviours spent less time on the centre and more faecal boli left on the arena after observation. It was expected for reduction of anxiety-like behaviours following the significant drops of PVN c-Fos activation. However, the mice on experiment 2 did not show any changes on anxiety-like behaviours following OR37 ligands exposure.

There were plenty of shortcomings of using open field tests as a measurement of anxiety-like behaviours (Seibenhener & Wooten, 2015). Despite being quite commonly used as an anxiety-like behaviours test, it is difficult to compare between studies due to the difference in protocols, illumination of the arena, including drug treatment that affect different aspects, not only anxiety-like parameters. One of good example was that the benzodiazepines, one popular anxiolytic drug and the golden standard that was used for the open field test, was also known to affect appetite, which prove to be a problem when being tested on a novelty-suppressed feeding test (Cooper, 1980). Further analysis on measured behavioural parameters, it is also important to control for confounding factors such as food consumption regarding the faecal boli which was not performed on experiment 2. Even though defecation is a measure of negative affective state in mouse measure of anxiety, due to uncontrolled variable of food consumption it is not clear whether the amount of faecal boli found was due to lower anxiety level or due to not enough faecal matter to be expelled (Ramos, 2008).

The behavioural aspect of experiment 3 did not show any difference between groups, which was expected, but because the PVN c-Fos result was unreliable, the experiment was inconclusive for the most part and needs to be controlled further for the confounding factors. The attempts to control the confounding factors were applied on experiment 4 by singly housing the animals and moving the animals to the experimental room 3-5 days prior the testing day. Other light sources were also applied to increase the illumination in the centre of the open fields in order to increase the stressor. The results on the behaviours were expected to not show any differences. However, there was also a problem with some of the PVN c-Fos results, making the results unreliable. However, the PVN c-Fos of the cage baseline was quite low, which

might suggest that the attempt to control the stressor of the cage baseline animals was successful. In addition, despite the time spent in the centre within 5 minutes observation not differing between experiment 3 and 4 (figure 3.4C and figure 3.5A), when the observation time was extended to 30 minutes, the time spent in the centre became significantly different (figure 3.5D), which suggests that the setup from experiment 4 was more anxiogenic.

Following the behavioural results in the open field, meta-analysis was performed using the time spent in the centre between the solvent group and the OR37 ligands group. The meta-analysis was first performed at the end of the experiment 4 and the results suggested that there was an increased effect on the time spent in the centre. However, the questionable methodological error on experiment 5 has the results of the solvent and the OR37 ligands in totally opposite directions to the previous results and made the behaviour of time spent in the centre to be inconclusive. Interestingly, the behaviour of the time spent in centre follows the PVN c-Fos trendline. This might suggest a connection between PVN c-Fos activation and the anxiety-like behaviour, however, with a very small effect.

Regarding the behaviours on zero maze arena, the results were as expected to be no difference between the OR37 ligands group and the solvent/the 1ng/1µl group from both the experiment 6 and experiment 7. These results also summarised that there were no anxiety-like behavioural differences following the reduction in the PVN c-Fos activation within anxiogenic behavioural tests using an open field test and a zero maze test.

Chapter summary

In summary, this chapter has provided new evidence that the OR37 ligands reduction effect to the PVN c-Fos activation can be repeated within anxiety-like behavioural context using an open field arena and an elevated zero maze arena. In addition, there was also evidence that shows the reduction effect started from a concentration of 2 ng/ 10µl and did not show a higher reduction effect at 10 ng/ 10µl. There was no sufficient evidence in this chapter regarding the neuronal type within the PVN being influenced by the OR37 ligands, whether it was the oxytocinergic or the vasopressinergic neurons. There was also no sufficient evidence regarding the different OR37 ligands mixture combination having an effect on the PVN c-Fos activation. This chapter also provided evidence of the dissociation effect between the changes within the PVN due to the OR37 ligands and the plasma CORT level. There was no sufficient evidence regarding the OR37 ligands effect to the anxiety-like behavioural output and there was evidence that this is not affected by mice preference or avoidance to the solvent.

Chapter 4: OR37 Ligands and Social Isolation

4.1. Chapter Aim and Objectives

The aim of this chapter is to investigate the hypothesis of whether OR37 ligands can be used chronically as olfactory enrichment in animal welfare refinement within the case of chronic social isolation. To achieve this aim, this chapter will compare the physiological and behavioural changes between chronic socially isolated mice and pair-housed mice, and whether these changes can be ameliorated by the OR37 ligands. In addition, this chapter will also explore alternative methods of exposure to the OR37 ligands for chronic exposure settings. This chapter will also validate an experimental setup to socially isolate the mice with a configuration that prevents the treatment groups from cross contaminating each other. Specific experimental objectives within this chapter include:

- To validate the use of a scantainer as an enclosure for social isolation compared to individually housed mice within a communal scantainer.
- To confirm the social isolation induced physiological and behavioural changes related to anxiety and aggression.
- To investigate the effectiveness of chronic OR37 ligands exposure in counteracting the physiological and behavioural changes due to social isolation.
- To validate various methods of exposure of OR37 ligands.

4.2. Introduction

Previous chapter's results showed no evidence for the OR37 ligands effect on anxiety-like behaviour in acute context but is consistent for the effect on the PVN activation. It is possible that there is more of a chronic effect of exposure, or maybe that there is an effect on different output, such as aggressive behaviour which is a form of social interaction. Klein et al. (2015) found the OR37 ligands had similar effects to the reduction of PVN c-Fos activation as being together in the novel environment with a conspecific or their secretions. This result raised a question whether OR37 ligands might actually affect social behaviours instead of anxiety-like behaviour. Aggression is one of the common social behaviours and one common problem in husbandry procedures between male mice in group housing. Aggression between group-housed male mice has been found to be influenced by several different factors such as strain, environmental enrichment, group and cage size, weaning age, cage cleaning regime, and scent marks (Gaskill & Pritchett-Corning, 2015; Lockworth et al., 2015; Van Loo et al., 2000; Van Loo et al., 2003; Van Loo et al., 2001). The issue with an aggression study itself as implied in a review by Van Loo, Van Zutphen, and Baumans (2003), is the lack of control and predictability of aggression.

Aggression in mice in general could be affected by different social contexts. Previous studies listed in Table 4.1 show how socially isolating mice can induce aggression and other detrimental effects. Many studies have investigated the social isolation paradigm in mice; however, it is often unclear what social isolation means to mice. According to Cacioppo et al. (2015), social isolation is objectively defined as the absence of social environment, which from the animals' perspective, is the contact with a conspecific. It is interesting that the social isolation seems to be viewed from a very human centric perspective even for a study on rodents which raised the question regarding the ethological validity of the social isolation treatments. One key point of social isolation itself is the term "loneliness" which is defined as perceived social isolation and this topic itself is very complicated in animal study henceforth will not be discussed further. Humans, as primates, have a more developed visual pathway compared to mice (Kaas & Balaram, 2014). Different dominant senses will result in different communication strategies which in turn, raises concerns in relation to the social isolation paradigm that many of previous studies have conducted. Most studies equated individually housing the animals within a single cage with social isolation. A study by Thaker et al. (2006)

went to the length of putting a divider between cages to limit visual communication, but it is still unclear whether the auditory and the olfactory cues could influence the results. However, previous studies did find effects associated with negative stress responses despite the paradigm not being true social isolation. In this chapter, we will try to validate a social isolation paradigm that minimised auditory, olfactory, visual, and tactile communication within an individual scantainer and compare it with a group of individually housed mice within a communal scantainer.

Table 4.1: Previous social isolation studies and their effects on mice

Strain	Sex	Age at start of experiment	*n*	Social Isolation Method and cage type	Social Isolation Duration	Parameter/Test	Effect of social isolation	Year	Reference
Athymic nude	Female	10 - 12 weeks	10 per group	Individually Housed with a wall between cages and distance of 24 inches, unclear cage type	28 days	In vivo ovarian cancer growth	Significant increase of tumour weight and number of nodules	2006	(Thaker et al.)
C57BL/6JOlaHsd	Male	4 weeks	15 per group	Individually Housed, unclear cage type	7 weeks	Dark-light exploration	Higher latency to enter light area	2004	(Võikar et al.)
DBA/2OlaHsd	Male	4 weeks	15 per group	Individually Housed, unclear cage type	7 weeks	Open field	Less time in centre		
						Novelty suppressed feeding	Higher latency to eat		
BALB/c	Male	3 weeks	10 per group	Individually Housed, standard cage (M1), unclear lid type	2 weeks, 4 weeks, 8 weeks	Resident-intruder test	Latency to attack significantly lower than respective control group	2017	(An et al.)
NIH swiss mice (ovariectomised)	Female	5-6 weeks	10 per group	Individually Housed with and without enrichment vs pair housing, acrylic static isolation cage	2 weeks	Light-dark exploration	Significant increase in frequency of transitions in pair-housed	2020	(Briones-Aranda et al)
						Elevated plus maze	Significant increase in time spent in open arm in pair-housed		
C57Bl/6N	Male	8 weeks	16 per group	Individually housed with or without enrichments, type II cage for individually housed and type III cage for group housed, unclear lid type	7 weeks	Open field	Significantly lower time in centre in singly housed with or without enrichment compared to their respective group housed control	2005	(Chourbaji et al.)
						Dark-light box	Significantly higher latency to enter light area in singly housed with or without enrichment compared to their respective group housed control		
C57BL/6	Female	6-8 weeks	10 per group	Individually housed with or without chronic	21 days	Tail suspension test	Significantly higher immobility time than	2019	(Gu, Chu, & Han)

				unpredictable stress, unclear cage type			control group without chronic unpredictable stress		
						Forced swimming test	Significantly higher immobility time than control group without chronic unpredictable stress		
						Sucrose consumption	Significantly lower sucrose preference index to control group without chronic unpredictable stress		
C57BL/6N	Male	4 weeks	8 per group	Individually housed, unclear cage type	4 weeks	Resident-intruder test	Significant reduction in latency to attack in individually housed mice compared to group housed control	2019	(Y. Liu et al.)
CD-1	Male	24 days	45 mice in total, unclear how many per group	Individually housed, black opaque polypropylene cage, unclear lid type	10 weeks	Resident-intruder test	Significant reduction in latency to attack and significant increase in attack duration in individually housed mice compared to group housed control	2019	(Z.-W. Liu et al.)
C57BL/6J	Female	8 weeks	12 per group	Individually housed, clear plastic cage, stainless steel food hopper	16 weeks	Body weight	Isolated mice were heavier than group housed control	2010	(Martin & Brown)
						Forced swimming test	Significantly higher immobility time than control group		
NMRI	Male	4-5 weeks	12 per group	Individually housed, 30x20x10cm for group housed, 24x10x8cm for individually housed, unclear lid type	4 weeks	Plasma CORT	Significant increase in plasma CORT (control: 33.68 ng/ml, Isolated: 140.08 ng/ml)	2000	(Francès et al.)
C57BL/6N	Male	8 weeks	Control 11, Isolated 19	Individually housed, unclear cage type	6 weeks	PVN CRH	Singly housed animals had significant increase of CRH activation after acute restraint stress	2020	(Heck et al.)

From the studies within table 4.1, the terms of individual housing and social isolation are often used interchangeably, which will be continued within this chapter. Although the definition of both terms is different, the studies in table 4.1 were individually housing their mice and there were behavioural and physiological changes observed and they consistently showed that it was potentially detrimental to the mice. As previously mentioned, social isolation is defined as the absence of a social environment while individual housing is defined as putting an individual mouse within a cage. Different studies have different durations of social isolation and different outcomes, but in summary from table 4.1, the shortest isolation was 2 weeks before changes in anxiety-like behaviours were observed. Despite the differences in isolation period, social isolation itself has been known to induce aggression. Social isolation may also influence different types of aggression. According to a review by Takahashi and Miczek (2014), aggression type can be subdivided by the context in which it occurs, such as territorial aggression, dominance-related aggression, and maternal aggression. Lin et al. (2011) also showed that aggression towards an intruder, which is a type of territorial aggression, can be induced by an optogenetic stimulation on the ventromedial region of the hypothalamus. Considering that odour cues also play an important role in inducing aggression via atypical MUPs in male mice, it is also possible that odour cues such as the OR37 ligands could play a role in social context and modulating aggressive behaviours. This chapter aims to confirm the effect of social isolation on aggressive behaviours in different context, the neural circuit involved in the aggressive behaviours (the ventromedial hypothalamus), and other stress responses such as stress induced hyperthermia, PVN c-Fos activation, and anxiety-like behaviours.

Social isolation, or individually housing male mice, is unavoidable in certain animal husbandry or experimental procedures. It has been a question for a while regarding the cost versus benefit of group housing or individually housing male mice. A review by Kappel, Hawkins, and Mendl (2017) highlighted some key points regarding the pros and cons related to individually housing or socially isolating the male mice. Individually housing the mice for a long period in consideration to avoid intermale aggression could possibly result in detrimental effects caused by social isolation. It is necessary to find a way to counteract the detrimental effects caused by the social isolation in male mice, which is where the potential usage of the OR37 ligands comes into the picture. However, it should be noted that the concept of mice social isolation

is viewed from a human perspective, in which less attention is given to isolating the mice from chemosignals communication. It has therefore become an issue where simply individually housing the mice does not necessarily prevent them from having social communications. On the other hand, one question that this chapter is trying to ask is whether the OR37 ligands, which was suggested to work in similar effect as a conspecific presence during novel environment stress, can be applied in the husbandry procedures related to social interaction. To tie things together, this chapter will also try to investigate whether the presence of OR37 ligands during social isolation can be used to mitigate the negative effects of social isolation. It is important to note that it is within consideration whether the effects might be different when it comes to female mice. Previous studies showed that the response to social isolation is sexually dimorphic (Guo et al., 2004; Rodgers & Cole, 1993; Senst et al., 2016). In addition to the differences between sex, the effects of social isolation also different between different age of the mice within the same sex. However, this chapter is focusing on the situation where male mice have to be individually housed, resulting in social isolation, and refining the situation by using the OR37 ligands.

New methodology was required to achieve chronic exposure using the OR37 ligands. The current setup from the previous chapter was using an enclosure which worked but proved to be a limitation since the exposure chamber is not practical. Therefore, an experiment was conducted to validate a more practical method of exposure using a tea ball infuser. The use of tea ball infuser is also relevant to potential routes of administering OR37 ligand exposure and potentially other volatile chemosensory compounds in animal welfare applications However, it is to be noted that the volatility and airborne concentration of the OR37 ligands were unknown. It was known from the previous chapter experiment that heated up crystals of the OR37 ligands were effective in reducing the PVN c-Fos activation. This piece of data suggested that the OR37 ligands can be delivered without using a solvent. As described in Bautze et al. (2014), hexadecanal is present on the surface of the mouse faeces. A review by González-Mas et al. (2019) did mention that hexadecanal was a constituent of lemon essential oil. This finding suggested that hexadecanal is produced within biological processes and quite possibly that the other components of OR37 ligands were naturally available. It just makes sense that the OR37 ligands' volatility and airborne concentration must have been able to be delivered to conspecifics with an ethologically

relevant context. To mimic the ethologically relevant context of the delivery method, one example was by heating the crystals to 37°C, which mimics the animal body temperature and mimics the condition of the ligands when they were deposited on the faeces' surface. Similarly, the Klein et al. (2015) study demonstrated that conspecific secretions and had PVN c-Fos activation reduction, if the OR37 ligands meant to be part of natural secretions of the mice, the method of delivery should not be overly complicated in order to relay the communication signal in an ethologically relevant manner. It seems likely that the tea ball exposure should be effective, but another aim of this study is to confirm whether delivering the OR37 ligands using a tea ball will produce similar reduction effect to the PVN c-Fos activation within a novel environment context to the exposure method using airflow as shown by Klein et al. (2015).

4.3. Methods

4.3.1. Animals and Housing

All mice used in this chapter were the males of the C57BL/6 strain (Harlan). The mice arrived at the animal housing unit at 7 weeks old as a group housed with a size of 3, housed in a conventional mouse cage with an open top lid within the Ventilated Cabinets ScanTainer Classic (Scanbur), and acclimatised for 1 week before any experiments. All animals were handled using their cage's tube for transferring between cages or from cage to testing arena. All animals were kept at a 12:12h conventional lighting cycle (lights on at 8 AM) with the scantainer temperature controlled (22±1 °C). All housings were enriched with standard enrichment as described in Chapter 2, section 2.1. Animals and Housing unless described otherwise. The animals had free access to food and water except during behavioural testing or 24 hour food restriction prior to specific behavioural testing. Further details regarding the animals used in this chapter are specified in table 4.2.

Table 4.2 : List of experiments and animals used in chapter 4

Experiments	Animals used	
	Pair housed	Singly housed
Experiment 1 : Validation of social isolation in scantainer	0	22
Experiment 2 : Chronic social isolation and OR37 ligands	30	29
Experiment 3 : Validation of OR37 ligands exposure methods	0	24*

*the pair housed animals from the previous experiment.

4.3.2. Experiment 1: Pilot study on validating social isolation in scantainers.

This experiment was done to confirm that the acute social isolation procedure in a Ventilated Cabinets ScanTainer Classic (Scanbur) did not incur a significantly different stress response compared to individual housing. This experiment was done because the individual housing was used as means of socially isolating the mice in most literatures, despite that individual housing did not fully isolate the mice from chemo signal communications. Therefore, it was necessary to validate whether individually housing a mouse within a communal scantainer (with other individually house mice) will be different to socially isolating an individual mouse within an individual scantainer.

Animals were individually housed in the communal scantainer 1 using the same cage and enrichment as described in Chapter 2 section 2.1 Animals and Housing. A day before the tissue collection day, one individually housed mouse was transferred to individual scantainer 2 (opposite to scantainer 1). A visual block was placed on the scantainer 2's door to prevent visual stimulus. On the experiment day, the animal from scantainer 2 was killed as a socially isolated animal, followed by an animal from the communal scantainer 1 as an individually housed control animal. The brain and the blood were collected for free floating 40µm c-Fos immunohistochemistry and blood plasma corticosterone as a measure of stress response following the procedures described in Chapter 2. The order of culling between scantainers and the placement of the animals taken out were counterbalanced. The experiments were done between 10.00-11.00 AM over 11 days. On the final day, due to the nature of the experiment,

instead of having 1 individually housed and 1 scantainer-isolated, 2 scantainer-isolated was obtained instead due to no other mice left in the scantainer 1.

4.3.3. Experiment 2: Social isolation and chronic exposure to OR37 ligands

This study was done to confirm the anxiety and aggression induced by the chronic social isolation paradigm and to investigate whether the chronic exposure to OR37 ligands can be used to mitigate the responses induced by the chronic social isolation. Based on experiment 1, it was known that there was no difference between socially isolating the mice and individually housing the mice. Henceforth, the scantainers were used to prevent cross contamination of the treatment solutions between groups.

The animals came to the animal unit in a group of 3 with the exception of 1 cage with only 2 animals. The animals were housed using the same cage and enrichment as described in Chapter 2 section 2.1 Animals and Housing. One animal from the randomly selected group of 3 was moved into a new cage to make: an individually housed animal and a pair housed, making up 20 pair housed cages and 19 cages of individually housed mice. Then, 5 randomly selected pair housed cages were then separated into individually housed animals making up a total of 15 cages of pair housed animals and 29 cages of individually housed mice. The 15 cages of pair housed animals were allocated to scantainer A, 14 cages of randomly selected individually housed animals to scantainer B, and 15 cages of randomly selected individually housed animals to scantainer C. All scantainers were placed within the same room and the output vent directed to the exhaust van on the ceiling. A tea ball infuser (d=7 cm) was added to all cages with a filter paper (1cm x 1cm) inside it. All pair housed cages in scantainer A and all individually housed cages in scantainer B were treated with the solvent for OR37 ligands. All individually housed cages in scantainer C were treated with the OR37 ligands mixture. The OR37 ligands mixture was prepared following the protocol on Chapter 2, section 2.2. OR37 Ligands preparation.

The study was done within 9 weeks as shown in figure 4.1. On Mondays, the animals were weighed and cage cleaned, and the filter papers inside the tea ball infusers were replaced. The order of scantainers for weighing was counterbalanced. On the filter papers, 100µl of the solvent or 100µl of the OR37 ligands were topped up every day between 10.00 AM – 12.00 PM. The tea ball infusers were replaced if damaged by the

mice. The eye temperature recordings were done at the weekend starting from week 2 to week 7. The zero maze test and the novelty suppressed feeding tests were done over 4 days and the treatment groups were blocked and counterbalanced. The tube test was done over 4 days with 2 days of training phase and 2 days of testing phase. The urine marking test was done over 1 day. The standard opponent test and the resident intruder test were done over 3 days. After the resident intruder test, all individually housed mice were killed at 90 minutes from the start of the test with cervical dislocation and the brains were collected for free floating 40μm c-Fos immunohistochemistry following the procedure described in Chapter 2, section 2.6. Immunohistochemistry. Only 6 animals were culled from the pair housed mice, which were the dominant mice, for the brain collection. From the pair housed animals, the 15 subordinate mice and the 9 dominant mice were all individually housed in 1 scantainer at the end of this study to be used for experiment 3.

