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**The Investigation of Age-related Changes in Apathy-related Behaviour and Biological Rhythms**

# The Investigation of Age-related Changes in Apathy-related Behaviour and Biological Rhythms

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## Abstract

Apathy is a multidimensional psychiatric syndrome that often occurs alongside neurodegenerative disease but also otherwise healthy ageing. Despite its clinical importance, little is known about its underlying mechanism in normal ageing. Ageing is associated with disrupted biological rhythms, which can have a significant impact on health, including the development of psychiatric disease. The development of a rodent model for apathy could allow for its underlying neurobiology to be investigated and for specific treatments to be developed. Improving our understanding of changes to the aged circadian system could allow for a potential relationship between apathy and disrupted biological rhythms to be tested in the future. This thesis therefore investigates the effects of ageing on apathy-related behaviour and biological rhythms in rodents.

Apathy-related behaviour was assessed in mice using a battery of behavioural tests and physiological measures that map onto human apathy domains. It was found that aged mice show behaviours relevant to multiple domains of apathy. To account for potential species differences and to explore more complex behaviours, apathy-related behaviour was assessed in aged rats. Aged rats did not show the same deficits as aged mice, revealing striking species differences. Under *ad libitum* feeding conditions aged rats showed blunted circadian activity and corticosterone release but not core body temperature suggesting a system-specific disruption. Feeding in the light phase disrupted normal nocturnal activity to a greater extent in aged versus young rats, suggesting that feeding time may be a stronger zeitgeber than light in aged rats. Aged mice showed a weaker activity circadian rhythm driven by a reduction in activity immediately following lights off.

This thesis provides evidence that aged mice may be a suitable apathy model and both rats and mice show some evidence of biological rhythm disruption. Future work should test a relationship between the two for potential therapeutic benefit.

## Dedication and Acknowledgements

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## 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.

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## List of Abbreviations

| | |
|---|---|
| AA- | Anticipatory activity |
| Ab- | Antibody |
| ABS- | Automated blood sampling |
| AD- | Alzheimer's disease |
| AD- | Antidepressant |
| AES- | Apathy Evaluation Scale |
| AMI- | Apathy Motivation Index |
| Arc- | Arcuate nucleus |
| AUC- | Area under the curve |
| AVP- | Arginine vasopressin |
| BAC- | Bacterial artificial construct |
| BMAL1- | Brain and muscle Arnt-like protein-1 |
| CLOCK- | Circadian Locomotor Output Cycles Kaput |
| CORT- | Corticosterone/cortisol |
| CRF- | Continuous reinforcement |
| CRH- | Corticotropin releasing hormone |
| CSD- | Cornell Scale for Depression |
| CUMS- | Chronic unpredictable mild stress |
| EBDM- | Effort-based decision making |
| EfR- | Effort for reward |
| FAA- | Feeding anticipatory activity |
| FR- | Fixed ratio |
| FrsBE- | Frontal System Behaviour Scale |
| FST- | Forced swim test |
| GR- | Glucocorticoid receptor |
| HAMD- | Hamilton Rating Scale for Depression |
| HB- | Hybridisation buffer |
| HD- | Huntington's disease |
| HPA- | Hypothalamic pituitary adrenal axis |
| HPC- | Hippocampus |
| LD- | Light-dark cycle |