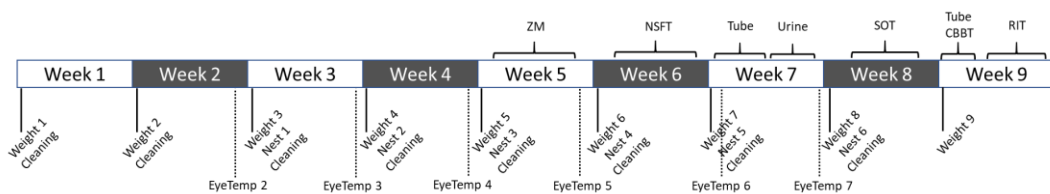

Figure 4.1: Social isolation experiment timeline diagram. ZM: Zero Maze Test, NSFT: Novelty Suppressed Feeding Test, Tube: Dominant Tube Test, Urine: Urine Marking Test, SOT: Standard Opponent Test, CBBT: Cotton Bud Biting Test, RIT: Resident-Intruder Test

4.3.3.1. Nest building test

The nest building test was conducted based on the Deacon (2006) protocol. The test was conducted in the middle of the light phase, within the mice home cage, and with a nestlet square cotton pad used as a nesting material. Prior to the test, the mice's cage was cleaned by transferring them to a clean cage filled with sawdust, approximately 1 cm from the bottom of the cage, and they were provided with one new nestlet square per cage. The enrichments were also transferred with exception to the cardboard tunnels, which were placed above the cage lid during the testing phase to encourage nest building. The order of the cage cleaning was counterbalanced for every week. The nestlets were novel in the first test and had not been experienced during husbandry. The nest was observed at 3 hours and 24 hours after the cage was returned to the scantainer and the scantainer was returned to the original position. The nest

observation was performed by rating the quality of the nest without disturbing the scantainer following the scoring in table 4.3.

Table 4.3: nest building test scoring criteria

Score	Criteria
1	Nestlet was noticeably untouched
1.5	Nestlet was shredded but still more than half of the nestlet pad left
2	Half of the nestlet was shredded
2.5	More than half of nestlet was shredded and scattered all over the cage
3	Most to all nestlet was shredded without any identifiable nesting area
3.5	Most to all nestlet was shredded with vaguely identifiable nesting area
4	Most to all nestlet was shredded with identifiable but flat nesting area
4.5	Most to all nestlet was shredded with small burrow on the nesting area
5	Cup shaped nesting area with walls

4.3.3.2. Elevated zero maze test.

The elevated zero maze test was conducted following the protocol in Chapter 2, section 2.4. Elevated zero maze test.

4.3.3.3. Novelty suppressed feeding test.

24-hours prior to the novelty suppressed feeding, the mice were put on food restriction by taking all the food pellets away from the cage. The novelty suppressed feeding was conducted during the light phase in a white Perspex arena (80 cm x 80 cm x 40 cm) covered with sawdust. A filter paper was placed on the centre of the arena with 2 food pellets placed on top of it.

The test was done following the project license within the home office regulation and was approved by the local ethics committee of the University of Bristol. On the test day, the mice were transported from their scantainer into the test room. During the test, the mice were recorded and observed using a video camera for 10 minutes or until the mice started eating the food pellets in the centre of the arena. The mice were then returned to their home cage and transported back to their scantainer. Observed

parameters were latency to approach food which was defined as the first time the mice nose or whiskers touched the food pellets and latency to eat which was defined as the first time the mice ate the food pellets for more than 3 seconds.

4.3.3.4. Dominant tube testing

The dominant tube testing was used as the main reference to determine the social hierarchy between animals in one cage. Only the pair housed mice were tested on this test to classify the status within the pairs. The test was conducted during the light phase using a custom-made clear plexiglass tube with 30 cm length and 3 cm wide diameter with supports to stabilise the tube on a flat surface. The tube size was designed to ensure only one adult mouse can pass through the tube without turning back or walking past the other mouse. The test was conducted in two phases which were the training phase and the trial phase.

During the training phase, the mouse was placed at one end of the tube and continued until the mouse walked to the other end of the tube. The training phase was conducted in 8 trials and the mouse was placed on alternating ends of the tube for every successive trial. The training phase was done in 2 consecutive days for each mouse with 5 trials for each mouse.

During the trial day, each mouse was given additional 3 training trials prior to testing. During the trial phase, both mice from the same cage were released simultaneously at both ends. The mice were observed until one of the mice walked back outside the tube or was pushed out by the other mouse. The mouse that left the tube was scored as the loser and the mouse that stayed inside the tube was scored as the winner. The test was repeated 3 times with the entry ends for the mice being swapped on every trial. The mouse with 2 or 3 wins was categorised as the dominant and the other was categorised as subordinate. The tube was cleaned with 70% ethanol followed with water and dried between each mouse pair.

4.3.3.5. Urine marking test.

The urine marking test was performed following the Drickamer (2001) protocol with modification. The test was conducted on the pair housed mice during the light phase on a modified mouse cage following the figure 4.2 (a clear perspex conventional tecniplast 1284L cage, 365 x 207 x 140 mm). The flooring of the cage was raised 2 cm

by inserting a grid flooring to allow urine to pass through but still supporting the mice body weight with the mice still able to walk around on top of it. The open top cage lid was divided midway using a perforated metal plate to physically separate the two mice while still allowing visual, auditory, and olfactory contact. Two filter papers (d=30cm) were placed under the grid floor with one filter paper under each mouse side to capture the urine as shown on figure 4.2. The mice were placed on each opposite side of the perforated metal plate and left undisturbed for 2 hours. After 2 hours, the mice were returned to their cage and the filter papers taken out. The filter papers were labelled, drawn with 1 cm scale, and air dried for 1 hour before being stored in a plastic bag in -20ºC freezer. The test was done over 2 days due to logistic and timing limitations. The grid flooring and the lid were cleaned using 70% ethanol followed with water, dried, and placed in a new clean mouse cage before used for another testing session.

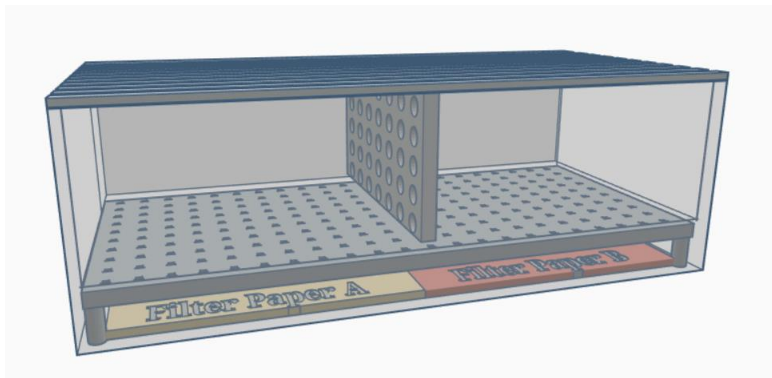

Figure 4.2: diagram representative of modified mouse cage for urine marking test.

For analysis, filter paper was visualised using a UV gel reader and the images were saved for further analysis. Image analysis was performed using Fiji software's grid tools (Analyze > Tools > Grid) with lines grid type and 1 cm^2 grid size after calibration to the scale drawn on the filter paper. The urine marking was measured as the total area of urine and the number of 1 cm^2 squares divided into the perimeter of the filter paper and the interior of the filter paper.

4.3.3.6. Standard opponent test

The standard opponent test was used as a measure of social aggressive behaviour without the territorial context. The test was conducted during the light phase in a clean cage as a neutral arena. The clean cage was filled with sawdust to approximately 1 cm high without any enrichment or cage lid. Prior to the test day, the mice were weighed, and the body weight was used to match the body weight for pairings. The dominant

mice from the pair housed from the scantainer A and the individually housed mice from the scantainer B and C were used as the subject animals. The subordinate mice from the pair housed within the scantainer A were used as the opponent and the test was conducted for 5 minutes or until a fight happened. The test for all the 44 subjects was spread over 3 consecutive days and each animal was only tested once. Counterbalancing was done to ensure: no pair from the same pair housing cage and the body weight difference was within 2.5 grams. A video camera was used to record the test for offline analysis. Observed behaviours were: latency to fight, total duration of anogenital sniffing, total duration of digging, and total frequency of rearing. The digging duration parameter was added due to the obvious amount of digging behaviour occurring across all treatment groups. According to Deacon (2006) and Njung'e & Handley (1991), digging is a species-specific behaviour and was found to be sensitive to different treatments, including to anxiolytic drugs.

4.3.3.7. Resident intruder test

The resident intruder test was used as a measure of social aggressive behaviour in the context of territorial protection. The test was done during the light phase in the resident's home cage. The dominant mice from the pair housed animals within scantainer A and the individually housed mice from the scantainer B and C were the subject for this test. The subordinate mice from the pair housed in scantainer A were used as the intruders and the test was conducted for 10 minutes. The test was spread over 3 consecutive days with counterbalancing to ensure: no pair from the same pair housing cage, the body weight difference was within 2.5 grams, and none of the pairings were the same pairings as the standard opponent test. The cage was left uncleaned for 7 to 9 days and all enrichments were taken out briefly during the test. Prior to the first test day, all mice were weighed. A video camera was used to record the test for offline analysis. Observed behaviours were: latency to fight, total duration of anogenital sniffing, total duration of digging, and total frequency of rearing.

4.3.3.8. Cotton bud biting test

The cotton bud biting test was done following the protocol in Chapter 2, section 2.10. Cotton bud biting test.

4.3.3.9. Eye temperature

The eye temperature recording method and analysis were done following the protocol in Chapter 2, section 2.9. Eye thermal recording.

4.3.4. Experiment 3: Validating OR37 ligands delivery methods.

This study was done to verify whether the tea ball infuser can be used to deliver the OR37 ligands. This study also investigated other methods of exposing the OR37 ligands such as by smearing on surfaces of the closed environment without any airflow. A novel box test as described in Klein et al. (2015) was used in this study. The study showed that the reduction effect of the PVN activation due to OR37 ligands exposure was achieved by carrying the ligands using airflow from the input pipe. However, the exposure method using the tea ball infuser and the smearing method, both without the use of airflow, have not been validated, which will be done in this experiment.

The 24 animals used came from the previous experiment (experiment 2) and were randomly allocated into 4 different groups of treatments which were: the solvent control carried within airflow group, the OR37 ligands carried within the airflow group, the OR37 ligands inside a tea ball infuser without airflow group, and the OR37 ligands smeared on the arena lid without airflow group. The solutions were placed on a filter paper for the airflow groups and the tea ball infuser group. 100 µl of solutions were used on each group.

The experiment was spread over 6 consecutive days and the treatment group orders were counterbalanced. The animals were moved to the experimental room a day prior to the experiment. On the experimental day, the animals were transferred using their cage's tube to the novel box arena and were left for 30 minutes. The animals were killed at 90 minutes from the start of the novel box test (or 60 minutes after taken out and placed back at their home cage) and the brains were collected for c-Fos immunohistochemistry using free floating 40µm sections following procedure described in Chapter 2, section 2.6. Immunohistochemistry.

4.3.4.1. Novel Box Test

The mouse was placed individually for 30 mins into an unfamiliar closed Perspex box (approximately 9cm height × 15.5cm width × 24cm length) as reported in Klein et al. (2015). On one side of the box, a metal pipe was attached for connecting the gas pipe input and a small hole on the opposite site for allowing the air to flow outside. A pressurised tank of medical air was connected to a valve to regulate 1 psi air pressure which then connected to the metal pipe of the box. A 1cm x 1cm filter paper was blotted with 100µl solution of solvent or OR37 ligands or none (for no airflow groups) according to the treatment group and placed in the tube that connected the valve and the metal pipe. After the test, the mice were returned to their home cage and killed by cervical dislocation 90 minutes after the start of the test.

4.3.5. Tissue Preparation and Immunohistochemistry

A 40µm section with a free-floating method was used. The tissue preparation and immunohistochemistry protocol were conducted following the described method on Chapter 2, section 2.5. Tissue collection, preparation, and storage, and section 2.6. Immunohistochemistry.

4.3.6. Corticosterone Radioimmunoassay

The corticosterone radioimmunoassay was conducted following the protocol on Chapter 2, section 2.8. Radioimmunoassay.

4.3.7. Data Analysis

All statistical procedure and graph producing were done as described on chapter 2, section 2.11. In experiment 1, PVN c-Fos data was analysed using an independent t-test. The plasma CORT data was analysed using an independent t-test.

In experiment 2, the dominant and the subordinate mice within the pair housing's data were analysed separately to see whether there were differences between dominant statuses. All the data from the pair housed males were analysed using a paired t-test when the data was normally distributed or Wilcoxon Matched-Pair Test when the data

was not normally distributed. The difference was checked using repeated measures with WEEKS as the within-subject factor and HIERARCHY as the between-subjects factor. Due to there being no difference between the two, the data from two were averaged to represent the observational unit as a cage to avoid pseudoreplication. The body weight progression and changes were analysed using a Repeated Measures ANOVA with WEEKS as the within-subject factor and TREATMENTS as the between-subjects factor. The assumption of sphericity was violated and a Greenhouse-Geisser correction was used. The total body weight was checked for normality and analysed using a One-Way ANOVA. The eye temperature data was analysed using a Repeated Measures ANOVA with WEEKS as the within-subject factor and TREATMENTS as the between-subjects factor. The assumption of sphericity was violated and a Huynh-Feldt correction was applied. A post-hoc analysis was performed using the Bonferroni correction. The Nest Building Test data was analysed using a non-parametric analysis due to the nature of the ordinal data type. Both the 3 hour and the 24 hour data was analysed using the Friedmann test on each group individually over the week 3 to week 8 data. A Kruskal-Wallis test was performed to test the difference between groups on week 3 and week 8 data (the start and the end of observational data) and on week 6 and week 7 (the time point after the ZM and NSFT weeks). The dominance testing data and the urine volume was analysed using a Mann-Whitney test, while the urine area, total body weight gain, and the body weight changes were analysed using independent t-tests. The Zero Maze was analysed using the Kruskal-Wallis test. The Novelty Suppressed Feeding Test data was analysed using Kruskal-Wallis test. The Standard Opponent Test data, the latency to attack, duration of anogenital sniffing, and the duration of digging data were analysed using Kruskal-Wallis analysis and a pairwise-comparison using the Bonferroni correction was used when the overall group differences were significant, while the rearing frequency data was analysed using a One-Way ANOVA. The Resident-Intruder Test data, the latency to fight, duration of anogenital sniffing, and the duration of digging were analysed using Kruskal-Wallis test and the frequency of rearing was analysed using a One-Way ANOVA. The Cotton Bud Biting Test data were both analysed using the Kruskal-Wallis test.

In experiment 3, One-Way ANOVA analysis was performed for PVN c-Fos cell counts after checking the normal distribution. A post-hoc analysis using Dunnett correction was done on the PVN c-Fos cell counts data with the solvent group being compared to

every other group. The MeA c-Fos cell count was analysed using Kruskal-Wallis test due to violation of normality assumption.

4.4. Results

4.4.1. Experiment 1: Pilot study on validating social isolation in scantainer.

This experiment was done to validate that acutely (24h) isolating mice in a scantainer alone did not induce different levels of stress responses to individually housing mice in a communal scantainer. Due to technical difficulties during the sectioning process, 1 brain from the individual housing group was lost. Due to no c-Fos expression at all even on the areas that were expected to have c-Fos expression, 1 brain from the individual housing was excluded from the analysis. One data point from the scantainer-isolated was omitted from analysis due to its value of standardised residual being higher than 3SD. Isolating a mouse in an individual scantainer compared to individual housing in a communal scantainer did not affect PVN c-Fos activation changes ($t_{(17)}$=-0.323, p=0.750, figure 4.3 Left). Meanwhile on the plasma CORT results, 1 sample from the individual housing could not be processed due to the level being unrealistically too low to be detected by the gamma counter. The mean plasma CORT level was not significantly different between the individual housing and the scantainer-isolated groups ($t_{(19)}$=1.758, p=0.095, figure 4.3 Right).

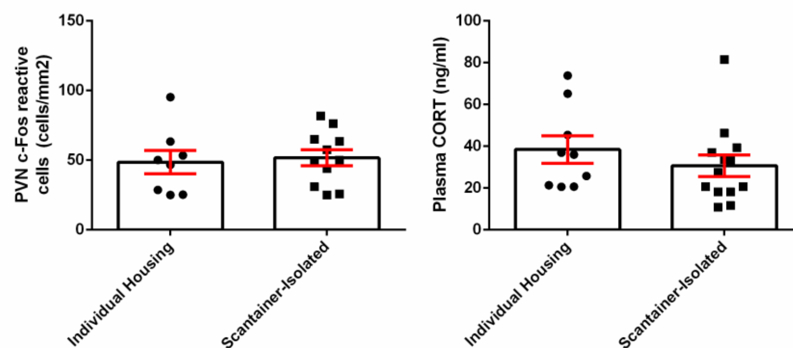

Figure 4.3: Individual housing mice did not differ to scantainer-isolated mice on PVN c-Fos activation and plasma CORT level. (Left) PVN c-Fos activation level, (Right) Plasma corticosterone level. Data shown as mean ± SEM

4.4.2. Experiment 2: Social isolation and chronic exposure to OR37 ligands

Dominance testing

Although this part of the experiment does not directly address the main question regarding the effect of the OR37 ligands, the dominance testing was conducted in order to assign dominant status to pair-housed males. The dominance status was used to categorise the animals within the pair-housed males and to ensure that there were no significant differences between the two, henceforth the data between the two can be averaged to represent the unit of a cage. In addition, the comparison between the tube test and the urine marking test was done to see whether there were differences between dominant status assigned from the tube test and the quality or the quantity of the urine marks on the urine marking test. Body weight was also added whether there was a difference from the body weight and their dominance status.

The total urine volume did not differ between the Dominant and Subordinate animals that were categorised by the dominant tube test (Wilcoxon Signed-Rank Z=45, p=0.394, figure 4.4A). There were also no differences on urine marking on inner area ($t_{(14)}$=0.358, p=0.726, figure 4.4B), marking on perimeter area ($t_{(14)}$=1.865, p=0.083, figure 4.4B), body weight at week 7 ($t_{(14)}$=0.940, p=0.363, figure 4.4C), and body weight changes from week 6 to week 7 ($t_{(14)}$=0.673, p=0.512, figure 4.4D).

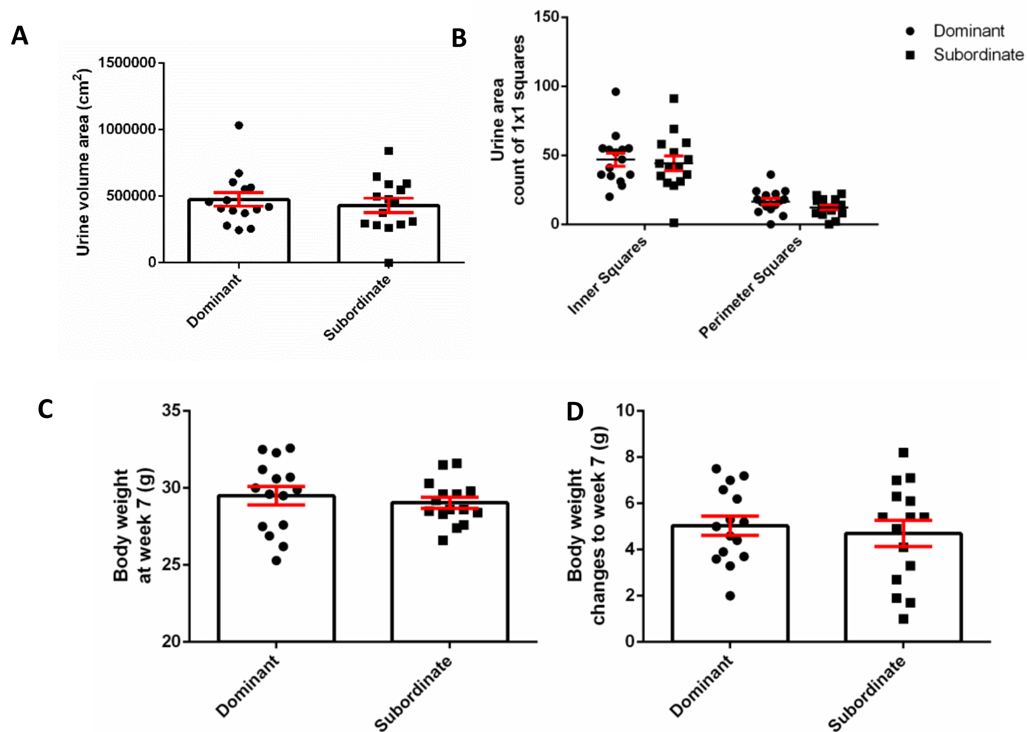

Figure 4.4: Comparisons between dominant and subordinate mice based on the dominant tube test to their urine marking test and body weight. (A) total urine volume during urine marking test in cm^2, (B) total marked area in 1x1cm square grids during urine marking test, (C) body weight at week 7, (D) body weight changes from week 6 to week 7, Data shown as mean ± SEM.

Body weight

The body weight data was recorded to investigate whether the social isolation affected their body weight growth. There was no difference between the dominant and the subordinate males from the pair-housed over 9 weeks of observation ($F_{(2.1,57.3)}=1.062$, $p=0.391$), henceforth, the data for the dominant and the subordinate from the pair-housed males was averaged for this data set. The body weight progression shows a significant effect of WEEKS ($F_{(2.2,90.5)} = 377.96$, $p < 0.001$, figure 4.5A) which signifies the animals' body weight growth over time. However, there was no effect of treatment ($F_{(2,41)} = 0.222$, $p=0.801$) and the social isolation with or without OR37 ligands seemed to have no effect on growth rate ($F_{(4.4,90.5)} = 1.914$, $p=0.108$). The body weight changes data each week (which was a particular week with previous week subtracted from it and analysed due the RM ANOVA does not consider correlation between neighbouring timepoints) also shows a significant effect of WEEKS ($F_{(5.9,240.7)} = 13.646$, $p < 0.001$, figure 4.5B). In addition, the mean week to week weight change was affected by treatments ($F_{(11.7,240.7)} = 3.335$, $p< 0.001$) without an effect of

treatment on its own ($F_{(2,41)} = 0.008$, p=992). A post-hoc test was done and there was a significant difference in body weight changes between week 3 and 4 ($p<0.0001$), week 4 and 5 (p=0.010), and week 5 and 6 (p=0.003). Simple effect analysis between week 3 and 4 showed that the significant decreases were driven by the Singly Housed – Solvent group (p=0.002) and the Singly Housed – Ligands (p=0.043) but not for the Pair Housed – Solvent (p=1.000). On the other hand, only the Singly Housed – Solvent group was driving the changes on week 4 and 5 (p=0.011) and week 5 and 6 ($p<0.0001$). As for the total body weight gain, there was no effect of social isolation on total body weight gain across 9 weeks of study ($F_{(2,41)} = 0.008$, p= 0.992, figure 4.5C).

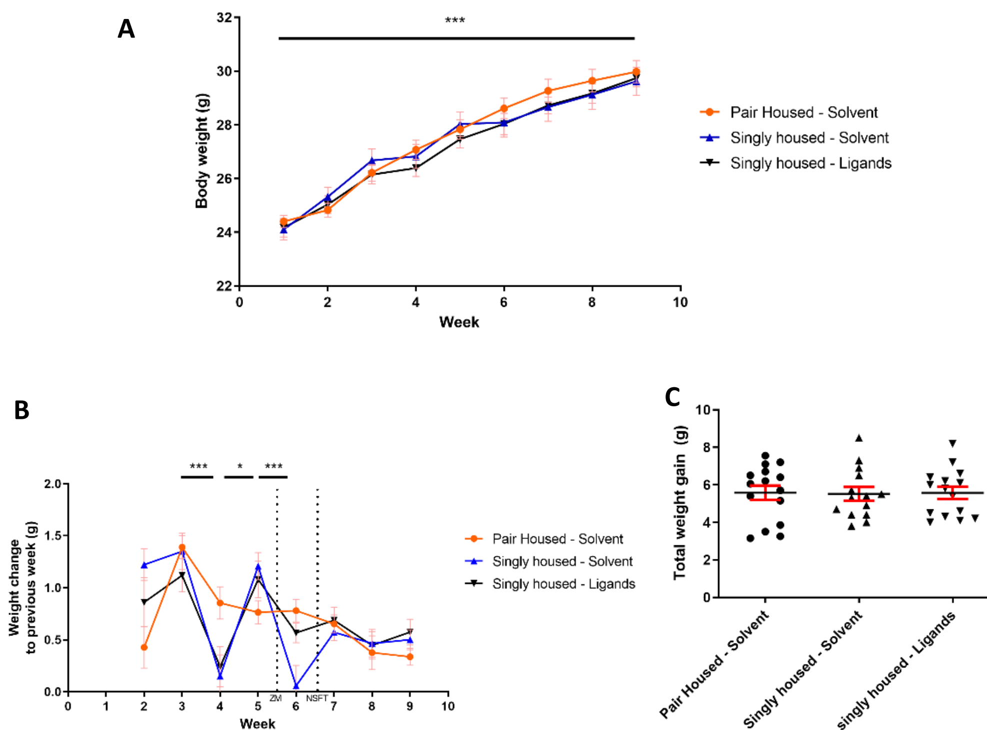

Figure 4.5: Normal growth curve of mice during 9 weeks of experiments without significant overall differences between pair housed mice and socially isolated (singly housed) mice. (A) body weight progression, (B) body weight change compared to previous week, (C) total body weight gain over the study, ZM: Zero Maze Test, NSFT: Novelty Suppressed Feeding Test, Data shown as mean ± SEM, *p<0.05, ***p<0.001

Eye temperature

The eye temperature recording was used as a measure of peripheral indication of core body temperature changes during the experiment using a non-invasive method. There was no difference between the dominant and the subordinate males from the pair-housed over 6 weeks of observation ($F_{(4.5,121.4)}$=1.187, p=0.320), henceforth, the data for the dominant and the subordinate from the pair-housed males was averaged for this data set. The eye temperature results (figure 4.6) showed that the treatment significantly affected the differences in mean eye temperature across weeks ($F_{(9.6,196.6)}$ = 6.131, $p < 0.001$). There were also significant differences between the treatment groups ($F_{(2,41)}$ = 7.987, p= 0.001) and between the weeks ($F_{(4.8,196.6)}$ = 13.274, $p < 0.001$). A post-hoc test on the main effect of WEEKS showed a significant overall decrease of eye temperature after the Zero Maze Test ($p<0.001$), a significant overall increase after the Novelty Suppressed Feeding Test ($p<0.001$), and significant changes between week 6 and week 7 ($p<0.001$). Further analysis shows that the drops in eye temperature on recording day after the Zero Maze Test week was driven by the Pair Housed – Solvent (p=0.019). Similarly, the increase in the eye temperature on recording day after the Novelty Suppressed Feeding Test week was driven by the Pair Housed – Solvent ($p<0.001$). However, the changes from week 6 to week 7 were driven by both the Singly Housed – Solvent ($p<0.001$) and the Singly Housed – Ligands (p=0.043). Furthermore, on week 7, the Pair Housed – Solvent was significantly different with both the Singly Housed - Solvent ($p< 0.001$) and the Singly Housed – Ligands (p=0.003). In addition, on week 7, there was also a significant difference between the Singly Housed – Solvent and the Singly Housed – Ligands (p= 0.003).

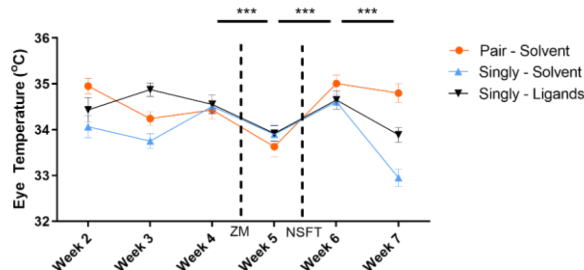

Figure 4.6: Recordings of mice eye temperature showed a decrease after zero maze test, an increase after novelty suppressed feeding test, and an effect after 7 weeks social isolation with the OR37 ligands buffering the eye temperature drop due to social isolation. ZM: Zero Maze Test, NSF: Novelty Suppressed Feeding Test, Data shown as mean ± SEM, ***p<0.001

Nest Building

The Nest Building Test was done to see whether social isolation, which is a form of stressor, influenced the nest quality. The Zero Maze Test week and the Novelty Suppressed Feeding Test week did not significantly affect the nest quality of any of the treatment groups either on 3 hour (post-ZM χ^2=1.16, p=0.561, post-NSFT χ^2=5.87, p=0.053) or the 24 hour observations (post-ZM χ^2=3.53, p=0.171, post-NSFT χ^2=0.74, p=0.692). There were no differences between treatment groups' nest qualities on 3 hour observations at week 3 (χ^2=3.32, p=0.191) and week 8 (χ^2=5.25, p=0.073, figure 4.7A), however, the Pair Housed cage had significant reduction of nest quality on 24 hour observation (p=0.032, figure 4.7B). Analysis on the group differences did not show significant differences except on week 3 both on 3 hour and 24 hour observations. On the 3 hour observation, the Singly Housed – Solvent group was significantly lower than the Pair Housed (p=0.009) and to the Singly Housed – Ligands (p=0.046). On the other hand, the 24 hour observation only showed a significant difference between the Singly Housed – Solvent and the Pair Housed animals (P=0.024).

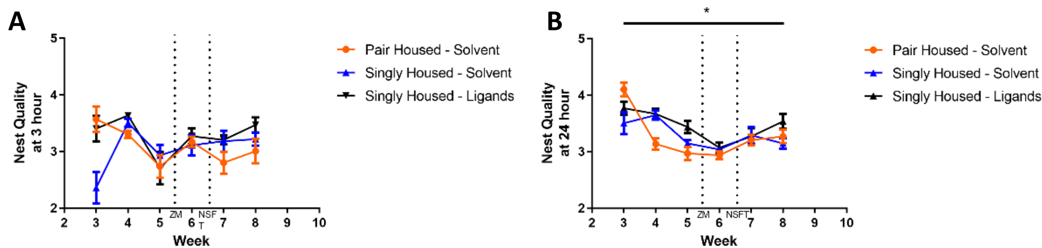

Figure 4.7: Social isolation for 8 weeks did not affect nest qualities on 3 or 24 hours observation. (A) observation at 3 hours after cage cleaning, (B) observation at 24 hours after cage cleaning, ZM: Zero Maze Test, NSFT: Novelty Suppressed Feeding Test, Data shown as mean ± SEM, *p<0.05 main effect of week

Anxiety tests

Anxiety-like behaviours were tested using a Zero Maze Test and a Novelty Suppressed Feeding Test to see whether the social isolation induced anxiety-like behaviours. There was no difference between the dominant and the subordinate males from the pair-housed on latency to open arm (Wilcoxon Signed Rank Z= 79, p= 0.281), duration in the open arm (Wilcoxon Signed Rank Z= 73, p= 0.460), frequencies of closed arm return (Wilcoxon Signed Rank Z= 45, p= 0.634), and frequencies of transition ($t_{(14)}$= 0.459, p= 0.653). Henceforth, the data for the dominant and the subordinate from the pair-housed males was averaged for this data set. On the elevated zero maze test, the social isolation by singly housing did not affect the anxiety-like behaviours using the parameters latency to the open arm ($\chi^2_{(2)}$=0.528, p=0.768, figure 4.8A), frequency of transitions between arms ($\chi^2_{(3)}$=1.581, p=0.664, figure 4.8C), or frequency of returning to the closed arm from the open arm ($\chi^2_{(3)}$=4.118, p=0.249, figure 4.8D). There was a significant difference between treatment groups on time spent in the open arm ($F_{(2,41)}$=4.792, p=0.013, figure 4.8B), however, on post-hoc analysis, there was no difference between Pair Housed – Solvent vs Singly Housed – Solvent (p= 0.262) and between Singly Housed – Solvent vs Singly Housed – Ligands (p= 0.245).

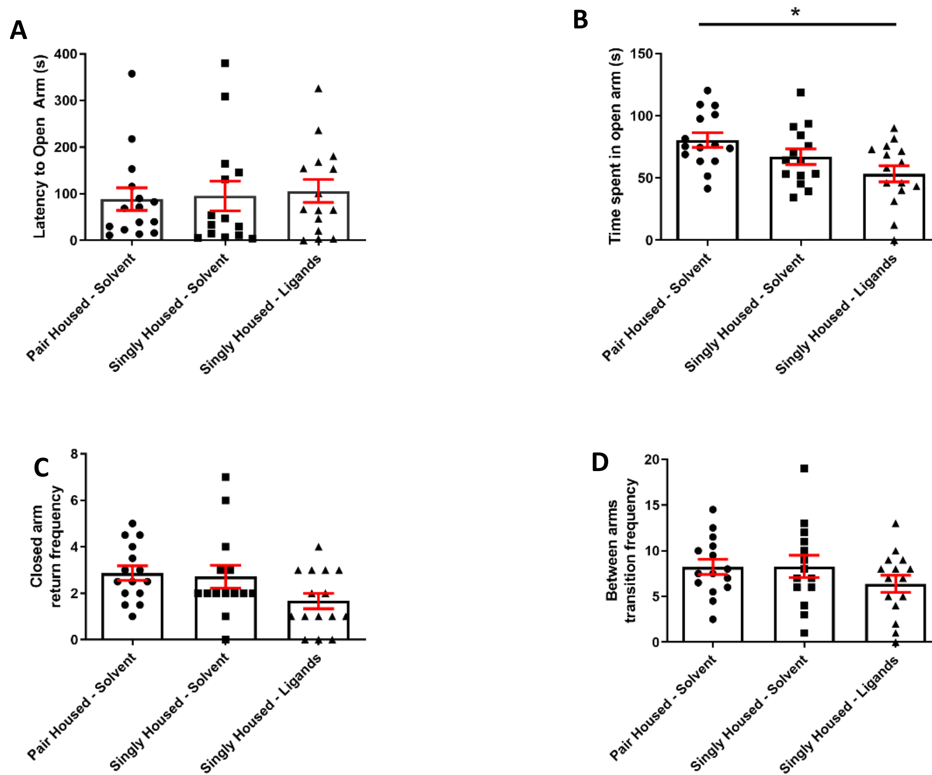

Figure 4.8: Socially isolated mice did not show significant differences to group housed mice on the anxiety-like behaviours using the elevated zero maze test. (A) latency of first entry to the open arm, (B) total amount of time spent in the open arm, (C) total frequency of return to the closed arm, (D) total frequency of transitioning between arms, Data shown as mean ± SEM.

The Novelty Suppressed Feeding Test was done as another anxiety-like behavioural measurement that has a different motivational drive to the Elevated Zero Maze Test. There was no difference between the dominant and the subordinate males from the latency to approach (Wilcoxon Signed Rank Z= 63, p= 0.865) and the latency to eat (Wilcoxon Signed Rank Z= 77, p= 0.334). Henceforth, the data for the dominant and the subordinate from the pair-housed males was averaged for this data set. The Novelty Suppressed Feeding Test also did not show any anxiety-like behaviours induced by social isolation. There were no significant differences between the four groups on either the latency to approach the food pellets ($\chi^2_{(2)}$=0.536, p=0.765, figure 4.9A) or the latency to eat the food pellets during the 600 seconds observation time ($\chi^2_{(2)}$=1.424, p=0.491, figure 4.9B).

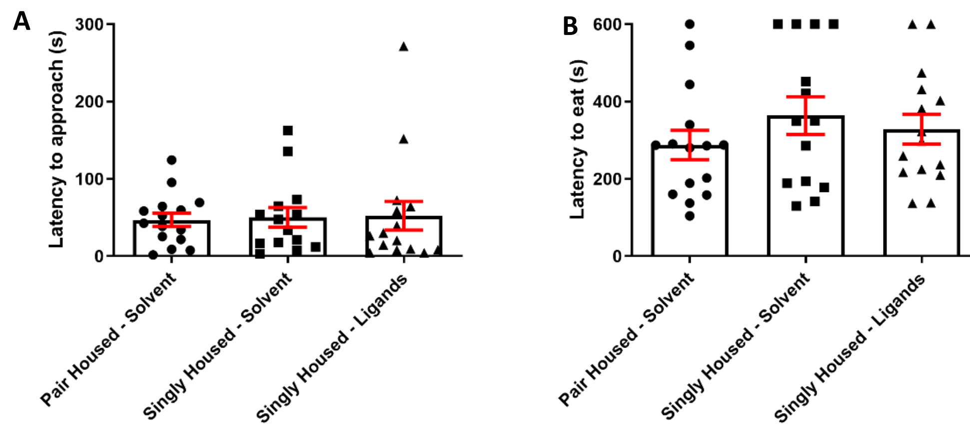

Figure 4.9: Socially isolated mice did not show significant differences to group housed mice on the anxiety-like behaviours using the novelty suppressed feeding test. (A) latency of first approach to the food pellets, (B) latency to eat the food pellets. Data shown as mean ± SEM

Aggression tests

Aggression tests were performed to see the effect of social isolation on aggressive behaviours. Only the dominant mice were used from the pair housed mice due to the subordinates being used for the opponent/intruder on the aggression tests. The standard opponent test was done to see the aggression in a neutral arena. On the standard opponent test, there was a lack of aggression as seen on the latency to fight, therefore, there were no significant differences between treatment groups ($\chi^2_{(2)}$=3.957, p=0.138, figure 4.10A). Similarly, there was no effect of social isolation on anogenital sniffing behaviour ($\chi^2_{(3)}$=1.804, p=0.406, figure 4.10B). However, there was a significant difference between treatment groups on rearing frequencies ($F_{(2,41)}$=3.532, p=0.038, figure 4.10C) and a planned contrast analysis showed that the socially isolated mice reared less compared to the pair housed mice (p=0.011) but there were no differences between the socially isolated mice with solvent and with the OR37 ligands (p=0.980). There was also a significant difference on the digging behaviour between treatment groups ($\chi^2_{(2)}$=7.718, p=0.021, figure 4.10D) and pairwise-comparisons showed that the difference was driven by the Singly Housed – Ligands group performed less digging compared to the Pair-Housed mice (p=0.022).

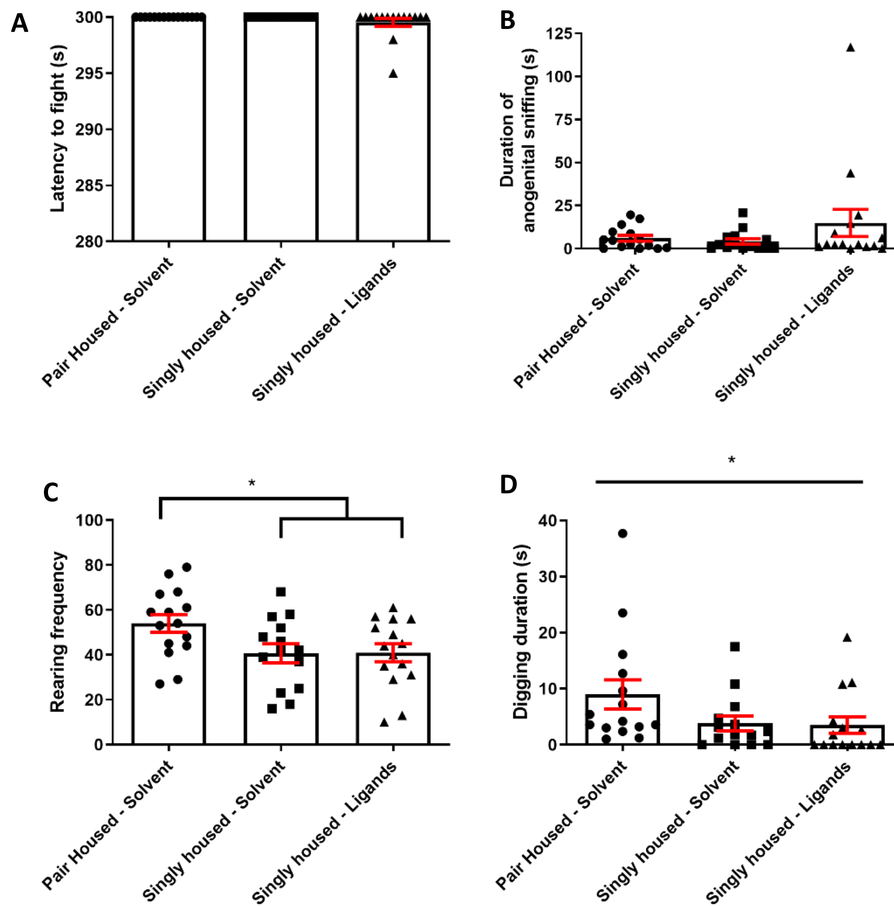

Figure 4.10: Aggression test on neutral arena using Standard Opponent Test with unexpected lack of aggressive behaviour did not show significant effect of social isolation but with significant differences on other behaviours during the test. (A) latency to attack on 300 seconds observation, (B) duration of anogenital sniffing, (C) frequency of rearing, (D) duration of digging, Data shown as mean ± SEM, *p<0.05.

Another aggression test was performed using the Resident-Intruder Test to see the effect of social isolation on aggression driven by territorial motivation. There was more aggression observed on this test compared to the Standard Opponent Test, however, there was no significant difference between treatment groups on the latency to attack ($\chi^2_{(2)}$=1.184, p=0.553, figure 4.11A) or the duration of anogenital sniffing both on overall data ($\chi^2_{(2)}$=5.549, p=0.062, figure 4.11B) and only including the mice that did not fight ($\chi^2_{(2)}$=2.456, p=0.293, figure 4.11C). There were also no effects of social isolation on other parameters which are the frequency of rearing ($F_{(2,41)}$ = 1.115, p= 0.338, figure 4.11D) and the duration of digging ($\chi^2_{(2)}$=0.439, p=0.803, figure 4.11E).