| | |
|---|---|
| MDD- | Major depressive disorder |
| MR- | Mineralocorticoid receptor |
| mRNA- | messenger RNA |
| NAc- | Nucleus accumbens |
| NORT- | Novel object recognition test |
| NSFT- | Novelty supressed feeding test |
| PBS- | Phosphate buffered saline |
| PD- | Parkinson’s disease |
| PER- | Period |
| PFA- | Paraformaldehyde |
| PFC- | Prefrontal cortex |
| POMC- | Pro-opiomelanocortin |
| PR- | Progressive ratio |
| PRL- | Probabilistic reversal learning |
| PTSD- | Post-traumatic stress disorder |
| PVN- | Paraventricular nucleus of the hypothalamus |
| RIA- | Radioimmunoassay |
| RLA- | Reward learning assay |
| RM ANOVA- | Repeated measures analysis of variance |
| SCN- | Suprachiasmatic nucleus |
| SD- | Sprague-Dawley |
| SEM- | Standard error of the mean |
| SHAPS- | Snaith-Hamilton Pleasure Scale |
| SPT- | Sucrose preference test |
| SSC- | Saline sodium citrate |
| SSRI- | Selective serotonin reuptake inhibitor |
| TdT- | Terminal deoxynucleotide transferase |
| TEA- | Triethanolamine |
| TST- | Tail suspension test |
| VTA- | Ventral tegmental area |
| ZT- | Zeitgeber time |

# Chapter 1: General introduction

## *1. Apathy*

Apathy is a complex neuropsychiatric syndrome that is operationally defined as a quantitative reduction in goal-directed behaviour (Levy and Czernecki, 2006). It accompanies a broad range of neurodegenerative, neurocognitive and psychiatric disorders, but also occurs in otherwise healthy ageing (Brodaty et al., 2009). Apathy is associated with higher mortality, poorer quality of life, cognitive decline, and significant caregiver stress (Ishii et al., 2009, Gerritsen et al., 2005). Apathy can result in the degeneration of basic self-care, leading to nutritional deficit, and failure to recognise early symptoms of impending medical issues. It can also negate the positive effects of therapeutic intervention by affecting adherence to medication and healthcare appointments (Padala et al., 2008). As such, its clinical relevance and research importance is unquestionable. Despite this, the underlying neurobiology of apathy remains unclear, and does not yet have a designated treatment.

### *1.1 The dimensions of apathy*

Apathy is a multidimensional construct with dissociable domains. The characterisation of these domains has shifted over the past two decades, largely driven by a surge in apathy research. The early work of (Levy and Dubois, 2006, Marin, 1991) conceptualised apathy into three domains of disrupted processing; 'emotional-affective', 'cognitive' and 'auto-activation'. Apathy relating to the emotional-affective domain refers to an individual who can no longer link emotional-affective signals with appropriate ongoing or forthcoming behaviour. Those with cognitive apathy can no longer elaborate a plan of action into forthgoing or ongoing behaviour, and auto-activation apathy refers to an individual who can no longer self-initiate thoughts or actions but show no deficits in externally driven behaviour. In the same work, attempts were made to map the domains onto different prefrontal cortex (PFC)-basal ganglia territories. Using mainly clinical observations of patients with brain lesions, it was posed that emotional-affective apathy was linked to orbital-medial PFC and ventral striatum, cognitive to the lateral PFC and the dorsal caudate nuclei, and auto-activation to the bilateral globus pallidus and dorsal-lateral PFC.

More recently, the diagnostic criteria and list of apathy dimensions were revised, incorporating work stemming from a newly developed Apathy Motivation Index (Ang et al., 2017). Three domains; behaviour-cognition, which encompasses the auto-activation and cognitive domains described above, emotion, which encompasses the emotional-affective domain, and finally, a new domain called social motivation. This was added as it was recognised that social behaviour was an important aspect of apathy-related behaviour (Robert et al., 2018, Ang et al., 2017, Sockeel et al., 2006). While the earlier classification described apathy at a more mechanistic level, the later classification describes apathy at a more clinical level, at which impairments can be observed and more easily scored (see **figure 1**).