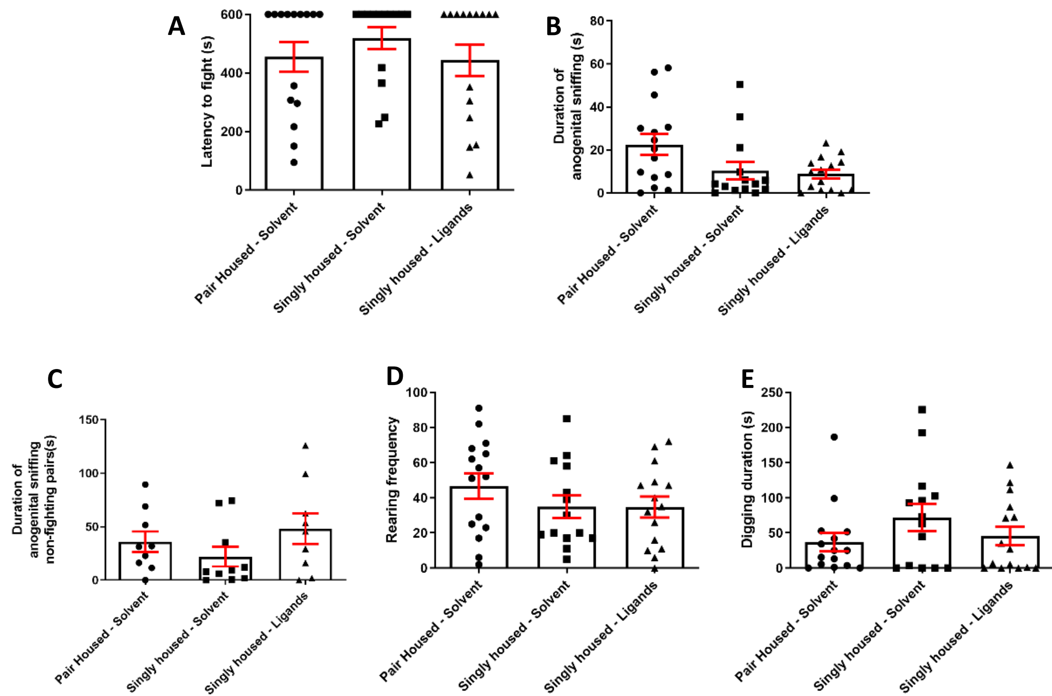

Figure 4.11: Social isolation did not increase aggression related parameters on resident-intruder test. (A) latency to fight, (B) total duration of anogenital sniffing in all mice, (C) total duration of anogenital sniffing in non-fighting pairs only, (D) total frequency of rearing, (E) total duration of digging. Data shown as mean ± SEM

The Cotton Bud Biting Test was conducted in order to assess the non-social aggressive behaviour. There was no difference between the dominant and the subordinate males on the biting frequencies (Wilcoxon Signed Rank Z= 17, p= 0.483) and on the biting duration (Wilcoxon Signed Rank Z= 16, p= 0.124). Henceforth, the data for the dominant and the subordinate from the pair-housed males was averaged for this data set. On the cotton bud biting test, it was shown that there was a significant difference in the biting frequencies among treatment groups ($\chi^2_{()}$=27.174, p<0.001, figure 4.12A). Pairwise comparison with Bonferroni correction shows that there was no difference in biting frequencies between the singly housed mice with solvent and with OR37 ligands (P=1). However, the biting frequencies of the pair housed mice with solvent were significantly lower compared to the singly housed mice with solvent (p<0.001) or to the singly housed mice with OR37 ligands (p<0.001). Results were also quite similar on the average duration of biting with significant difference between treatment groups ($\chi^2_{(2)}$=28.236, p<0.001, figure 4.12B). Pairwise comparison with Bonferroni correction also showed that there was no difference in average duration of biting between singly

housed mice with solvent and with OR37 ligands (P=1). Similarly, there were also consistent results on average duration of biting with the pair housed solvent were significantly lower compared to the singly housed mice with solvent (p<0.001) or to the singly housed mice with the OR37 ligands (p<0.001).

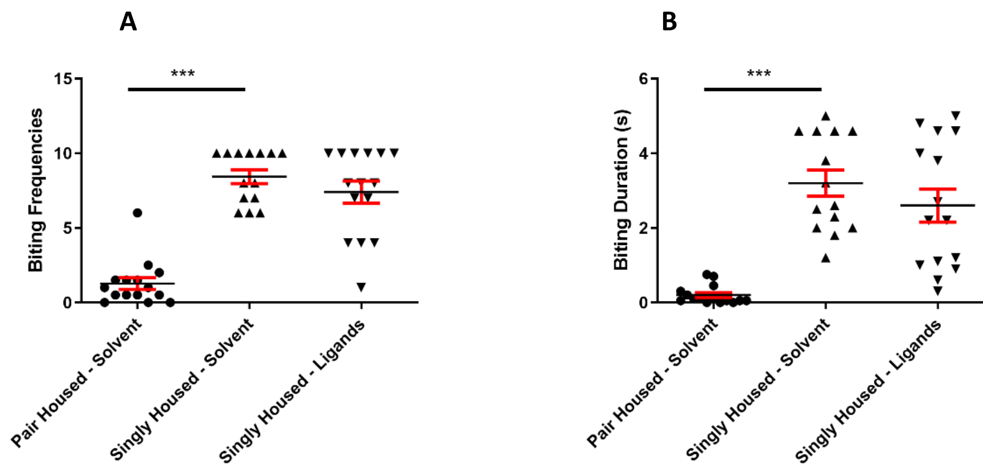

Figure 4.12: Individually housed mice significantly bite more frequently and for longer duration to pair housed mice without effects of OR37 ligands exposure. (A) frequency of biting, (B) duration of biting. Data shown as mean ± SEM, ***p<0.001

Immunohistochemistry

The c-Fos immunohistochemistry was performed to investigate activation of brain regions associated with the aggression (VM Hypothalamus) and stress response (PVN of hypothalamus) after the Resident-Intruder Test. Only 6 animals from the Pair Housed – Solvent (dominant) were sacrificed due to the experimental design to ensure the intruder would still be on a pair housing at the final day of experiment. Due to time constraint, only 7 sets of images were analysed from the Singly Housed – Solvent and respectively only 9 sets of images were analysed from the Singly Housed - Ligands. From the immunohistochemistry results, there was no significant difference between treatment groups on the ventromedial hypothalamus c-Fos expression ($\chi^2_{(2)}$=4.132, p=0.127, figure 4.13A). However, there was a significant difference between treatment groups on PVN c-Fos expression ($F_{(2,18)}$ = 6.223, p= 0.009, figure 4.13B) with one data point from the Singly Housed - Ligands group was removed from analysis (denoted with red triangular on figure 4.13B) due to being outlier based on the cook's distance value (0.53, higher than the criteria 4/n of the group). Planned contrast analysis on the

PVN c-Fos expression showed that the Pair Housed - Solvent was significantly higher than the Singly Housed - Solvent group (p=0.007). There was no significant difference between Singly Housed - Solvent and Singly Housed - Ligands groups (p=0.921).

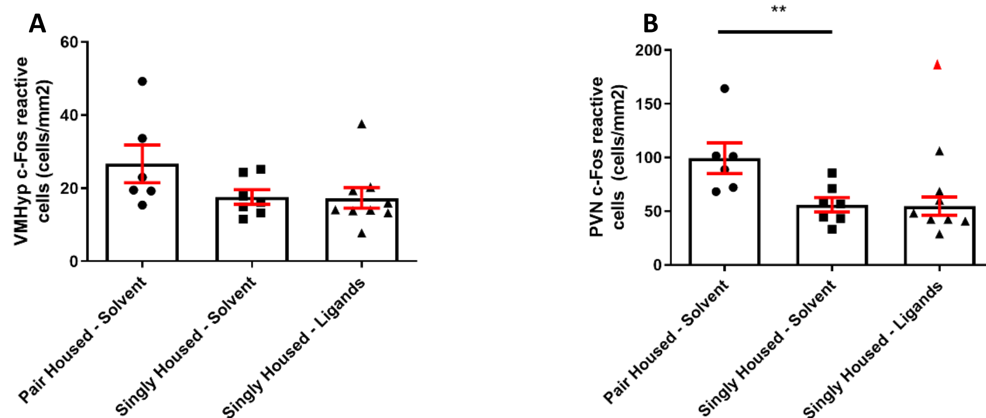

Figure 4.13: c-Fos expression levels in ventromedial hypothalamus and PVN between group housed mice with solvent, singly housed mice with solvent, and singly housed mice with OR37 ligands following the resident intruder test. (A) Ventromedial area of the hypothalamus c-Fos activation level, (B) PVN c-Fos activation level. Data shown as mean ± SEM, **p<0.01

4.4.3. Experiment 3: Validating OR37 ligands delivery methods

This final experiment on this chapter was done to validate that the OR37 ligands can be delivered using the tea ball infuser. As another alternative to the delivery method, the OR37 ligands solution were smeared in the Novel Box Test's Arena. During the Novel Box Test, there were significant differences among the means between different methods of exposing OR37 ligands groups and the solvent group ($F_{(3,16)}$= 5.697, p=0.008, figure 4.14A). Post-hoc test (Dunnett correction) shown that PVN c-Fos activation was significantly reduced when compared to the solvent group when OR37 ligands were delivered using an airflow (p=0.017), delivered in a tea ball infuser (p=0.012), or delivered by being smeared on the novel arena's lid (p=0.006). There was no sufficient evidence that shows changes in the medial amygdala's c-Fos activation between different methods of OR37 ligands delivery and solvent ($\chi^2_{(3)}$=3.800, p= 0.284, figure 4.14B).

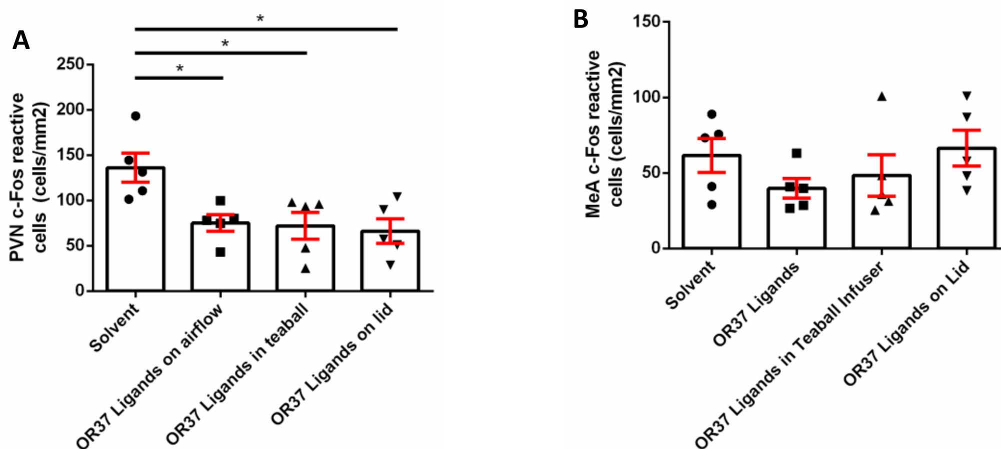

Figure 4.14: Significant reduction of PVN c-Fos activation using an airflow, tea ball infusers, and lid-smear without a change in the MeA following OR37 ligands exposure in novel environment test. (A) PVN c-Fos activation level, (B) Medial Amygdala c-Fos activation level. Data shown as mean ± SEM, *$p<0.05$

4.5. Discussion

Validation of OR37 ligands exposure methods

One of the challenges with the OR37 ligands as mentioned in the previous chapter is the inability to measure airborne concentrations of the ligands. However, it was clear that the OR37 ligands exposure did affect the PVN c-Fos activation. As for the results, it showed that the presence of OR37 ligands, no matter what method of exposure, significantly reduced the PVN c-Fos activation in a novel environment (figure 4.14A). The validation of the tea ball and the direct smearing methods showed how these results are potentially useful as delivery methods of the OR37 ligands in future experiments. Although to be noted that this setup was done for acute exposure. It was difficult to assess for chronic exposure due to no equivalent to the airflow procedure as a positive control. However, as it was done in the chronic exposure in the social isolation experiment, the solutions were topped up every day and it was noted that the filter papers were not fully dried since the previous addition when the addition of the solution was done. Within the assumption that the OR37 ligands were dissolved in the solvent, it is very likely that the regular top up should have ensured consistent presence of the OR37 ligands in the mice cage.

Social isolation using the scantainer did not differ with individual housing.

This experiment used a more complete form of social isolation than what the previous studies have done. It is important to check the baseline levels of the PVN c-Fos and the plasma CORT to see whether these stress response parameters were raised by socially isolating in scantainer housing. There was no evidence for increased baseline responses 24 hours after isolating the mice in the scantainer (figure 4.3). Previous studies have found that acutely isolating mice resulted in physiological responses such as: synaptic changes in dopaminergic neurons of the dorsal raphe (Matthews et al., 2016), social recognition memory impairment (Kogan, Franklandand, & Silva, 2000), and an increase of first spike latency and a decrease of neuronal excitability in the CRH neurons in females (Senst et al., 2016). There are limited references regarding the effect of acute social isolation in mice to PVN c-Fos and plasma CORT measurement. Although it is unclear whether the differences between the scantainer isolation and the individual housing will become more apparent over time, there were no changes observed after socially isolating the mice for 24 hours. As for the plasma CORT, the level was at an acceptable level for baseline stress level, hence, it suggested that acutely social isolating the mice did not affect the stress responses.

One important discussion point is regarding the relationship between social isolation and sensory deprivation. Sensory deprivation in mice is commonly achieved by cutting their whiskers. Previous studies have reported that sensory deprivation in mice resulted in reduced levels of oxytocin compared to non-deprived mice and an impairment within anxiety-like behavioural measurements (Vaidyanathan & Hammock, 2020; Soumiya et al., 2016). It is possible that socially isolating the mice does induce sensory deprivation. It is within consideration that social isolation also affects the outcome of anxiety-like behavioural measurements and as reported by Grippo et al. (2009), that injection of oxytocin does reduce the negative behavioural consequences from social isolation. However, there was limited literature that discussed the relationship between the two and comparing between socially isolated mice and sensory deprived mice. Although it is possible that the negative outcome from the social isolation caused by sensory deprivation, the connections between the two needs to be investigated further. In addition, the social isolation within scantainer was done within 24 hours, which is considered acute and most sensory deprivation or social isolation studies did their

study within a chronic or long term duration. This was the limitation of the study done, however, will be interesting to be investigated further.

Finally, this study was done within male C57BL/6 mice because of the social isolation in relation to separating fighting males. The results might be entirely different if the mice used were females. It is known that the effect of social isolation is sexually dimorphic (Guo et al., 2004; Rodgers & Cole, 1993; Senst et al., 2016). However, further experiment with possibly different experimental designs will be necessary, considering that the aggression between females has different means to aggression between males.

Effect of chronic social isolation and OR37 ligands on aggressive behaviours

The main objective of this experiment was to investigate the potential of the OR37 ligands in ameliorating social isolation-induced changes, primarily in aggression. There has been no lack of studies done on mice using social isolation to induce aggression. The Resident-Intruder Test (RIT) has been the most commonly used test in checking territorial aggression. In this experiment, we added a wider range of context for aggression, which includes the non-territorial aggression using the Standard Opponent Test (SOT) and the non-social aggression using the Cotton Bud Biting Test (CBBT). Unexpectedly, there was a lack of aggression on all treatment groups on both the RIT and the SOT. It was not clear the reason why there was a lack of aggression, however, considering that almost no animals fought on the SOT (2 out of 44 animals, figure 4.10A), it suggested that the animals were not motivated to fight other animals in the neutral arena. Interestingly, during the Resident-Intruder Test, more animals were fighting (16 out of 44 animals, figure 4.11A), but there was no significant effect of social isolation. According to Koolhaas et al. (2013), important components during an RIT consisted of the attachment to the territory of the resident and the threat that intruders brought to the resident. The attachment to the territory should not be a problem since the mice were not cage cleaned for at least 10 days before the testing day and a study by Mertens et al. (2020) showed that the mice that were moved to a clean cage 24-48 hours prior to the RIT still showed aggression towards the intruders. With regard to the intruders, the subordinate males were not castrated, which should not be an issue for inducing aggression to the resident. It is well documented how castrated male did not induce aggression in other males which

is possibly due to changes in the urine composition, notably to be dependent on the MUP in male mice urine, and therefore did not send a threat signal to other males (Chamero et al., 2007; Luttge, 1972; Mugford & Nowell, 1970). However, this was not the case in this experiment since the intruders used in this experiment were the subordinate animals from another cage and controlled for body weight. Although one thing to be noted, according to Kaliste-Korhonen and Eskola (2000), there are aggressive and non-aggressive subordinates, which were not accounted for in this study. In this study, there were few occurrences where the resident got attacked by the intruder (3 cases of total 44 observation) which might suggest that the aggressiveness of the residents was lacking. A study by An et al. (2017) shows that after 4 weeks of social isolation, the expected latency to attack for KM strain mice during an RIT was around 200 seconds. Despite there potentially being strain differences, the fact that most animals hit the 600 seconds ceiling suggested that most animals could be considered as not aggressive. Although the study did not investigate social isolation, the study by Strekalova et al. (2003) showed that the C57BL/6 mice did show aggressive behaviour within the resident intruder test. However, the main difference from the study and this experiment is that the intruder used during the resident intruder test was a mouse from a different strain (CD1 mice). This could be explained that the mice within the same strain have less genetic variability (Casellas, 2011). Although there might be some degree of variation and possible different phenotype between animals having the similar genotype, the degree of similarity between the animals from the same strain might be perceived as siblings. Therefore, it is possible that the intruders were perceived as siblings and from an ethological point of view, as it is not energy efficient to attack one's own siblings. Otherwise, it is possible that the C57BL/6 strains were "accidentally" selected within the captive environment to exhibit less aggressive behaviour due to the nature of animal's breeder to maintain animals that fought less and breed these animals.

Another concern related to the subordinate animals was whether the animals were properly assigned to their hierarchy in the pair housing. The dominant tube test is a common test to determine dominance hierarchy. In addition to the tube test, the urine marking test was also performed in this experiment to ensure the hierarchy between the pair housed animals. As for the result itself, there does not seem to be differences between the dominant and the subordinates assigned from the tube test to their urine

marking quality and quantity in the urine marking test (figure 4.4). However, as the results from the tube test itself, not all of the mice that were assigned as dominant won all 3 times from the tube encounters, which might suggest the dominance level was different between dominant mice. In addition, the urine marking may have been affected by drinking, which might confound the amount of urine that can be excreted. However, the study by Wang et al. (2011) did mention how the urine marking test did not fully correlate to the dominant tube testing with some animals. One possible explanation was the group housing size. According to Varholick et al. (2019), the stability of the hierarchy with a group size of 3 was quite volatile. It raised the question how the hierarchy system works on the pair housed mice. Jirkof et al. (2020) reported that the pair housed CD1 mice had more lesions on the skin that were caused by aggression compared to the group housed with a size of 3, which suggests that aggression happened more often in the pair housed mice. Apart from that, the data that was collected from the experiment suggested that the hierarchy on the pair housed mice was unstable which might be an explanation for why the subordinate mice attacked the resident mice. Although this does not account for the socially isolated mice, it is still unclear and needs to be investigated further why the socially isolated mice did not show aggression in the SOT and RIT.

However, on the CBBT results, it was very clear that the socially isolated mice were more aggressive than the pair housed mice (figure 4.12). Unfortunately, despite showing a sign of reducing the aggression, the OR37 ligands did not significantly reduce the aggressive behaviours. Things to be considered about this CBBT itself is that even though the original method was used to measure the aggression of the mouse knockout "schizophrenic" model that has been established to be aggressive, it was not clear what kind of aggression did this test measure (Park et al., 2015). It is also interesting that the aggression in this test is directed towards a non-social stimulus, whereas aggression is normally directed towards another conspecific producing pheromones. It is possible that the social isolation increased the propensity of the aggressive behaviour, which drives the mice to be aggressive even towards the non-social stimulus. Study by Yang et al. (2017) showed that chemo-genetic stimulation of the progesterone positive neurons in ventromedial hypothalamus could drive out of context aggression that occurred without opponents and chemo sensing involved. Although the increase of the propensity of the aggressive behaviour was expected to also be shown in the social aggression test, further investigation is needed to validate

this method in order to get a better picture of what happened to these mice in this experiment.

Anogenital sniffing behaviour measured in the RIT (figure 4.11B) and the SOT (figure 4.10B) is part of the ethogram of a fighting bout (Koolhaas et al., 2013). However, due to the nature of the experiment being stopped when fighting occurred, it was quite difficult to interpret the value of the anogenital sniffing since every animal will have a different total amount of time. There was an option to use the ratio of time spent on sniffing per total time observed across all animals, however, this analysis method was not used. Using a ratio has the potential to make the value to be smaller or larger than the actual time spent, which might bias the analysis, therefore, only the animals that hit the ceiling of the latency to fight were used for another analysis of the anogenital sniffing. It is within consideration that performing the analysis using the animals that hit the ceiling instead of using the ratio will reduce the n number and potentially lower the power. However, this was done to avoid a false positive conclusion. Even when the data was filtered to be only showing the pairs that did not fight, there did not seem to be any differences between groups. According to Leser and Wagner (2015), although the study was done in rats, it was expected that social isolation would increase the social investigation time, but that was not the case in this study. Moreover, the anogenital sniffing data on the RIT, despite being non-significant, was moving in the other way than expected, with the socially isolated mice expected to have higher anogenital sniffing behaviour. On the other hand, the amount of rearing and digging of the pair housed animals were significantly higher than the singly housed mice in the SOT. Considering this is a neutral arena, one interpretation of the data was that the pair housed mice explored more than the singly housed mice. A study by Sturman, Germain, and Bohacek (2018) mentioned that rearing behaviour in the open field arena is context dependent, which in the study was interpreted as a parameter of anxiety-like behaviours. It was unclear why and what it means, however, it might suggest that something other than aggression might have happened to these mice due to an effect of social isolation.

Lastly, the ventromedial nuclei of the hypothalamus (VMHyp) did not show significant c-Fos changes from the social isolation which was consistent with the lack of aggression between groups during the RIT (figure 4.13). Interestingly, the PVN c-Fos of the pair housed mice were significantly higher than the individually housed

mice. Study by Steinman et al. (2016) showed that a social defeat paradigm increased the c-Fos activation in the PVN that colocalized with the oxytocinergic neurons in male mice. Considering that the RIT did not show any behavioural differences, it is possible that there were more factors involved in this experiment other than the possible arousal type of response. It is to be noted that this c-Fos data was obtained from the RIT and not the SOT which means the data was not consistent for the rearing and the digging behaviour. It is very difficult to interpret the relationship between the behaviours and the PVN c-Fos activation due to the possible neuronal type being activated in the PVN. In conclusion, it was still unclear why the PVN c-Fos on pair housed mice were significantly higher than the socially isolated mice, however, this data could not be interpreted due to the complexity of the PVN activation and the RIT, especially that there were no behavioural changes on the RIT.