**Figure 1**

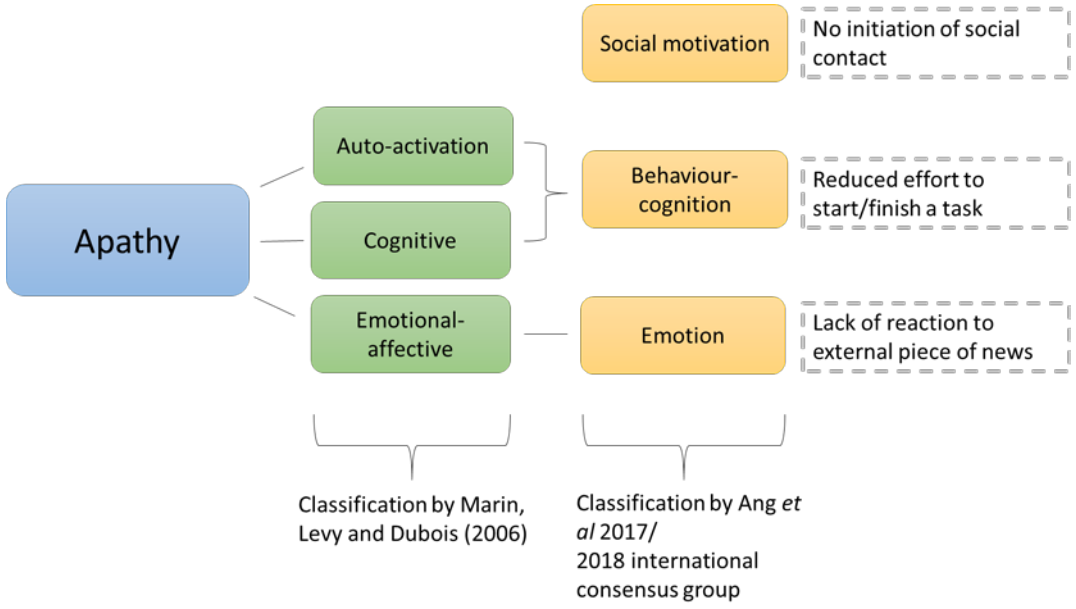


***Fig.1. The dimensions of apathy.*** *The constructs in green represent the classification developed by Marin, Levy and Dubois and embodies a mechanistic view of apathy. The constructs in yellow represent a more recent classification built from the initial classifications with the addition of social motivation. Examples of apathy behaviour linked to each of these constructs are shown in grey boxes.*

## *1.2 Diagnostic criteria of apathy*

A new set of diagnostic criteria for apathy was proposed in 2018 and can be viewed in more detail here (Robert et al., 2018). To be diagnosed with clinical apathy, the individual must show behaviours relating to at least two apathy dimensions i.e., behavioural/cognitive, emotional, or social for a period of at least four weeks, that must be present for most of the time. Apathy relating to behavioural/cognitive changes includes making less effort to start or finish a task and overall reduced levels of activity. Apathy relating to emotional changes includes lack of emotional response to external news, difficulty conveying emotion with physical/verbal expression and empathetic behaviour. Social apathy includes staying homebound more often, not initiating social engagements and being less likely to initiate conversation. Importantly, these behavioural changes must be a change from their own normal functioning and have a clinically significant impact on daily functioning e.g., occupational, social. These changes must also not be explained by other factors, such as a physical disability, effect of drugs or diminished consciousness.

Traditionally, apathy was treated as part of major depressive disorder (MDD) due to difficulties in distinguishing them clinically. Apathy and depression share multiple overlapping characteristics,

including psychomotor slowing, diminished interest in previously enjoyed activities and fatigue (Ishii et al., 2009). However, apathy and depression are distinct constructs that can be experienced together or singularly. They can be distinguished most clearly in the domain of affect, where depressed patients can experience extremely low moods while apathetic patients experience an emotionally blunted state. This was highlighted in work investigating the convergence of depression and apathy rating scales. A study conducted on 107 patients showed that the Apathy Evaluation Scale (AES) and the Hamilton Rating Scale for Depression (HAMD) converged only on symptoms in a subset of the HAMD that corresponds to disrupted motivation, including psychomotor slowing and lack of interest. When this specific subset was removed, the correlation disappeared (Marin et al., 1993). A similar case was found using the Frontal System Behaviour Scale (FrsBE) and the Cornell Scale for Depression (CSD). While they converged on a set of symptoms relating to motivation, they did not correlate in symptoms relating to negative state or anxiety (Ready, 2003).

Anhedonia is defined as a loss of pleasure or interest in a significant proportion of daily activities the patient once enjoyed. Apathy and anhedonia share intersecting characteristics, and forms of assessment for the two constructs are often overlapping. However, it was shown that scores from the Apathy Motivation Index (AMI) and the Snaith-Hamilton Pleasure Scale (SHAPS) do not perfectly correlate, suggesting there are aspects of anhedonia that are distinct from apathy. Furthermore, work has shown that apathy and anhedonia have distinct neural substrates (Lee et al., 2015).

## *1.3 Current approaches to the treatment of apathy*

The unknown aetiology of apathy makes it difficult to find the correct pharmacological or psychological approach. However, studies have explored a variety of different pharmacological treatments in the context of the wider co-morbid conditions most commonly associated with apathy. The wide variety of pharmacological approaches tested, some focusing on completely different neurochemical systems, highlights that the underlying neurobiology of apathy must be clearly understood before a treatment can be developed to treat it specifically. For example, Donepezil, an anticholinesterase inhibitor, Memantine, a NMDA receptor antagonist and Methylphenidate, a dopamine reuptake inhibitor have all been used in open label/randomised control trials (RCT) for the treatment of apathy in Alzheimer's disease (AD), with some improvement in apathy scores (Theleritis et al., 2019). With little knowledge of the underlying neurobiology of apathy, it is not clear whether these systems are involved in apathy or that improving the underlying condition improves the symptom domain.

Antidepressants are often prescribed as a treatment for apathy despite having little/no convincing clinical evidence of efficacy (Benoit et al., 2008). As described above, despite sharing some overlapping characteristics, apathy and major depressive disorder (MDD) are distinct constructs

and one can present with apathy without MDD. Treatment with the selective serotonin reuptake inhibitors (SSRIs) class of antidepressants has little to no effect, and can even exacerbate symptoms, known colloquially as 'SSRI-induced indifference' (Kim et al., 2019, Sansone and Sansone, 2010).

The diversity in the pharmacological approaches highlighted above emphasizes the importance of understanding distinct symptoms in neurological disease to produce a targeted treatment. With a growing movement to tackle neurological disease by treating symptoms rather than the disease as a whole, this is particularly pertinent.

## *1.4 Apathy in otherwise healthy ageing*

Apathy does not just occur in the context of disease. There is evidence to suggest that apathy is prevalent in otherwise-healthy ageing. A study showed that in a population of 76 healthy, community-dwelling elderly subjects, apathy increased with age over a 5-year period (Brodaty et al., 2009). Cross-sectional analysis of an elderly, dementia-free population of 4354 participants showed that 49% of them had $\geq 2$ symptoms of apathy. Furthermore, those with apathy symptoms were significantly older than those who did not (Grool et al., 2014). Using MRI, it was found that those with $\geq 2$ symptoms of apathy had a smaller total brain volume, as well as grey and white matter volume in all lobes and frontal/parietal lobes respectively, compared with those with 1 or no symptoms of apathy. However, due to its cross-sectional design it is not possible to tell whether these changes caused apathy or were driven by it. Functional brain activity changes have also been found in otherwise healthy individuals with apathy. These changes related to key brain regions involved in motivated behaviour, described in more detail in section 1.6. In a population of 100 healthy elderly participants, resting state fMRI revealed a deterioration of resting state functional connectivity between the ventral striatum and frontal regions in apathy (Kawagoe et al., 2017). See (Husain and Roiser, 2018) for a more extensive review of brain regions implicated in apathy pathophysiology.

## *1.5 The dopaminergic reward system*

While the results discussed in this thesis do not directly pertain to manipulation of the dopaminergic reward system, it is important to outline the role of dopamine in reward and motivated behaviour. Existing studies discussed in below sections have sought to probe apathy behaviour by disrupting the dopaminergic system via pharmacological manipulation or in the context of disease models where the dopaminergic system is altered.