Effect of chronic social isolation and OR37 ligands on anxiety-like behaviours

Another aspect that was explored in this experiment is whether the social isolation induced anxiety-like behaviours. Two tests were done to assess the anxiety-like behaviours which were the Zero Maze Test and the Novelty Suppressed Feeding Test. Both tests were assessing anxiety-like behaviours with different conflicting motivations, whereas the ZM was in relation to the motivation of exploration and the NSFT was in relation to the motivation of hunger. However, both tests did not show any significant behavioural differences in general resulting from social isolation. Despite that there was a significant difference between overall treatment groups on time spent in the open arm (figure 4.8B), there were no differences between the treatment groups of interest. The latency to eat on the NSFT data (figure 4.9B) suggested that the data was trending towards an increase of latency to eat due to social isolation, however, was not significant. Even though there might be a possibility that the effect was too small to be detected, in the end, this result did not align with the findings in the literature regarding the significant increase in the latency to eat in the socially isolated mice (Võikar et al., 2005). Although the study by Võikar et al. (2005) used DBA mice, it was expected for the C57BL/6 mice to respond better since according to by Bouwknecht and Paylor (2008), the C57BL/6 mice were commonly used on anxiety-like behavioural tests.

It was unclear why there was a lack of aggression and anxiety-like behaviours. It was expected for the socially isolated mice to have lower time spent in the centre of the open field and a higher latency to eat in the NSFT (Chourbaji et al., 2005; Võikar et al., 2005). It might be that the animals were following more of a social isolation-induced depressive-like paradigm in this experiment due to the frequent disturbance to top up the solvent/OR37 ligands, which was perceived as Chronic Variable Stress (CVS). However, this could not be verified due to no depressive-like behavioural measurements being taken. Interestingly, a study by Gu et al. (2019) did show that CVS paired with social isolation did induce depressive-like behaviours and the study done by Yoon et al. (2014) showed that Chronic Non-Social stress induced depressive-like behaviour via an increase of immobility time in a forced swimming task, but did not induce anxiety-like behaviours. This information raised the question whether our experimental design might be akin to putting the mice in an enriched and spacious cage rather than an environment that lacks social enrichment. One of the limitations from this study is that it was difficult to measure the amount of communications between cages within the same scantainer, which might suggest that the mice were not fully socially isolated. However, it was clear that the mice in the individually housed group were deprived of tactile stimulation from other animals, which might put the mice into some form of sensory deprivation. In addition, the solution addition every day might have caused the chronic non-social stress, hence putting them in a depressive-like condition. The relationship between sensory deprivation and chronic stress is unclear within the literature. However, none of these conjectures could be verified. In future experiments, the relations between the social isolation and sensory deprivation to aggression, anxiety, and depression needs to be clarified further.

Effect of chronic social isolation and OR37 ligands on physiological parameters and nesting behaviour

The final aspect from this experiment was the measurement of physiological stress response. The general stress response physiological measurements were observed including the body weight (figure 4.5) and the eye temperature (figure 4.6). An eye temperature recording can be considered as novel method in mice since it was not very common despite its being non-invasive compared to using an anal probe or a core body chip. Interestingly, measurements of eye temperature method have been commonly

used on cattle, birds, or even sea mammals (George et al., 2014; Jerem et al., 2015; Melero et al., 2015). George et al. (2014) found that both rectal and eye temperatures did correlate with a caveat on slightly different temperature value recording. Nest building quality data was also observed since nest building could be an influencing factor on thermoregulation. As for the nest building data itself, the first 3 hours observation within the first observation week seemed like that the singly housed animals with solvent reacted differently to the other 2 treatment groups to novel stimulus (figure 4.7A), however, this data was not significantly different.

The body weight progression data suggested no overall difference between groups and that mice are essentially healthy which suggests the animal welfare in general can be considered as good. Based on the study by Martin and Brown (2010), it was suggested that social isolation will increase mice body weight approximately 1.5 grams difference with the pair housed mice, which interestingly was not the case in this experiment. The study was done in 16 weeks, however, the data from this experiment did not show any sign that the socially isolated mice were gaining more weight than the group-housed mice despite having free access to the food. Interestingly, during week 4, the singly housed animals weight gain is significantly lower compared to the previous week but not associated with the eye temperature and changes in nest qualities. It was not clear what happened to the socially isolated mice on week 4, but it might be the tipping point where the social isolation might start to significantly affect the mice. It is interesting how the direction is totally opposite in regard to socially isolated mice gaining more body weight to pair housed mice, however, further investigation will be needed in order to draw conclusion from this.

After the Zero Maze Test on week 5, there was a significant drop of eye temperature in all groups but only the body weight of the Singly Housed – Solvent group that stayed constant to the previous week. This drop in body weight change could be explained by Jeong, Lee, and Kang (2013) that mentioned the chronic stress prevented ICR mice from gaining body weight compared to non-stressed mice. It is also interesting how the eye temperatures were dropping instead of increasing after the ZM week as one would expect to follow a stress-induced hyperthermia paradigm. Oka (2018) mentioned how stressed mice could have an increase of core body temperature up to 2°C. Oka (2018) also mentioned that a stress-induced hypothermia would happen instead as a long term adaptation to anticipatory to stress response. The nest quality

itself did not give more insight to this condition since it was not significantly different to the previous week. One interesting result from this after Zero Maze Test data point is the fact that the changes in body weight only occurred in the Singly Housed animals with solvent but not on animals that had chronic OR37 ligands exposure. There have not been any studies that investigate OR37 ligands' effect on body weight and it is still not clear what behavioural outcome of the OR37 pathway, however, this suggests that OR37 ligands might have effect either on metabolism or feeding behaviour which will need to be validated before jumping into the said conclusion.

Another interesting time point was the week 6 time point after the NSFT. The singly housed mice gained significant body weight compared to the previous week but not any other groups. There might be a possibility of a rebound, however it is interesting how the change only happened to the singly housed mice. Interestingly, all mice had a significant increase in eye temperature at the measurement point after the NSFT, which might suggest stress-induced hyperthermia or potential metabolic stress. However, it was quite difficult to interpret considering that a part of the NSFT did food restrict the mice for 24 hour which quite possibly caused metabolic stress. The metabolic stress was difficult to quantify since there was no data collection regarding food consumption and excretion, although it is possible that this parameter potentially connected to the reason for eye temperature increase. This could not be verified in this study, which could be another useful thing to be investigated in future experiments.

Lastly, in the final week of the observation, there were no differences in body weight changes or total body weight which were unexpected. Study by Martin & Brown (2010) showed that socially isolated mice have heavier body weight. It is interesting considering the pair housed animals supposedly have a lower level of thermal stress, and therefore a lower physiological stress, which consequently results in heavier body weight. However, despite no changes in body weight, there was a difference instead of the eye temperature observed on the week 7, which showed that the Singly Housed mice with solvent had their eye temperature significantly dropped compared to the Pair Housed animals. Interestingly, the Singly Housed mice with the OR37 ligands group were significantly different from both of the Pair Housed and the Singly Housed with solvent groups. This suggests that the social isolation that was done in this study induced hypothermia instead of hyperthermia. One of the possibilities of the Social Isolation paradigm that we did not consider was the possibility of social isolation-

induced depression which could explain the stress-induced hypothermia. Studies done Gu et al. (2019) did mention that the depression-like behaviours in mice did increase during social isolation when it was paired with CVS. It raises the question whether the solvent and the OR37 ligands being topped up every day could be considered as exposing the mice into a CVS paradigm. Unfortunately, this question could not be answered since no tests of depressive-like behaviour were conducted. Future experiments could potentially include these measurements and test whether there is an effect of OR37 ligands on depressive-like behaviours associated with social isolation. Nevertheless, this study shows that the OR37 ligands can be used to ameliorate the effects of the social isolation on body temperature even though still not on the same level as pair housing the mice.

Chapter Summary

In summary, this chapter provides an insight into application of the OR37 ligands as a chronic enrichment to mice within a social isolation context. Although it does not seem like the social isolation affected the mice behaviours both on anxiety-like and aggression as expected, there was an effect of social isolation on the eye temperature and the OR37 ligands did ameliorate the effects, despite not on the same level as pair-housing the mice. In addition, this chapter also provides additional insight on methods of exposing the OR37 ligands using a tea ball infuser and by smearing it on the arena, which is an applicable method of exposure for chronic application as an enrichment.

Chapter 5: Alternative methods (refinement) in laboratory animal welfare

5.1. Chapter Aim and Objectives

The aim of this chapter is to investigate potential refinements within animal welfare context without the use of the OR37 ligands. Specific cases and experimental objectives within this chapter includes:

- To investigate mice housing preference to hot or cold houses and the correlation to group size preference.
- To investigate the welfare benefits of a refined mouse handling method using thermal imaging and conditioned place preference.
- To investigate if a modified mouse restraint technique benefits welfare and its effect to stress responses.

5.2. Introduction

One of the components of 3R is the refinement concept and the previous chapter explores the use of the potential stress ameliorating effect of the OR37 ligands via PVN activation reduction. However, this chapter will address other potential refinement topics that are separate from the OR37 ligands application. Specifically, this chapter will investigate the refinement concept on mice housing temperature preference, mice handling method preference and temperature responses, and mice responses to different scruffing methods.

The housing temperature is an issue that has been quite problematic within mice husbandry procedure. According to the Code of Practice of the UK Home Office (2014), the recommended temperature for mice housing is between 20°C to 24° C. However, this temperature range is below the thermoneutral zone of the mice which is ≈30°C (McKie et al., 2019). Study by Gaskill et al. (2011) showed that mice spent less time in colder area (20°C) in comparison to warmer area (30°C), on the other hand the mice have better nest quality in the colder area to the warmer area. Despite having the option to build a nest to aid thermoregulation or being group housed to enable huddling behaviour, a study by Maher et al. (2015) showed that nest building and huddling only slightly ameliorate cold stress caused by being in 20°C conditions compared to being in thermoneutral zone on 30°C. Mice that were housed at the thermoneutral zone also showed better exercise performance and improved immune response (Jhaveri, Trammell, & Toth, 2007; McKie et al., 2019). There are more studies using female mice in studies involving temperature; however, the cold stress did happen to both sexes (Gaskill et al., 2011). These findings raised a question regarding “normal” mice behaviour when being housed in 20-24°C conditions which in fact is constantly exposing the mice to cold stress. This “normal” behaviour also raised questions regarding mice huddling and nesting behaviour. It is common knowledge that mice are social animals. However, being social animals is a separate issue with regards to the preference of living together when living together is necessary in promoting survival; in this case, huddling and nesting together for thermoregulation. Interestingly, there are vast amounts of studies regarding temperature preference in mice but there is a gap in literature regarding the change in temperature to their nesting preference. Hence, one aim of this chapter is to explore mice preference to different houses for nesting’s temperature and their preference to group nesting. Having better understanding in mice

preference towards nesting temperature and group nesting size preference will in turn provide more context for making decisions and designing the optimum housing for laboratory mice.

Another common husbandry procedure which has more recent refinement impact is on mouse handling and restraint procedures. Notably, the works of Hurst and West (2010) showed refinement in mice handling protocol proposing benefits could be achieved by replacing the tail-handling methods with either the hand-cupping method or using the tunnel enrichment. The tail-pickup as a common husbandry protocol poses a questionable welfare perspective in itself as quite a few studies (reviewed on Balcombe, Barnard, and Sandusky (2004) found that being picked up by the tail increases the parameters associated with mice stress responses such as an increase in body temperature, an increase in corticosterone levels, and a decrease in immune levels (Moynihan et al., 1994; Seggie & Brown, 1975; Zethof, Van Der Heyden, Tolboom, & Olivier, 1994). The study done by Deuis, Dvorakova, and Vetter (2017) mentioned about the tail and paws of the mice being sensitive to nociception. It is difficult to measure the nociception of the tail due to the variability of the handling method's protocol between handlers, however, according to Hurst and West (2010), the tail-pickup method may be more likely to induce a naturally selected behaviour of avoidance to predator passed down from the ancestor. This issue does pose a question regarding a change of behaviour due to the handling method; henceforth, the refinement of the handling protocol which avoids the tail entirely is in favour in an attempt to avoid inflicting stimulus on mice which might elicit the avoidance behaviour. More recently, there were more studies validating the different handling methods with tail-handling showing a reduction in lick cluster sizes in the mice sucrose preference tests as well as a reduction in time spent in the centre of the open field (Clarkson et al., 2018; Nakamura & Suzuki, 2018). There was also a study by Mertens et al. (2019) that showed handling the tail by using forceps increased body temperature compared to tunnel handling. However, there has not been a study that investigates the effect of the cup handling method with regards to change in body temperature and whether the mice have a preference for refined handling. This chapter will also investigate how body temperature that is being observed via eye temperature changes based on the handling methods. This study will also try to investigate the feasibility of using eye temperature as a non-invasive alternative method in measuring mice bodily

temperature. Furthermore, as a separate study, an investigation of mice preference to the handling methods will be assessed.

Finally, one other aspect of the mice husbandry protocol is physical restraint which commonly involves a scruff-based method; this is an important part of experimental procedure used for particular treatments. The scruffing protocol involves holding down animals by the base of the tail followed by the scruffing of the skin behind the head and along the dorsal surface of the mouse in order to safely deliver some treatments. In the Hurst and West (2010) study, it was mentioned that handling the mice by the tail after being picked up using the cupping method did not reverse the taming effect. it is another question itself how the orthodox scruffing method impacts the mice's "normal" behaviour. Although, from the handler's perspective, it is quite common for mice to struggle and bite the handler hand during the scruffing protocol, there is a lack of studies mentioning the detrimental effect of the scruffing itself. In this chapter, we will investigate an alternative way of scruffing that avoids holding the tail altogether and attempt to validate the method against the orthodox method to see whether the proposed method is less stressful from the mice's perspective and safer for the handler.

5.3 Methods

5.3.1. Animals and Housing

The mice used on the studies were either from CD1 strain or C57BL/6J. The mice were housed at a 12:12h conventional light-dark cycle (lights on at 08:00h) or a reverse light-dark cycle (lights off at 08:00h), in a temperature controlled room (23±1 ^{o}C). Animals were given standard enrichment of cardboard tube, bedding material, plastic houses, and wooden blocks. All mice were on *ad libitum* feeding regime and had free access to water. All experimental procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the local ethical review committee (University of Bristol). Details on animals' usage for every study in this chapter is specified on table 5.1.

Table 5.1: Experiments and animals' usage

Experiment	Cohort	Strains	Animal Number		Housing	Lighting
			Male	Female		
1 : Nesting study	1	CD1	24	0	Group (3)	Conventional
	2	CD1	0	16	Group (4)	Conventional
2 : Handling study	1	CD1	0	10	Group (4/4/2)	Reverse
	2	CD1	12	0	Singly	Reverse
3 : Scruffing study	1	CD1	8	8	Group (2)	Reverse
	2	CD1	62	0	Singly/Group	Conventional/Reverse
		C57BL/6J	15	17	Singly/Group	Conventional/Reverse

5.3.2. Experimental Designs

5.3.2.1. Experiment 1: Do mice prefer warmer house temperature and does the temperature affect their decisions on group nesting?

The nesting study was done to see whether the male and female mice have preferences towards warmer nesting sites and whether this preference affects their group nesting size. It is a question itself regarding the optimum group nesting size and which combination of the group nesting size from the available number of animals within a group to focus on. As the question is the relationship between the nest temperature and their group nesting size, there was an option to choose whether to go with no group nesting at all (all mice live separately) or total group nesting (all mice live together). The option of two male mice living together and one living on its own was avoided due to the possibility that this might be influenced by the dominance hierarchy. Following this, the group nesting size of three (all mice nest together) was used with the assumption that if the nesting in the group was done for preservation, the drive for survival should precede the drive for dominance. Although it is acknowledged that females have different drives in a social dominance hierarchy between females, the "all mice nest together" perspective was applied in line with the male nesting group size.

The study was conducted using a modified housing arena as depicted on figure 5.1. The arena wall was made from metal shaped in a circle with 80cm diameter and 50cm height. Drinking bottles were taped to opposite sides of the circle. A ceramic bowl was placed in the centre of the arena and normal food pellets were placed *ad libitum*. The

flooring of the arena was made of Perspex and covered with sawdust. Heating pads (commercial reptile heating pad, 28 x 15 x 0.5 cm) were placed under the Perspex of the designated hot houses. Houses were made from opaque red polycarbonate material (10 x 5 x 5cm with holes on each width side and on the top) and the number of houses was adjusted such that there were twice as many houses as mice to allow the options of cold and hot houses for nesting for each mouse. 6 wooden blocks were provided for male mice. Mice were also given plenty of nesting materials (paper shavings) which covered the area between the houses and the food bowl. A thermal camera (FLIR C2) with maximum temperature locked at 31°C was installed above the arena to monitor the housing temperature for the first cohort. A thermometer was placed on the hot house to monitor the house temperature for the second cohort. The study was done every 2 weeks (10 days of observation, 19 observational time points due to the animals being placed in the arena during day 1 morning) for the first cohort and every 1 week for the second cohort. The second cohort study was done by an independent researcher and laboratory technician (Julia Bartlett). Observations were performed in the morning (09.00-10.00, AM) and in the afternoon (17.00-18.00, PM). During the manual observation, the location of each mouse was noted down as the house number and the mice were observed by peeking through the holes with minimal disturbance. The average temperature of the house box was also noted down during the observation. The preference to the hot house box was calculated by dividing the number of animals in the hot house by the total animals in a cage during an observation period and multiplied by 100%. For example, there were 2 animals in cage 1 residing in a hot house during the observation period will result in a 66% preference for cage 1 on one observation time point. On the other hand, the group nesting with a size of 3 preference was calculated with the number of cages that have 3 animals nesting together during the observation time divided by total number of cages and multiplied by 100%. For example, during morning observation on day 2, only 1 cage was observed nesting in a size of 3 from 12 observed cages. This will result in 8.3% preference in day 2 morning observation.

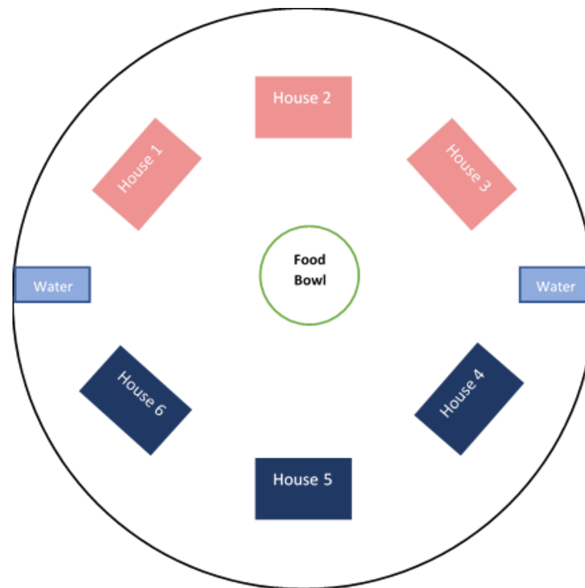

Figure 5.1: Representative diagram of the modified housing arena for 3 mice. Red boxes (house 1-3): warm houses, Dark blue boxes (house 4-6): cold houses.

5.3.2.2. Experiment 2: Do various handling methods induce hyperthermia stress responses and do mice prefer one handling method to the others?

This study was done to explore the impacts of refined handling methods on stress induced hyperthermia and mice preference towards the refined handling method. The female mice cohort was used for the handling methods and the stress induced hyperthermia study. Following Hurst and West (2010) with slight modification, the three handling methods used were: tail-handling (mouse was picked up by the base of their tail using the thumb and index finger, hind legs were suspended in the air with the front legs supported on the arm for 30 seconds), cup handling or cupping (mouse was picked up with two hands, one hand held in a cupping position and the other hand used to guide the mouse into the cup, and suspended in the air for 30 seconds with the guiding hand used to loosely cover the mouse), and tube handling (mouse was picked up using their cardboard tube and suspended in the air for 30 seconds). The first cohort in the handling study was used to see how the mice eye temperature was affected by different handling methods. The study was done over 3 experimental days with every mouse experiencing each handling method once. The handling order was counterbalanced and done during the dark phase in a separate testing room to the animal holding room. In the testing room, the mice were handled in accordance with

their assigned handling treatment and then moved into a clean cage filled with sawdust with a thermal camera positioned 30cm above the cage. The sequence of images was captured every 5 minutes for 30 minutes, after which the mice were returned to their cage and then transported back to their holding room. A fresh cage was used for the next group of mice. The data was then analysed following the protocol described in Chapter 2, section 2.9. Eye Thermal Recording.

The male mice cohort was used for the conditioned place preference study. The mice were trained and tested within a 2 week period following the protocol described below. The video recordings from the conditioned place preference test were blinded for the offline behavioural analysis.

5.3.2.3. Experiment 3: Does the modified scruffing method have a better taming effect and does it induce a reduced stress response relative to the common scruffing methods?