### *1.5.1 Defining motivated behaviour*

The term 'motivation' is used to describe how an organism's internal state, external environment and past experiences produce goal-directed behaviour. Motivated behaviour is a series of cost-benefit analyses, in which the organism weighs up the cost (physical and cognitive effort, lost time, potential danger) against the benefit (fulfilment of physiological/psychological needs e.g., feeding or escape from harm). For a more detailed review of this see (Simpson and Balsam, 2016). Goal-directed behaviour has two components: directional and activational behaviour. Within the directional aspect there are two phases, instrumental (also referred to as 'seeking' or 'wanting') and consummatory (also referred to as 'taking' or 'liking'). The instrumental phase refers to the way in which the organism gains access to the stimuli or moves away from it, be it physical or psychological. The consummatory phase refers to the way in which the organism directly interacts with the goal stimuli (Salamone et al., 2016, Salamone and Correa, 2017). The activational or 'energising' aspect of motivation refers to the effort the organism makes to achieve its goal i.e. the speed, vigour and persistence (Salamone and Correa, 2017). To place this in the context of rodent behaviour research, the instrumental phase refers to the rodent making operant responses (lever pressing, nose poking) for reward. The consummatory phase refers to the rodent collecting the reward. The activational aspect refers to the rate of response, the length of time and level of obstacle the rodent is willing to overcome to obtain reward.

### *1.5.2 The role of dopamine in motivated behaviour*

There are three major dopaminergic systems within the brain; the mesolimbic, mesocortical and nigrostriatal dopamine projections. The mesolimbic projection originates in the ventral tegmental area (VTA) and innervates mesolimbic regions, primarily the nucleus accumbens (NAc), while the mesocortical projection also originates in the VTA and projects to the prefrontal cortex (PFC). The nigrostriatal dopamine projection originates in the substantia nigra and innervates the striatum (Cools, 2008). The striatum, in turn, innervates the basal ganglia, where body movements are facilitated.

It is well established that dopamine is crucial for motivated behaviour, but in exactly what way, is still debated (for a comprehensive review see (Wise, 2004)). For some time, it was thought dopamine modulated the hedonic experience of a reward. That is, the enjoyment or 'liking' of a pleasurable stimulus. However, more recent research suggests that it is in the instrumental/seeking/wanting of goal-directed behaviour that dopamine (DA) plays a key role (Salamone and Correa, 2012, Berridge and Robinson, 1998). Modulation of DA transmission in the nucleus accumbens (NAc) affects instrumental behaviour without changing food-induced hedonia or appetite, and is important for behavioural activation, effort expenditure, spontaneous and novelty-induced exploration (Salamone and Correa, 2012). The NAc is thought to serve as a point

of functional integration for limbic and motor systems (Morrison et al., 2017) thereby facilitating movement towards reward.

Drugs that modulate the dopaminergic system in the treatment of neurodegenerative and neuropsychiatric disease have been shown to modulate apathy symptoms suggesting that this system is relevant to its underlying neurobiology. For example, treatment of Schizophrenia with antipsychotic D2 antagonists have been shown to cause or exacerbate apathy symptoms (Stępnicki et al., 2018). Treatment of Parkinson's Disease (PD) patients with levodopa has been shown to improve symptoms (Czernecki et al., 2002, Rosqvist et al., 2018) though little/no effect has also been reported (Lhommée et al., 2018). This may be due to differences in the way in which apathy is assessed, including which domains are tested.