This study was done to explore if there are differences in the effects of a modified restraint method compared to common tail handling approaches, and whether there were differences in the animals' stress responses. The common tail handling approach being the method where the mice were held by their tail on a cage lid and scruffed from the base of their tail to their neck, and the modified restraint method being the method where the scruffing was done by putting the mice on the lab gown's sleeve and the mice were covered by the other hand without pressing down too hard. After being covered, the thumb and the index finger were loosened slightly to allow a gap such that the mice were able to poke their heads out slightly. Once the head poked out, the hand covering the mouse would grip the loose back skin into a scruffing position with a firm grip, ensuring not to exert too much force. The first cohort for the scruffing study was used for the overt behaviour observation, voluntary interaction test, elevated zero maze, and corticosterone radioimmunoassay. All mice were handled using the cupping method except during the pickup for the scruffing and were habituated to the cupping method from 2 weeks prior to the study. The hand approach test was done over a 2 week period (only on day 1, day 5 and day 10) with one cage pair undergoing the same scruffing method treatment and the allocation for scruffing method was randomised between cages and between mice within the same cage. The mice were scruffed for 10 days during the observational weeks (only during the weekdays). On

week 3, the mice were tested in the elevated zero maze after being scruffed using the same scruffing method as the hand approach test. Following the elevated zero maze, the mice were sacrificed after 20 minutes using the schedule 1 method (cervical dislocation) and then the blood was collected for corticosterone radioimmunoassay analysis. All experimental procedures were done during the dark phase. The hand approach test data collection and the scruffing on the first cohort was done by an independent researcher and laboratory technician (Julia Bartlett). The second cohort was used for the cotton bud biting test and consisted of various mice with CD1 and C57BL/6J strains which were being used in another study. Factors considered within the mice were age, sex, and strains. The handling background for the mice from the second cohort was unclear, however, all mice were handled using their home cage's tube for the scruffing study prior to their respective randomly allocated scruffing method.

5.3.3. Conditioned place preference (CPP)

The conditioned place preference test was done using a rectangular clear Perspex arena (75cm x 30cm x 20cm) that was divided into 2 testing chambers (30cm x 30cm x 20cm) and a middle chamber (13cm x 30cm x 20cm) by a clear Perspex divider with a gap (5cm x 5 cm). The gap was intended for the mice to travel between chambers but also had a slot for a clear Perspex trapdoor to prevent the mice travelling to the other chamber. The arena was placed on a table (1m high) and facing the entrance to the testing room ensuring the left and right hand side chambers were at a similar distance to the door.

The conditioned place preference test was divided into pairing sessions and test day. During the pairing session, the mice were paired between substrate (chopped timothy hay/wood chips), treatment (cup handling/tail handling), and chamber side (left/right). The pairing sessions were counterbalanced for all substrates/treatments/chambers for the 8 days pairing sessions. A day of pairing session was done by placing the mouse in the middle chamber with the trapdoor to the day's unused chamber closed. Once the mouse moved to the pairing chamber containing the pairing substrate, the trapdoor to the middle chamber was closed and the mouse was picked up with their respective handling method to the substrate pairing. The mouse was picked up (approximately

10cm from the substrate) for 10 seconds and then released and left for 90 seconds in the chamber. The picking up was repeated 5 times and the mouse was returned back to its home cage. The arena was then rinsed and dried before adding the next substrate for the next mouse. Every mouse had 4 days of pairing session with substrate 1/treatment 1 and 4 days of pairing with substrate 2/treatment 2.

During the test day, one chamber's floor was covered with one substrate of the two substrates while the opposite chamber's floor was covered by the other, the two substrates being chopped timothy hay or wood chips. The substrates' positions were counterbalanced for all animals for an unbiased conditioned place preference design. The animals were then placed in the middle chamber of the arena with both trap doors opened and animals had free access to explore both sides of the chambers for 30 minutes. Animals were recorded and left undisturbed during the testing period and the observer was standing by just outside the door during testing.

5.3.4. Thermal imaging

The recording and data processing protocols of the thermal imaging followed the protocol described in Chapter 2, section 2.9. Eye Thermal Recording.

5.3.5. Modified scruffing methods and overt behaviour observations

In principle, the proposed refinement was done by avoiding the touching and/or pulling the tail of the mice while trying to scruff the back of the mice in order to get a firm grip on the loose back skin. During the scruffing, manual observations for overt behaviours were recorded with the following parameters: vocalisation made during the grip (yes/no), urination during and/or after the grip (yes/no), struggle to be released from the grip (yes/no), and an escape behaviour indicated by running or avoiding the hand of the handler when released in the cage (yes/no). The data presented was an average of 10 days of observation, given the mice were scruffed 10 times and each observation resulted in a value of 1 or 0 which then averaged for each mouse to give one data point. Manual observations were also performed on the mice scruffed with the common tail scruffing method.

5.3.6. Voluntary Interaction Test

The voluntary interaction test was performed following the protocol described by Hurst and West (2010). As mentioned above, the voluntary interaction test was performed on day 1, day 5, and day 10 on the 2 weeks study. Prior to the test, all enrichments (cardboard tube, plastic house, and chewing blocks) were taken out from the cage and the cage lid was removed. For 60 seconds, the observer would stand in front of the cage and subsequently place a gloved hand into the front-half of the cage for 60 seconds. Both mice were then picked up using the cupping method, scruffed following their designated scruffing treatments, and returned to their cage. The experimenter would then stand back from the cage for 60 seconds. This was followed by standing in front of the cage for 60 seconds and putting the gloved hand into the cage for 60 seconds. All steps during the test were recorded using a webcam and analysed offline using The Observer XT software. Analysed mice behaviours were time spent in the front half of the cage during the 60 seconds when the experimenter stood at the front of the cage and time spent interacting with the gloved hand (sniffing, climbing, biting, and putting limbs on the glove) during the successive 60 seconds. All videos were blinded for analysis.

5.3.7. Elevated zero maze test

The elevated zero maze was performed following the protocol described in Chapter 2, Section 2.4. Elevated Zero Maze Test.

5.3.8. Corticosterone radioimmunoassay

The blood collection protocol and the radioimmunoassay protocol were done following the protocol described in Chapter 2, Section 2.8. Radioimmunoassay.

5.3.9. Cotton bud biting test

The cotton bud biting test was performed following the protocol described in Chapter 2, Section 2.10. Cotton Bud Biting Test.

5.3.10. Data Analysis

All data was analysed following the procedure described in Chapter 2, Section 2.11. Data Analysis and Statistics.

In the nesting study experiments, the preference data was analysed using a linear regression to test whether the trendline was significantly different from 0. For correlation data, a Pearson correlation analysis was done, and the coefficient was tested against the value of 0. The correlation was performed between the average of the hot house temperature preference and the value of group nesting preference. The preference between male and female during the same observation time were analysed using a Mann-Whitney non parametric test.

In the handling experiments, the eye temperature data was analysed using a repeated measure ANOVA with TIME as a within-subject factor and GROUP as a between-subject factor. For time series data, a repeated measure ANOVA was performed on the data. The preference data from the conditioned place preference was analysed using a one-sample t-test against 0 for column analysis and a paired t-test for habituation vs testing day preference analysis.

In the scruffing experiments, the overt behaviour data was analysed using a Mann-Whitney U test. The voluntary interaction test data was analysed using a Repeated Measures ANOVA with WEEKS as within-subject factor, and GROUPS and SEX as between-subject factors. The data from the time spent in the zero maze's open arm was analysed using an independent t-test. The plasma CORT's data was analysed using a Mann-Whitney U test. The cotton bud biting test's data was analysed using a Kruskal-Wallis test followed by a pairwise comparison analysis using a Bonferroni correction.

5.4. Results

5.4.1. Experiment 1: Do mice prefer warmer house temperature and does the temperature affect their decisions on group nesting?

5.4.1.1. Nesting study on male mice (Cohort 1)

The male mice nesting study results show that the male mice did have a significant negative trendline of preference to hot houses for nesting over the time (figure 5.2A). On the other hand, the male mice show a significant positive trendline of preference to group nesting size of 3 over the 10 days of observation (figure 5.2B). A correlation analysis (Pearson correlation) shows a significant negative correlation (p=0.0078) between preference to hot houses and group nesting (figure 5.2C). The CORT data was not shown as a figure, however, have the mean at 18.10, SEM at 2.62.

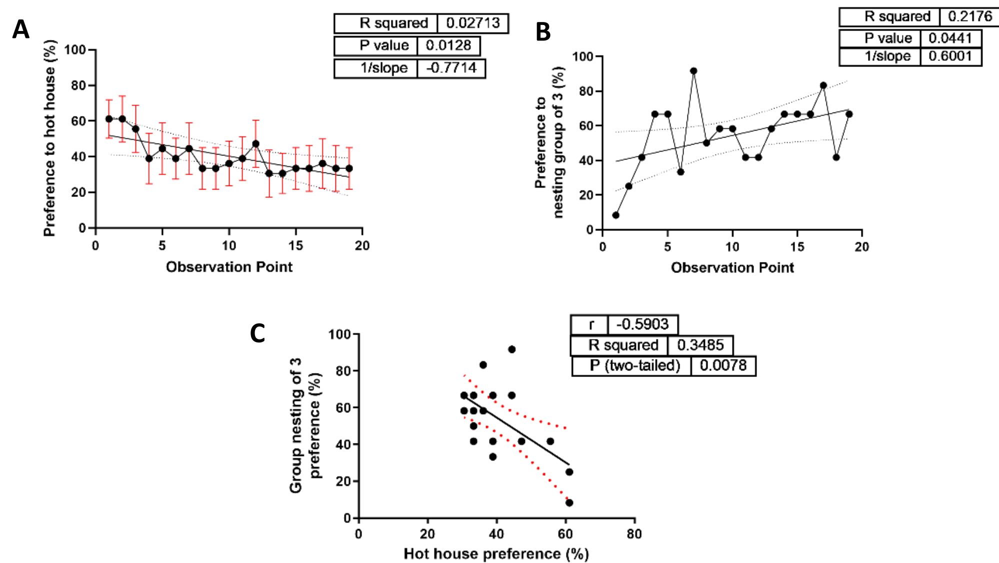

Figure 5.2: (A) male mice preference to hot houses trendline over 10 days of observation; (B) male mice preference to group nesting with a size of 3 trendline over 10 days of observation; (C) correlation between hot housing preference and group nesting with a size of 3 preference over the 10 days observation. 10 Days of observation were divided into 2 observation points for each day except on day 1 with only 1 observation point (total 19 observation points). Data shown as mean ± SEM

4.4.1.2. Nesting study on female mice (Cohort 2)

The female mice nesting study results show that the female mice did not have a significant trendline over 5 days of observation period to hot house (figure 5.3A). Similarly, female mice also did not have a significant trendline towards group nesting size of 4 over time (figure 5.3B). There was also no correlation between the average housing box temperature preference and the group nesting with a size of 4 preference (figure 5.3C). In addition, there was no difference between male and female mice preference to hot house on day 1's afternoon observation (Mann-Whitney U= 22.5, p= 0.981, figure 5.3D). However, there was a significant difference between male and female mice preference to hot house on day 5's afternoon observation (Mann-Whitney U= 5. p= 0.017).

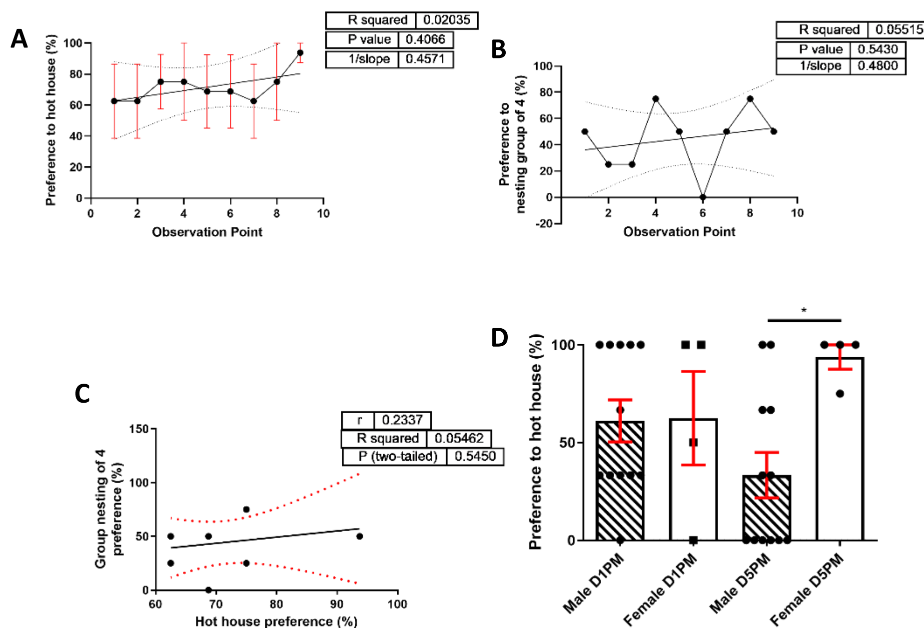

Figure 5.3: Female mice preference to hot houses (A), preference to group nesting with size of 4 (B), correlation between hot houses preference and group nesting of 4 preference (C), and (D) comparison between male and female preference to hot house on day 1 afternoon observation and day 5 afternoon observation. 5 Days of observation were divided into 2 observation points for each day except on day 1 with only 1 observation point (total 9 observation points), *p<0.05, Data shown as mean ± SEM

5.4.2. Experiment 2: Do different handling methods induce hyperthermia stress responses and do mice prefer one handling method to the others?

5.4.2.1. Thermal imaging on mice handling study (Cohort 1)

There were no significant differences of the eye temperature (figure 5.4) between the time points after the treatment (repeated measure ANOVA $F_{(2,52)}=2.008$, $p=0.145$) or between groups (repeated measure ANOVA $F_{(2,26)}=1.170$, $p=0.326$). There was no interaction between time and group (repeated measure ANOVA $F_{(4,52)}=0.606$, $p=0.660$).

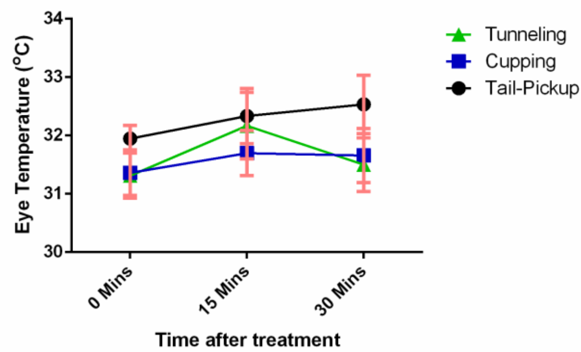

Figure 5.4: Mice eye temperature over different time points after various handling methods. Data shown as mean ± SEM.

5.4.2.2. Conditioned place preference to cupping and tail-handling methods (Cohort 2)

The conditioned place preference test showed a significant change in preference to the right hand side chamber from the habituation phase to the testing phase ($t_{(11)}$=3.048, p=0.011, figure 5.5). However, during the testing phase, there were no significant preferences to either substrates (one sample t-test $t_{(11)}$=0.044, p=0.965), chamber sides (one sample t-test $t_{(11)}$=1.020, p=0.329), or handling methods (one sample t-test $t_{(11)}$=1.774, p=0.103).

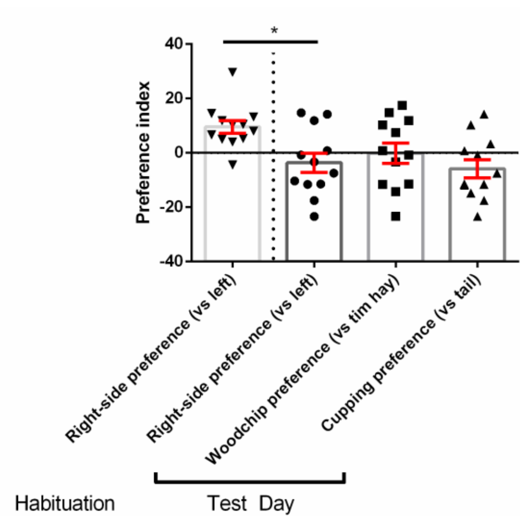

Figure 5.5: Mice preferences to handling methods (tail-handling vs cupping) on conditioned place preference. Data shown as mean ± SEM.

5.4.3. Experiment 3: Does the modified scruffing method increase voluntary interaction time and does it induce a greater reduced stress response relative to the common scruffing methods?

5.4.3.2. Overt behaviour observation from mice scruffing study (Cohort 1)

During the overt behaviour observation, the arm-scruffed (modified scruffing method) mice showed significantly lower levels of struggle (Mann-Whitney U=2.500, p=0.0008, figure 5.6A), occurrences of urination (Mann-Whitney U=4.500, p=0.0028, figure 5.6B), occurrences of vocalisation (Mann-Whitney U=1.500, p=0.0006, figure 5.6C), and aversion on release (Mann-Whitney U=0.0, p=0.0002, figure 5.6D) to the tail-scruffed (common scruffing method) mice.

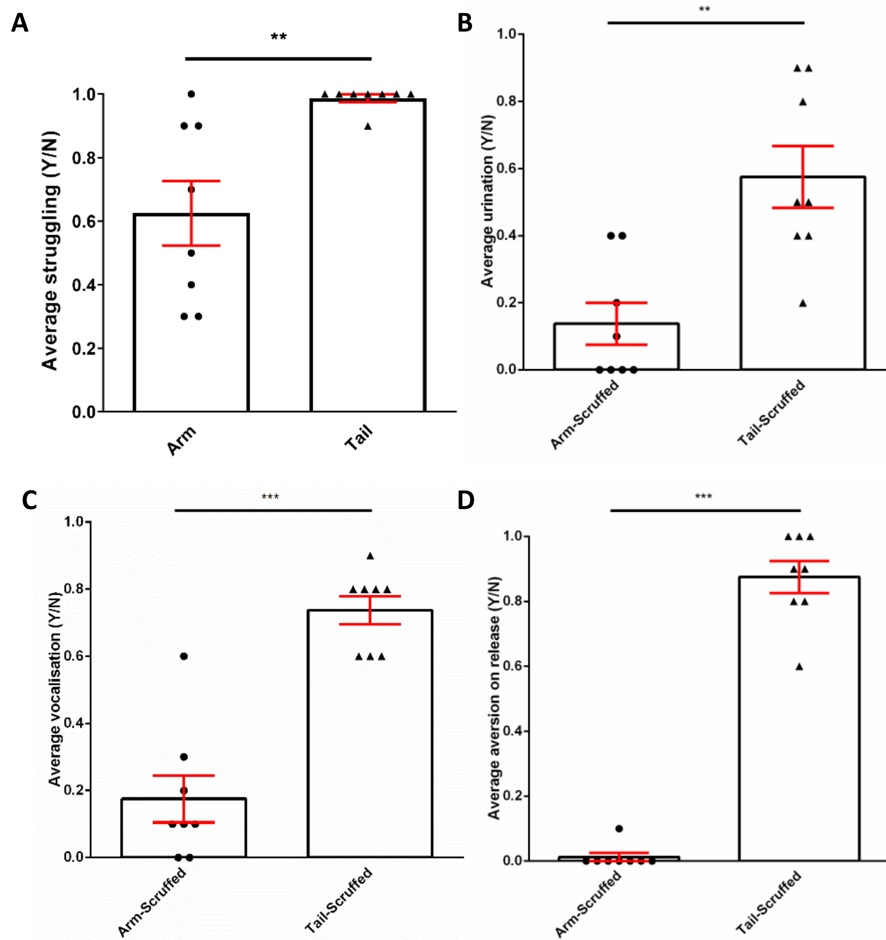

Figure 5.6: Average score of overt behaviour observations during the different scruffing methods over 10 days of observations. (A) average struggling, (B) average occurrence of urination, (C) average occurrence of vocalisation, (D) average aversion on release. Data shown as mean ± SEM

5.4.3.3. Voluntary interaction test on mice scruffing study

Mice voluntary interaction (figure 5.7 top) during the hand approach test showed a significant effect of day (RM ANOVA $F_{(2,24)}$=8.609, p=0.002) without significant differences between arm-scruffing or tail-scruffing methods (RM ANOVA $F_{(1,12)}$=1.593, p=0.231) and between male and female (RM ANOVA $F_{(1,12)}$=3.523, p=0.085). There were no interactions between day and different scruffing methods (RM ANOVA $F_{(2,24)}$=1.476, p=0.249) and between day and sex (RM ANOVA $F_{(2,24)}$=2.887, p=0.075) on the voluntary interaction behaviour. On the other hand, the approach on the observer-side of the cage (figure 5.7 bottom) showed no significant effect of day (RM ANOVA $F_{(2,24)}$=2.483, p=0.105) and sex (RM ANOVA $F_{(1,12)}$=2.618, p=0.132) but with the tail-scruffed mice significantly approached the observer-side of the cage than the arm-scruffed mice (RM ANOVA $F_{(1,12)}$=6.170, p=0.029). There were no interactions between day and different scruffing methods (RM ANOVA $F_{(2,24)}$=1.333, p=0.282) and between day and sex (RM ANOVA $F_{(2,24)}$=2.615, p=0.094) on the approach behaviour.