## *1.6 A translatable approach to assessing motivated behaviour in rodents*

Clinical studies investigating the underlying neurobiology of apathy in healthy ageing are limited. This is in part driven by the method of clinical assessment of apathy, which is traditionally performed using questionnaires, performed either by the clinician or as self-report. This approach is limited when trying to assess the underlying neurobiology of apathy, as questionnaires do not track changes over time, and rely on the patient or the caregiver's perception of their symptoms. Furthermore, this form of assessment cannot be translated to rodent tasks, where pre-clinical research into apathy and potential treatments can be tested. With the ability to control the animal's environment and ease of tissue collection, the use of aged rodents could provide a valuable route to assess apathy-related behaviour in ageing and could allow for a more rigorous assessment of its underlying neurobiology. The dissociation of apathy into behavioural, cognitive, and emotional domains presents an opportunity to investigate whether these same deficits arise in animals at a behavioural level. A commonly used way to assess motivated behaviour in rodents is the use of translational effort-based decision-making tasks (EBDM).

### *1.6.1 The progressive ratio task*

In the progressive ratio task (PR) the rodent must make an increasing number of operant responses (e.g., lever pushes or nose pokes into an aperture) for each successive food reward. The number of responses the rodent is willing to do before giving up (a period of inactivity in operant responding) is defined as the breakpoint, and this measure is used to quantitatively assess the rodent's motivational state. In addition, aspects of the instrumental behaviour, such as time take to complete lever presses for reward are also used as a metric of motivation, with prolonged latencies indicating a lower motivational state (Hernandez et al., 2010).

The PR task has been used to measure motivational state in many pharmacological studies, including the investigation of psychostimulant and opiate effects (Stafford et al., 1998), drug self-

administration (Richardson and Roberts, 1996) and dietary manipulations (Sharma et al., 2012) among many others.

This task has been back translated to human study, where button pushes, clicking a computer mouse, and timed riding of a bike have all been used as physical efforts to receive a reward (Stoops, 2008). The PR task has been used for the past 30 years to examine drug reinforcement in humans (McLeod and Griffiths, 1983, Stoops, 2008). Beyond drug studies, it has also more recently been used to study the negative symptoms of schizophrenia, where it was shown that schizophrenic patients had a lower breakpoint than healthy controls, and that breakpoint correlated with amotivation levels scored in a negative symptom questionnaire (Wolf et al., 2014).

### *1.6.2 The effort for reward task*

In the effort for reward (EfR) task, the rodent must choose between a more difficult to obtain (higher effort) but higher value reward, or an easier to obtain (lower effort) but lower value reward. In the operant box version of the task, developed by (Salamone et al., 1991), the higher effort option is a set number of operant responses for a palatable reward, and the lower effort option is to consume freely available standard lab chow. By offering a choice to the rodent, it can tease apart changes to hedonic processing/appetite from motivation. While a shift from the high effort to low effort reward option indicates a change in motivational state, an overall suppression of consumption indicates a change in appetite. This task has been pharmacologically validated as a measure of motivational state, where administration of haloperidol, a dopamine D2 receptor antagonist, directly into the NAc decreased responding on the lever and increased consumption of freely available chow, demonstrating a key role for dopamine in the instrumental response for food reward (Salamone et al., 1991). Variations of the task exist, including a T-maze set up where the rodent must choose whether to traverse a wall for a higher value reward, or choose the easily accessible but lower value reward (Cousins et al., 1996). This task is restricted by a small number of trials however and is not suitable for studies where overt motoric changes may be a confound.

The EfR task has also been back translated into a human study. The Effort Expenditure for Rewards Task (EEfRT) is a multi-trial task, associated with varying levels of monetary rewards. The higher effort task involves higher levels of button pressing with a less dominant finger, and the lower effort task involves less button pushes with the dominant finger. This task was used to show that trait and state anhedonia is associated with less willingness to put in greater effort for greater reward. The task has also been validated pharmacologically, where it has been shown that d-amphetamine increases willingness to exert higher effort for higher reward (Treadway et al., 2009, Treadway et al., 2012).

It should be noted that these are quantitative tests of motivated behaviour, and while they do test an aspect of apathy-related behaviour, motivated behaviour is not the same as apathy behaviour, which is multidimensional and should not be generalised. This is discussed in more detail in section 1.9.