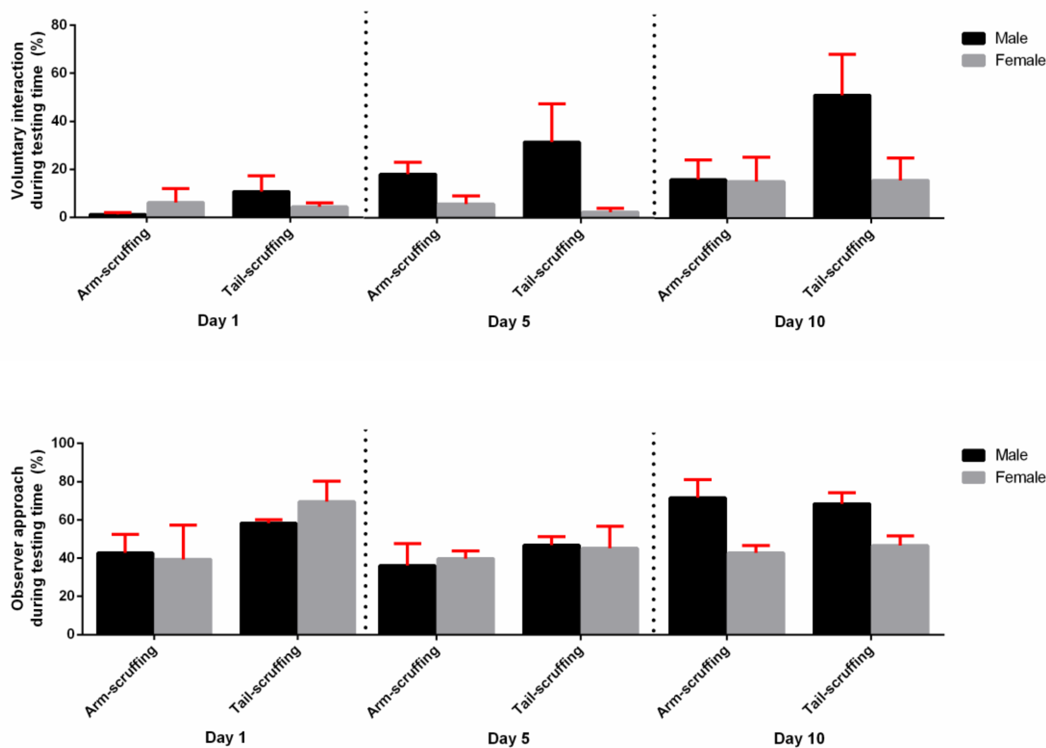

Figure 5.7: Mice voluntary interaction (top figure) and observer-side of the cage approach (bottom figure) after different scruffing methods on day 1, day 5, and day 10. Data shown as mean ± SEM

5.4.3.4. Elevated zero maze test behaviour and plasma CORT on mice scruffing study

Different scruffing methods did not affect the time spent in the open arm of the zero maze ($t_{(14)}$=-0.386, p=0.705, figure 5.8 left) nor the plasma CORT level after the zero maze (Mann-Whitney U=29.00, p=0.776, figure 5.8 right).

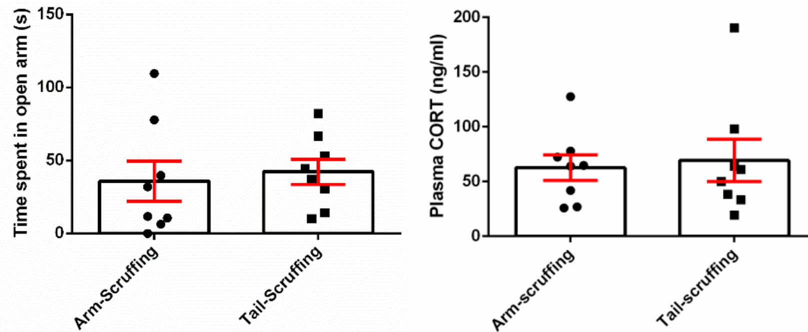

Figure 5.8: Time spent in the open arm of the zero maze (left figure) and plasma CORT level (right figure) to different scruffing methods. Data shown as mean ± SEM

5.4.3.1. Cotton bud biting test on mice scruffing study (Cohort 2)

There were no significant differences in biting frequencies (figure 5.9) following the various scruffing methods whether on C57BL/6 mice strain (Kruskal-Wallis pairwise comparison X^2=-1.000, p=1.000) or CD1 mice strain (Kruskal-Wallis pairwise comparison X^2=-4.028, p=1.000). However, there was a difference between strains on both arm-scruffing (Kruskal-Wallis pairwise comparison X^2=-25.793, p=0.005) and tail-scruffing (Kruskal-Wallis pairwise comparison X^2=-22.764, p=0.013).

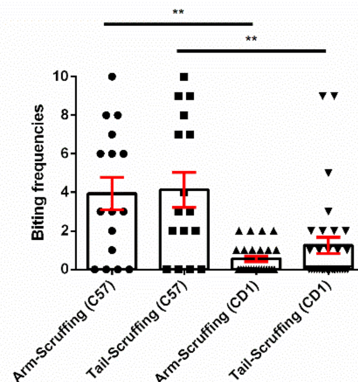

Figure 5.9: Mice biting frequencies on cotton bud biting test from different scruffing methods. Data shown as mean ± SEM.

5.5. Discussion

This chapter explored different refinement aspects on routine husbandry protocols. The main findings from the studies in this chapter showed that: (1) for the conditions tested, male mice have smaller preference to hot house for nesting compared to female mice on day 5 of observation, and (2) modified handling and/or scruffing refinements show overt behaviour differences, but not when looking at more specific differences in effect and physiological stress responses.

Male and female mice nesting preference

The nesting study was done to investigate the mice nesting temperature preference and whether this preference affects their decisions relating to group nesting. This study was done in two separate cohorts of male and female mice. The male mice cohort showed a negative trendline for preference to the hot house boxes (figure 5.2A), whilst the female cohort showed a constant preference trendline towards the hot house boxes (figure 5.3A). On the other hand, there was a positive trendline to preferences towards group nesting (group size of 3) in male mice (figure 5.2B) but not for female mice (group size of 4, figure 5.3B). The difference in the nesting temperature preference trendline might be due to differences in metabolism, which causes a difference in heat generated by different sexes and hence their chosen nesting option (Touma et al., 2003). A study by Bridges et al. (2012) also shows that the female mice have a higher tolerance to chronic heat exposure than male mice. Perhaps the males would show different behaviour if they were given housing boxes at a slightly lower temperature compared to the females; this hypothesis could be validated by further investigation through comprehensive testing over a range of temperatures and analysing their nesting preferences when they were given the various options. It would also be better if the study for both sexes was done in parallel to minimise variability.

Further analysis on the male mice cohort's preference over the observation period showed a significant negative trendline, which suggests that the preference towards the hot house boxes decreases over time. This significant negative trendline also shows a negative correlation in group nesting with a size of 3 preference. The negative correlation suggests that the group nesting preference of male mice is simply due to the huddling behaviour used to achieve a thermoneutral zone. Plenty of studies mention that the mice thermoneutral zone is ≈30ºC (McKie et al., 2019), which might explain the initial higher preference value for the hot house boxes on the first and

second day. However, as the time went by, the heat from the heating pad accumulated under the hot house boxes and the hot house boxes temperature presumed to exceed 30°C, which might have become too hot for the male mice. Unfortunately, due to the limitation of equipment, it was not clear what the temperature was over time to accompany the preference data. Despite the limitation, this simple preference value of a cold or hot house box itself provided an insight into how male mice actually have initial preference to be in the hot house boxes, but not too hot, which raised another question to the knowledge of a housing room's ambient temperature. Although the preference to nest temperature per se might not be beneficial to animal welfare, knowing more details in precise preference to temperature could possibly show the optimum housing setup for mice cage. It is a common practice in husbandry to have a nesting box as an enrichment and some nesting materials. However, knowing the optimum temperature preference will help in deciding to achieve the "ideal" cage in regard to providing the said nesting temperature for the mice either by adding a heating unit under their nesting box or having different nesting materials. Knowing that this preference to nesting temperature also correlates with their group nesting size might also suggest that it might be a good idea to put the male mice in slightly colder temperatures to avoid fighting between males. There is definitely a serious consideration to be weighed between exposing them to constant cold stress and having the male mice fighting. However, at the end of the day, fighting males are known to be quite detrimental to their welfare status and in the worst case scenario could result in accidental death, which in turn needs to be avoided. Although, more work needs to be done before reaching a solid conclusion. Future experiments could potentially include the parameter of dominance status to see whether this drives to huddle in order to promote survival precedes dominance status.

Another limitation to testing male mice group nesting preference and the negative correlation with the group nesting size is the difficulty to analyse the group nesting size. There might be a different answer if the analysis was performed on a group of 2 instead of 3 or any other different combinations of group nesting sizes, however, we decided not to dig too deeply into these details as the research question was whether the nesting temperature affects mice decisions to live together. The R^2 value from the linear regression is considered to be low (under 0.8), which suggests that there was an unexplained factor that could influence the correlation. One possible factor which

might influence the group nesting size is the dominance level between mice. There was an opportunity to perform dominance testing (such as using the tube test) at the beginning or the end of each group test, however, the problem with the dominance hierarchy itself is that it is not stable and would add another layer of complexity to this simple preference study. Although, the CORT level data (figure not shown, mean at 18.10, SEM at 2.62) shows relatively similar CORT levels. Larrieu et al. (2017) mentioned that for dominant mice, their baseline plasma CORT level will be higher compared to their subordinates. It was expected that within the group size of 3, there will be a dominant mouse which will have a higher CORT level compared to the other 2. Despite there was no dominance testing being done on this experiment, the CORT data might suggest that the dominance hierarchy might not have been properly established within these mice. This dominance hierarchy is also important to consider when analysing the group nesting behaviour between male mice, considering that the hierarchy might be an influencing factor in group nesting preference.

The main difference in the female mice cohort was instead of using the thermal camera, a thermometer was used to record the temperature. The temperature of the house boxes was maintained at an average of 30°C. Due to similar limitations to the group nesting size analysis as the male mice, there were no conclusive results for their nesting preferences. Kappel et al. (2017) mentioned how female mice live more harmoniously in group housing situations than male mice, but the "optimum" group size and the group nesting size preference is unclear for female mice. The hot house boxes preference result validates the female mice preference towards a temperature akin to the thermoneutral zone. This result then poses the question whether the group nesting behaviour at ambient temperature is considered to be "normal" behaviour due to the mice needing to be nesting in group in order to promote survivability. It is also to be noted that the nesting material of paper shaving was available during the housing study and was abundant (based on the plenty of nesting materials untouched by the end of the study), but the mice still prefer the hot house boxes rather than cold house boxes with an option to build a warm nest. Looking at it from a survival point of view, this does make sense since building a nest requires the mice to expend more energy, rather than staying in the available warm house boxes. Hence, it would be interesting for a future study to look at the actual group nesting preference, housing temperature, and quantitative nesting material availability.

Mice handling and scruffing refinements

The main finding from the modified handling studies is the difference in overt behaviour measurements between the modified restraint method by avoiding the tail and the common restraint method involving the tail. Despite the differences on the overt behaviours during and after the handling, further studies need to be done to draw a definite conclusion on whether the modified restraint method for scruffing is better than the common restraint method. The overt behaviour data can be considered as a stress response, or a sign of a negative response to the handler (Bailey & Crawley, 2009; Mutungi et al., 2019; Whitney, 1969). The results on the tail-scruffed mice resemble an active attempt to avoid the stimulus (more struggle, more vocalisation, and aversion on release), while the arm-scruffed mice's behaviour suggests more passive responses.

According to Hurst & West (2010) and Gouveia & Hurst (2017), the tail handling method was found to affect their willingness to interact with handlers and increase their anxiety-like behavioural responses in elevated plus maze tests. Although it was suggested that this mechanism was due to the evolutionary adaptation of mice to avoid the predator that picks them by their tail, it was still unclear within the literature and there has not been a definite study elaborating the mechanism of why tail handling is considered to be aversive by the mice. Although, an alternative perspective could be viewed from the known function of the tail itself. A study by Siegel (1970) showed how a mouse without a tail fell more on the rod walking test compared to the control group, which suggests that the tail has a function in relation to balance. In addition, a study by Silverman and Hendricks (2014) showed the presence of presumed nociceptive neurons within the mouse tail since post-partum day 3. It is possible that the reason why tail handling is aversive to the mice was simply because it stimulated the nociceptive neurons and also affected their balance, especially when they were lifted from the ground. However, these ideas need to be further validated.

In addition, the arm-scruffed mice showed less active overt behaviour, which might suggest that the animals were coping passively to the brief restraint method. Although passive coping paradigms were commonly studied using behavioural tests related to a depressive-like state, the review by de Kloet and Molendijk (2016) suggested that the

passive coping behaviour does not reflect depression. On the contrary, a passive coping mechanism could potentially be beneficial since this behaviour conserves energy, which is normally mobilised during active coping mechanisms (de Kloet & Molendijk, 2016). However, it is important to do further investigation in relation to animal's affect to conclude whether this modified method is detrimental to an animal's affective parameter. In general, scruffing the animal for administering the treatment is most likely to be stressful. It is a welfare perspective question regarding which method is more humane, which is the main objective of the refinement.

The attempt to address the affective parameters was done using the voluntary interaction test as described by Hurst and West (2010). Instead of the handling methods, the treatment was changed into scruffing to see whether the test can also be used to check the taming effect on the scruffing methods. The result showed a significant increase in voluntary interaction over time, which suggests a taming effect has been successfully found in this study. However, there was no treatment group difference and no interaction between treatment group and time which suggests the treatment itself did not affect the taming effect. This result also suggests that the taming itself was due to the repeated testing over the 10 days period and not the scruffing itself. It is interesting how the statistical results showed that the mice with tail-scruffing spent more time in the front half of the cage during the observation, which suggests the tail-scruffing is less aversive to the arm-scruffing. Although, it is to be noted that the mice were handled using the cupping method. This becomes important as the study by Gouveia & Hurst (2017) showed that the mice handled using the cupping method have more variable responses to stimulus. Despite the test with the cupping was not performed in the voluntary interaction test, it is possible that there was a carryover effect which affected the results of the voluntary interaction test. However, it is too soon to draw the aversive conclusion since to show whether a treatment is aversive or preferred, it is necessary to pair the treatment as a conditioned stimulus with an unconditioned stimulus.

To address the aversion of tail-handling as a conditioned stimulus, a conditioned place preference test was done between the tail-handling and refined handling (cupping method). There was no conditioned aversion to the tail-handling method. It is to be noted that the mice were handled daily using the cupping method. According to Wilde et al. (2017), the various handling methods were potentially preceding the treatment,

which in this experiment, the mice were accidentally put in various handling methods paradigm. It is possible that the absence of the aversion could be attributed to the alternating handling methods within the same subject situation, which in future experiments, more caution should be taken as not to mix handling methods. The unbiased paradigm was used for the conditioned place preference to avoid a false positive result, hence, the preference during the habituation phase was ignored and the animals were fully counterbalanced for the treatment method. However, according to Rosecrans, John, and John (2009), assignment of compartments without assessing the baseline preferences might result in no CPP at all. It was unclear whether the lack of result was due to the absence of preference/avoidance to the treatments, or there were not enough pairing sessions done to condition the mice. It is recommended for future study to do longer pairing sessions and possibly incorporating a positive control.

An anxiogenic behavioural test using the zero maze was done to measure the anxiety-like behaviours between the modified restraint and the tail-restraint. There was no significant difference between both groups in latency to approach the open arms. However, according to Hurst and West (2010), the tail-handled mice showed increased body extension behaviour, which signifies an anxiety-like behaviour phenotype. However, due to time constraints, the body extension behaviour was not analysed at the time.

One last behaviour that was investigated was the aggression of the mice using the cotton bud biting test. Although as discussed in the previous chapter regarding the limitation of the test, the test was quick and easy to do. Scruffing was also an integral part of this test, which should reflect the differences between the modified restraint method and the common tail-restraint method. It is also within consideration that the cohort of mice for this study was very mixed and not controlled for their previous handling experience. The test did not show differences between scruffing methods; however, it did find a significant difference between strains. Despite the animals used were coming from various handling backgrounds and ages, there is still a significant difference between the strains. This result is interesting; however, it will be discussed further in the general discussion.

The physiological parameters of the stress responses, including the eye temperature recording and the plasma CORT level, also did not show significant differences between tail-handling mice with the other handling/restraint methods. This lack of

difference in the eye temperature measurements is still within expectations since a previous study done by Zethof et al. (1994) did not find a significant effect of handling on rectal temperature changes. Despite the study not specifically investigating the effect of tail handling on mice, the handling itself might not induce significant stress induced hyperthermia. However, it is also possible that this result was due to mixed handling methods being performed on the same mice. It is necessary to do this study with a better design where the handling methods do not get mixed on the same mice, and also care is taken to ensure the daily handling did not affect the experimental design. In addition, this eye temperature result is in line with there being no significant differences in CORT results. Although the parameters were done over two separate studies, CORT level and body temperature are closely linked. It is possible that these results were affected by the mixed handling methods. As shown by Ghosal et al. (2015), tail handling did affect the CORT level. The study was done by not mixing the handling methods within the same mice. This suggests that the impact of mixing handling methods might affect the results, which in turn means that further investigation is necessary while ensuring the mice did not experience mixed handling methods in order to get clearer measurement results.

It was clear from previous studies that tail-handling should be a relic of the past. However, the modified restraint method, despite not showing affective/behavioural/physiological benefits, does not necessarily mean that this modified scruffing method is more detrimental. However, further study needs to be done regarding the active escape/passive coping mechanism differences attributed to the tail-handling and the modified scruffing method.

Chapter Summary

To summarise this chapter, the refinement studies showed that: (1) male mice preference to hot houses trends negatively over time and correlates negatively to their group nesting size of 3 whilst the female mice showed no change to their hot house preference trendline for nesting without any correlation to their group nesting size, and (2) the modified scruffing method that avoids tail-handling shows overt behaviour differences during and immediately after handling to the common scruffing method, which includes tail-handling without further specific differences in effects, anxiety-like behaviour, aggression, and physiological stress responses. The housing study could be taken further with a more comprehensive study involving different temperature gradation and with both sexes done at the same time and having the dominance hierarchy for the male mice tested. Whilst the modified handling/scruffing study could further investigate the affective parameter of the stress responses and further investigation to passive coping mechanism.

Chapter 6 General Discussion

This thesis provides further insight into the general knowledge of the OR37 subsystem and its application, specifically in the context of animal welfare. Prominent findings regarding the OR37 subsystem are that the OR37 ligands can be acutely applied to produce similar results as the Klein et al. (2015) using more practical methods of exposure with a tea ball or smeared on a surface. The application of the OR37 ligands for welfare refinement showed preliminary findings of an effect on eye temperature that was potentially caused by social isolation, which requires further investigation before drawing any conclusions regarding using the OR37 ligands as a form of olfactory enrichment.

Apart from the OR37 ligands, this thesis also provided further perspective in relation to animal welfare refinement regarding mice preference towards housing temperature and mice responses to tail handling. The mice preference on housing temperature also investigated whether the housing temperature affected mice, especially male mice, regarding their group housing preference. The mice's responses to tail handling supported published findings by demonstrating how their overt behaviours showed negative responses when compared to the non-tail handling methods.

OR37 Subsystem and its acute effect: now and then.

Previous studies, notably by Klein et al. (2015), provided the foundation of the acute application of the OR37 ligands mixture in a novel arena test and its effect on the PVN c-Fos activation. The study Klein at al. (2015) study showed a significant reduction in c-Fos activation compared to the solvent control group and this reduction was suggested to be associated with the CRH neurons activation. Previous studies have shown how a significant reduction in PVN c-Fos activation were followed with other measurements in different contexts such as: an increase of immobility duration during a forced swimming task in a knockout mice model for depression (Gaszner et al., 2012), a change in food intake behaviour in a knockout mice model for anorexia (Rowland et al., 2010), and reduction in marble burying activity and corticosterone level (Choi et al., 2013). Despite the significant reduction in PVN activation, no published findings on wider effects of OR37 ligands on functional application, and whether this reduction

affected other parts of the HPA axis, or other stress responses as an effect of PVN activation reduction. This thesis extended the effect to reduce c-Fos expressions in the PVN to conventional anxiogenic behavioural tests, specifically in an open field test (figure 3.2) and in a zero maze test (figure 3.7A). Despite the significant reduction of the PVN activation, there was no evidence regarding the OR37 ligands affecting anxiety-like behaviours (figure 3.3 and figure 3.7).

The lack of anxiety-related behavioural output from the OR37 ligands exposure might suggest an alternative hypothesis regarding the OR37 ligands' effect on the HPA axis. The processing of an anxiogenic context is known to involve the basolateral region of the amygdala and then be relayed to the PVN (Dore et al., 2013). On the other hand, it was also known that the stress response output from the PVN and the amygdala have a bidirectional processing relationship during anxiogenic context (Tye & Deisseroth, 2012). The PVN plays an important role in providing an integrated response to anxiety-inducing stressors, including behavioural responses. A study by Zhang et al. (2017) showed that a knockout of the CRH1R within the PVN neurons in mice shows an anxiolytic effect on anxiety-like behavioural tests (open field test and hole board test). This context highlights the importance of the CRH neurons within PVN in stress responses to anxiogenic stimulus. Interestingly, although the effects of the OR37 ligands on the reduction of the PVN activation by Klein et al. (2015) was suggested to be associated with CRH neurons activation, the study did not show a direct colocalization between the CRH and c-Fos in the OR37 ligands exposure study. The study showed that the CRH neurons were the dominant neurons colocalized with the c-Fos activation in the novel arena exposure instead of the oxytocinergic neurons and the vasopressinergic neurons. Considering the significant c-Fos reduction without a change in anxiety-like behavioural content, it is possible that the reduction in c-Fos was not fully attributed to a change in CRH neurons activation, but possibly due to other neurons such as oxytocin and vasopressin neurons in the PVN. Furthermore, considering that the Klein et al. (2015) study also showed that the OR37 ligands' effect was similar to natural chemosensory cues left by the mice, the OR37 ligands might possibly mediate behaviours involved in social behaviours, which could possibly be observed within a group living situation. The group living behaviours hypothesis also fits considering the study done by Bader, Breer, and Strotmann (2012), which showed that the OR37 subsystem also projects to the medial amygdala. Previous studies

showed that the oxytocinergic transmission within medial amygdala plays an important role in social behaviour with dimorphic sexual synaptic organisation (Cooke & Woolley, 2005; Ferguson et al., 2001). A study by Bosch and Neumann (2012) mentioned that the social behaviour in mice, including aggression, is modulated by the oxytocinergic and vasopressinergic transmission. In addition, a study by Mishor et al. (2020) used the hexadecanal (16 carbon long-chain aliphatic aldehyde), a component of the OR37 ligands mixture, which showed that the application of the hexadecanal changed aggressive behaviour differently between men and women. Considering the possibility that the PVN activation reduction due to OR37 ligands exposure is attributed to the oxytocin neurons or the vasopressin neurons, instead of the CRH neurons, it fits with the explanation for why there were no anxiety-like behavioural changes. However, this hypothesis could not be proven due to the low response level in the colocalization study done in this thesis (figure 3.8) and could be possibly pursued in future experiments.

Another consideration regarding the possibility that the OR37 ligands do not affect the CRH neurons was also supported by the response of the HPA axis. Other HPA axis measurements that were done in this investigation were the plasma ACTH level (figure 3.4B) and the plasma CORT level (figure 3.4A and figure 3.6B). It is to be noted that the data was collected using 6 animals per group. A study done by Le Cudennec et al. (2002) investigated the effect of saline intraperitoneal injections to the plasma CORT level and it was shown that the experiment was done by using 20 mice per group. It is clear that the intraperitoneal injection is not the same with the stressors that was used in this study, however, there were limited studies that investigated the CORT in mice with a big effect size. A calculation of the power based on the available data from the study resulted in an approximate effect size of 2.8. Although the effect size of the OR37 ligands to the plasma CORT was unknown, the study was done with the power calculation based on the PVN reduction data that showed the effect size of 1.8. It is quite possible that the plasma CORT study carried out within this thesis was underpowered, considering a study with an effect size of 2.8 employed 20 animals, and this study used 6 animals per group. Although the plasma CORT data was possibly underpowered, it does not change the fact that the plasma CORT data was not trending towards the same direction as the PVN c-Fos data in the final open field experiment (see figure 3.6A and figure 3.6B). Both plasma CORT and PVN c-Fos data were not obtained from the same animals, however, both parameters were collected at

appropriate time points for each parameter, which should reflect any changes that supposedly are affected by the OR37 ligands exposure. Although, according to Lightman (2008), despite that the vasopressin was also involved in the activation of the anterior pituitary, the vasopressin is more relevant in the context of chronic stress and changes in CRH is the more important signal in acute stress response. In future experiments, other than checking the colocalization of the PVN c-Fos activation changes, it will also be beneficial to check the changes in the anterior pituitary activity, since the activity of the anterior pituitary is affected by both the CRH and the vasopressin. It was unfortunate that the oxytocin and vasopressin colocalization study using the OR37 ligands within this thesis did not show a significant difference with their respective control group. A possible future investigation should continue to focus on colocalization of the CRH, vasopressin, and oxytocin neurons with the PVN c-Fos activation, and how this is associated with the anterior pituitary activity and the plasma CORT data.

Another finding from this investigation was regarding the method of the OR37 ligands exposure. The Klein et al. (2015) study used an airflow system to deliver the OR37 ligands for the treatments. Although the delivery method was proven to be effective, this raised a problem regarding the practical application of the OR37 ligands treatment. This investigation showed similar reduction in PVN c-Fos activation effect: by containing the OR37 ligands solution in an enclosure chamber (as done in chapter 3, experiment 2), by heating up the crystal form of the OR37 ligands in an enclosure chamber (as done in chapter 3 experiment 6), by putting the OR37 ligands on a filter paper in a tea ball infuser (chapter 4, experiment 3, figure 4.14), and by smearing the OR37 ligands solution on the arena lid (chapter 4, experiment 3, figure 4.14). Despite the enclosure chamber not being airtight, the groups being exposed to the OR37 ligands still showed a significant reduction on the PVN c-Fos activation, which showed that the OR37 ligands did not dissipate outside of the chamber and were still within effective concentration. The heated crystal also showed a good response level, although having to be admitted that this approach was not practical financially due to the amount of the crystal used. Despite showing a good PVN response, the enclosure chamber was still not practical for the objective of applying the OR37 ligands for animal welfare refinement. To address this issue, this investigation added the OR37 ligands to a filter paper inside a tea ball. The use of the tea ball is not novel since

previous studies, such as one done by Mandairon, Didier, and Linster (2008), used a tea ball as a delivery method for odour enrichment in animal cages. A similar volume of 100µl was used in this investigation and the exposure using the tea ball method showed significant reduction in PVN c-Fos activation. Although it was difficult to determine the volatility and the concentration in the air of the OR37 ligands due to the very low concentration in the treatment solution, it suggests that the OR37 ligands were at least volatile enough to be delivered utilising this method. In addition, considering the Klein et al. (2015) study, which used the mice natural secretion, and the study done by Bautze et al. (2014), that showed a component of the OR37 ligands was found on the mouse faecal surface, it is ethologically relevant for the OR37 ligands to be easily delivered to other recipients. This consideration then was used as a reason for using the smearing method of exposing the OR37 ligands and the exposure using this method also showed significant PVN c-Fos activation reduction. There was a concern regarding the availability of the OR37 ligands in mouse secretion. The study by Bautze et al. (2014) showed different hexadecanal concentrations found on the faecal pellets during different times of the day, which suggested that this component of the OR37 ligands were secreted in either a time or possibly context dependent manner. At this point, the natural source of the pentadecanal and heptadecanal is unknown. Either way, further studies regarding the OR37 ligands chemical properties still need to be carried out. Although there were plenty of unknowns relating to the chemical properties of the OR37 ligands, with both the tea ball and the smearing methods, it was shown that they were of a similar effect as the airflow exposure method, both the tea ball and the smearing methods were more practical to be used for OR37 ligands application.

Another question that this investigation tried to answer was regarding the concentration of the OR37 ligands. The study done by Klein et al. (2015) used the 1ng/10µl and the 10ng/10µl concentrations with the 10ng/10µl as the effective concentration. This study tried to investigate whether the immediate concentrations would produce a concentration-response curve and whether a concentration higher than the 10ng/10µl will have greater effect size or not. As the data shown previously (figure 3.7), it is quite possible that the OR37 receptors might have an all or none response, which needs to be investigated further. It is possible that the 4.5ng/10µl concentration response being in between the 1ng/10µl and the 2ng/10µl, might be due to random variation. The fact that the response level was the lowest at the 1ng/10µl

and the highest response level was achieved around the 2ng/µl and the 10ng/µl, this suggested that the OR37 ligands concentration response graph was plotted between 1 log unit, or possibly even lower, which is considered to be a small range for a concentration response. Although, it is to be noted that the response was measured in the mid-pathway response instead of the input response (such as olfactory receptor type of response) or the output response (such as behavioural output changes). There was limited available literature that shows a concentration response type investigation on the level of mid-pathway measurement such as PVN measurements using the inhalation method of exposure in mice. To make things even more difficult, the unusual characteristics of the OR37 subsystem pathway also makes it difficult to compare, either with a typical MOS type of receptor's concentration response, or with a typical vomeronasal system type of receptor's concentration response. However, as a comparison, an example of concentration response of an olfactory sensory neuron's response to eugenol stimulation was between 3 log units (Oka et al., 2004), while an example of concentration response of the vomeronasal system's receptor, a V1r to 2-heptanone stimulation was between 3 log units (Boschat et al., 2002). On the other hand, a study done by Hu et al. (2007) investigated a concentration response of CO2 concentration to a learning behaviour output and the concentration response was between 2 log units. Once again, although it was not a direct comparison, these previous studies data put the OR37 ligands concentration response in perspective regarding the small range of concentration response. It might suggest that the response of the OR37 ligands exposure might be quite specific, but further investigation is needed before jumping to conclusions and this concentration response data fits as a preliminary data for the investigation. In addition, the concern regarding the problem in measuring airborne concentrations also need to be addressed.

Other than the unusual concentration response, the OR37 subsystem's receptors themselves were quite unusual. It was unfortunate that the study on different mixtures of the ligands did not show any conclusive results due to technical problems (figure 3.4 and figure 3.5). However, a future similar investigation is important to elucidate the characteristics and to answer the function of this subsystem in nature. One of the unusual features of the OR37 receptors are the presence of different receptors (OR37A, -B, -C, and some others in the OR37 cluster I) that respond to different ligands but still within the same chemical family. The common paradigm of the olfactory receptor is

that it is either a very highly specific receptor that only responds to a specific information about stimuli, or via overlapping responses of broadly tuned receptors (Katada et al., 2005). It is unusual how the OR37 receptors were specific to the long-chain fatty aldehydes, but one type of long-chain fatty aldehyde, such as hexadecanal, can activate the receptors OR37A, -B, and -C. There is also the question of the other functional OR37 receptors such as the OR37E within cluster I and the other OR37 receptors within cluster II. One interesting fact is that the study done by Mishor et al. (2020) showed the effect of hexadecanal exposure to human participants and how it changed the aggression measurement was different between males and females. Apart from the sex difference, it is interesting since according to Hoppe, Breer, and Strotmann (2003), the OR37 receptors in human were homologous to the mice OR37 receptors in cluster II, which is different cluster to the OR37A, -B, and -C. It is important to understand the natural source of the OR37 ligands and how these ligands were biologically produced to elucidate the function. Considering the high level of conservation between species (Davies et al., 2018; Hoppe et al., 2006), these receptors might play an important role. It would also be interesting to see how the ligand binding specificities of the receptors themselves and whether there is population coding of a pattern of chemosignals from different sources or a certain pheromonal blend is optimal for eliciting an effect. It is also interesting to see whether this response was innately hardwired or involved a learning process from social situations.

To summarize the findings of the acute exposure of the OR37 ligands from this investigation, it was found that the OR37 ligands did affect the PVN activation in an anxiety-like context without endocrine and anxiolytic behavioural output, the OR37 ligands can be delivered more practically using a tea ball method or being smeared on the arena lid, and the PVN measurement of the concentration response showed a narrow concentration response curve within 1 log unit. There are plenty of things that can be done in future investigations regarding the OR37 subsystem, however, this study also investigated the effect of chronic exposure of the OR37 ligands in mice.

OR37 Ligands in Chronic Exposure and as a potential refinement

The investigation of chronic exposure of the OR37 ligands has not been studied previously in the literature. Considering that the hexadecanal was found on the surface of the mouse faecal pellets, and assuming the pentadecanal and heptadecanal were also naturally available, it is possible that in the natural environment, the mice were more likely to be constantly exposed to the OR37 ligands. Moreover, the chronic exposure investigation is more relevant as an enrichment for mice as captive animals due to laboratory situations forcing the mice to be deprived of their natural living condition, including olfactory cues.

It is important to establish the fundamentals regarding the factors that affect the odour profiles within a captive environment. One of the main differences within the captive environment and the natural environment regarding the odorant profile is the speed of air replacement and the regular cleaning as part of the husbandry procedure. The problem with the cleaning of air is that it replaces the familiar odour environment with a neutral odour environment, which potentially also removes the OR37 ligands that were available within the environment previously. Considering this, if the OR37 ligands are indeed necessary for mice in perceiving a social environment, laboratory mice were constantly deprived from this due to hygiene concerns. Addressing this issue, it gives the rationale of using the OR37 ligands as a form of enrichment for laboratory mice. Putting the OR37 ligands as a form of enrichment means that the exposure method needs to be practical, such as using the tea ball method (apart from the tea ball itself already a form of enrichment as a toy for the mice), and it is important to understand whether maintaining the OR37 ligands in chronic exposure poses a detrimental effect to the mice.

The air circulation system is another potential problem for a chronic exposure of olfactory chemosignals type of study. Other than the air circulation system replacing odorants, it also potentially contaminated other animals within the same unit with other species smells and other group treatments. This investigation did not check the details regarding the full specification of the air circulation/recirculation within the animal unit, however, it will be useful information to be accounted for when doing future investigations. On the other hand, this investigation used a scantainer cabinet to control the chemosignals exposure and the air output was directed to the ventilation system to prevent the air circulating within the same room. Looking back, it would be a good

idea to use carbon filters on the air input to remove any effect of air circulation within the animal unit.

Although there was evidence for a potential buffering effect of chronic OR37 exposure on social isolation-induced temperature change (figure 4.6), one of the main aims of the chronic exposure study, to study the effects on male aggression could not be achieved due to the lack of aggression in the socially isolated mice. This study was based on the social isolation-induced aggression paradigm, but only 35% of the socially isolated mice, combined between solvent and the OR37 ligands treatment groups, were fighting (based on figure 4.11A). Which suggested the social isolation-induced aggression paradigm was not as robust as expected. A possible reason why the mice were not as aggressive as expected was instead of putting the mice in a social isolation-induced aggression paradigm, the mice were put into a chronic mild stress paradigm and possibly approaching a depressive-like state. Although this could not be proven from this study due to no behavioural testing being done to assess depressive-like behaviours, one obvious thing is that there were clearly no behavioural changes in both aggression test (the resident-intruder test, figure 4.11) and the anxiety-like behavioural tests (the elevated zero maze test, figure 4.8 and the novelty-suppressed feeding test, figure 4.9). The problem with a depressive-like state is that it is commonly comorbid with an increase in anxiety-like state and an increase in aggression, which makes it difficult to pinpoint the causal mechanism between the three. A study done by Ma et al. (2011) showed how the mice that were exposed to aggression and chronic mild stress, compared to the chronic mild stress only group, were showing lower sucrose preference, higher immobility time in forced swimming tasks, and lower time spent in the centre of an open field. In addition, the study done by Mineur et al. (2003) showed how the mice that were subjected to chronic mild stress had increased aggression in the resident-intruder test. These studies did show the opposite direction of what this investigation found, which was that the aggression level should be increased along with depressive-like behaviours. There was a concern related to the separation line between isolation-induced aggression and the chronic mild stress-induced depressive-like state paradigm, however, considering these two paradigms were linked, it is important to come up with a way to separate both when studying the effect of social isolation in one of the paradigms. This investigation did not measure parameters related to depressive-like state; therefore, it is difficult to interpret whether

this depressive-like state also became one of the factors affecting the aggression of the mice.

On the other hand, it is possible that the reason why the mice were not fighting was because of the opponent/intruder being used in the aggression tests. As previously mentioned, it was known that the C57BL/6 mice were found to be aggressive within the resident-intruder test (Strekalova et al., 2003). However, the main difference between the said study and this experiment is that the referenced study used CD1 mice whilst this experiment used the same C57BL/6 mice. The main issue with using the same strain of mice is that it is possible that the inbred mice have very low genetic variability, which in turn makes them consider each other as siblings. In addition, as it is energy inefficient to fight with siblings compared to fighting with intruders, it might be another explanation why the mice within this experiment did not fight.

To summarise, there were plenty of factors that need to be considered when aiming to use the chronically exposed-OR37 ligands as a potential enrichment, including the air circulation system within the laboratory and the relationship between aggression, anxiety-like state, and depressive-like state. More parameters also need to be investigated to assess the effect of chronic exposure of the OR37 ligands.

Refinements of Laboratory Technique

The first refinement investigated in this thesis was the nesting temperature preference and whether the temperature affected mice preference in group nesting. A number of studies have investigated mice temperature preference and thermoneutral zone. Mice prefer warmer temperature as their thermoneutral zone (closer to 30ºC rather than to 20ºC) and there is a sex difference when it comes to temperature preference (Gaskill et al., 2011; Kaikaew et al., 2017; McKie et al., 2019). The findings from this investigation showed that the male mice preference negatively trended over time to the hot houses (figure 5.2A), unlike the female mice, which have no change in their preference trendline to hot houses over time (figure 5.3A). However, the most interesting finding from this investigation is that the male mice preference to a hot house was negatively correlated to the group nesting preference (figure 5.2C), in other words, as the housing temperature increased, the mice preferred a colder house while at the same time the mice preferred to be nesting in group (size of 3). Interestingly,

there is a lack of literature that investigated the association between mice group nesting behaviour and how it changes due to temperature. To put into perspective, house mice that live together within the human population were commonly found to form a social breeding group (deme) that normally consists of one or two males (Singleton, 1983). This house mice population was found to disperse and/or spread out less compared to the more feral house mice that live within a field (Pocock, Hauffe & Searle, 2005). Although it is uncommon for these mice to have more than one or two adult males within one deme, male laboratory mice were commonly housed in a situation where adult male mice were forced to be housed and nest together. It is possible that this condition was made possible due to domestication and being forced to live together, which made the male mice being accidentally selected for the one with lower aggression traits and live harmoniously with other adult males. However, despite the domestication, it is still another question whether the laboratory mice still need to disperse and maintain their social deme. Considering how it is uncommon to find more than one adult male within a deme, it is a possibility that these laboratory male mice do not prefer to live together.

This raises the question: do sexually mature male mice actually prefer to live together with other sexually mature male mice? Or is this huddling behaviour simply because they act as such to enhance survival? If the later question is the current situation that is happening to all laboratory mice, this means that male laboratory mice were not only being forced to live below their thermoneutral zone, but also forced to live with other mice that they naturally would not prefer to. It is quite possible that this situation might actually affect their welfare state. However, the current findings are not deemed to be strong enough evidence to challenge the long-standing paradigm: mice are social animals, therefore both male and female mice must prefer to live in groups. There are plenty of improvements that can be done to the experiment design, however, the data itself showed promising preliminary data towards this alternative hypothesis.

The other refinement topic that was investigated was regarding whether the mice prefer not to be handled by their tail, which was a further investigation following on from the works of Hurst and West (2010). Although the findings from the study showed that the mice handled by their tail had higher anxiety-like behavioural measures and showed less approach to the handler, this investigation tried to expand the evidence regarding tail-handling being detrimental to mice on other measures. However,

physiological measurements using eye temperature (figure 5.4) and affective state measurements using a conditioned place preference (figure 5.5) did not show direct evidence between tail handling and other refined handling measurements. The current hypothesis regarding why tail handling is aversive to mice is because it was attributed to prey response behaviour, similar to when the mice are attacked by a predator. However, it is possible that this aversion could also be attributed to their tail being a bundle of nerves that play roles in balancing the body. Although the anxiety-like behavioural measure fits within this hypothesis, it is important to check more measurements when assessing animal welfare due to difficulties in interpreting welfare measures, and that most measurements that assess welfare are not exclusively measuring welfare.

Another approach that was taken to the tail-handling question within this investigation was using the alternative method of scruffing that does not involve using the tail. Although this was not an investigation to add more evidence for handling, this does add to the knowledge regarding the effect of using the tail within the procedure. Interestingly, there was no strong evidence of anxiety-like behaviour (figure 5.8) and plasma corticosterone (figure 5.8) that pointed to the scruffing method involving the tail being more detrimental. However, overt behavioural measurements (figure 5.6) that were commonly linked with anxiety-like parameters (vocalisation, urination, faecal bolus discharge) were higher in the tail scruffed group compared to the method that did not involve the tail (Deacon & Rawlins, 2005; Sánchez, 2003). There was a possibility that the other method was not more beneficial because there was no control treatment to compare this result to, however, it strengthened the evidence regarding handling the mice by their tail to be detrimental based on the overt behavioural responses. It is necessary to further investigate what the overt behaviour means, or more straightforward investigation could be done by assessing the mice preference for the method. Although a CPP was done within this study, the CPP was developed to assess a drug preference with a big effect of reward or aversion. However, the problem with handling the mice is that it was not clear regarding the level of aversion induced to the tail handling compared to the aversion level of being handled. When both treatments were not obviously different, it was difficult to assess using a CPP due to there not being a motivational drive to actively avoid the paired condition. One

possible idea for future investigation is by assessing such aversion or preference and including a reward motivation-based task, such as the affective bias test.

Future Perspective

Taken together, the OR37 subsystem was an unusual subsystem within the main olfactory system. It is still not fully understood regarding its function and why this subsystem is highly conserved across mammals (Davies, March, et al., 2018; Hoppe et al., 2006). Data from this investigation has added to the knowledge regarding the use of acute and chronic exposure of the OR37 ligands and its effects on mice. One important subject that still needs to be investigated with the OR37 subsystem is the neural pathway for the effects and the neuroendocrine responses during exposure. Although it was known that the OR37 subsystem affected the PVN c-Fos activation, there is still a gap in the knowledge regarding which neuronal type of activation is being affected within the PVN, because understanding this parameter will improve our understanding regarding the OR37 subsystem's potential function. This knowledge then can be used for further investigation regarding the acute and chronic effects of the OR37 ligands, and whether the OR37 ligands can be applied in an animal welfare context or possibly in other functions. Another direction for further investigation would be to investigate the effectiveness of different ligand mixtures and whether a particular pheromonal blend is required to elicit a maximum response. This investigation, in conjunction with the functional pathway investigation, will then support each other's conclusion to explain why these receptors are evolutionarily so highly conserved and the OR37 subsystem's unusual features. It is important to investigate a more focused study on the effects of the OR37 ligands on aggressive behaviour without the chronic disturbance.

There are more investigations needed before the OR37 subsystem can be used in animal welfare applications. But this thesis has provided preliminary results regarding mice nesting temperature preference affecting their group nesting size preference and more evidence for not handling mice by their tails. The mice nesting preference showed how the increase in housing temperature decreased the male mice preference towards group nesting, which raised the question regarding the long-standing paradigm regarding male mice group nesting behaviour. There is also the question whether the mice are driven to a negative affective state by the tail-handling. Apart

from more parameters of stress responses that need to be assessed, the mice innate preference and why mice show poorer measurements of overt behaviour to tail handling needs to be investigated further. At the end of the day, there are still a lot of things that we do not understand about natural mice behaviour. It is important to understand these points in order to develop better strategies in improving animal welfare in the interest of the animals themselves.

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