### *1.6.3 The probabilistic reversal learning task*

Reward learning (RL) refers to the ability of a reward to modulate future behaviour. Using action-based feedback to guide future action is an important part of effort-based decision making. In everyday life, reward availability is often dynamic and probabilistic. The ability to flexibly adjust behaviour in response to either positive or negative feedback to maximise likelihood of obtaining reward represents a healthy reward system. Impaired reward learning has been implicated in psychiatric and neurological diseases such as MDD, schizophrenia and Huntington's disease (HD) (Bari et al., 2010, Lawrence et al., 1999).

A common way to assess reward learning is with a probabilistic reversal learning task (PRLT), which has been used in both human and rodent studies, owing to its translational validity. In this task, the rodent or human is faced with two options (two different pictures, a left and right-placed lever etc). One option is associated with a higher probability of reward and the other with a lower probability of reward. First, the subject must learn this association, and stably choose the option with the higher probability of reward. The reward contingencies are then reversed. In this way, the task challenges the subject to discriminate between options, shift behaviour once the rule has changed and disengage from the previous behaviour (Bari et al., 2010), requiring cognitive flexibility and appropriate use of positive and negative feedback.

While the EfR and PR tasks require higher physical effort and lower cognitive effort, the PRLT requires cognitive flexibility, which could be used as a cognitive 'effort' to probe apathy behaviour. It also assesses sensitivity to positive and negative feedback, giving it an affective component. As with the tasks above, apathy has not been explicitly assessed with this task. However, disease states in which apathy often occurs has been. A study using PD patients off medication showed weaker adaptation to rule change compared to controls and were slower in the initial stages of phase of learning (Peterson et al., 2009). Patients with advanced HD completed less reversals in behaviour compared to healthy controls and had a lower performance level following each rule change (Nickchen et al., 2017). In both studies, these deficits are linked to changes in the dopaminergic system or gross changes in the reward centres of the brain. In addition, probabilistic reversal learning has also been shown to be impaired in healthy aged humans. Older participants showed impaired reversal learning and poorer acquisition (Weiler et al., 2008). Reversal deficits have also been reported in the preclinical literature, though these studies are largely confined to the non-human primate literature (Weiler et al., 2008).

## *1.7 Ecological and spontaneous measures of motivated behaviour in rodents*

### *1.7.1 Home-cage behaviours*

It has been suggested that a more ecological approach to assessing motivated behaviour could complement the operant tasks described above and provide some degree of translatability to the gold-standard practise of interviewing techniques in the clinical assessment of apathy (Cathomas et al., 2015) (see **figure 2**). Much of the interview relates to the impact of apathy symptoms on the daily living of the individual, including overall self-care. The behavioural observation of the rodent in a normal home-cage environment can provide similar 'self-care' and daily living information. Nest building, grooming and social interaction have all been suggested to be relevant to apathy-related behaviour. For a full review on these behaviours see (Cathomas et al., 2015).

### *1.7.2 Object exploration and general locomotion*

Impaired interest in novel experiences is also a part of apathy-related behaviour in humans. The natural exploratory behaviour in rodents has been utilised for many years to assess changes in cognition. For example, the novel object recognition test and the object in place tests, which rely on the rodent's natural drive to explore a novel object over a familiar one. Exploration of a novel object is a motivated behaviour, where the rodent must find the novelty of the object sufficiently arousing to overcome the cost of locomoting towards the object and overcoming potential threat (if the novel object is not placed in the home cage) and spend time exploring it (most often measured by time spent sniffing the object). It has been shown that intraperitoneal administration of a selective dopamine D4 receptor agonist increased time spent exploring a novel object (Powell et al., 2003). General locomotion may also be used to assess apathy-related behaviour in patients. Psychomotor slowing has been reported in patients with apathy and depression (Feil et al., 2003, Sawa et al., 2012). Placing a rodent in a novel environment and allowing them to explore for a set amount of time and assessing parameters such as velocity and distance travelled could provide more information about exploratory behaviour.

**Figure 2**

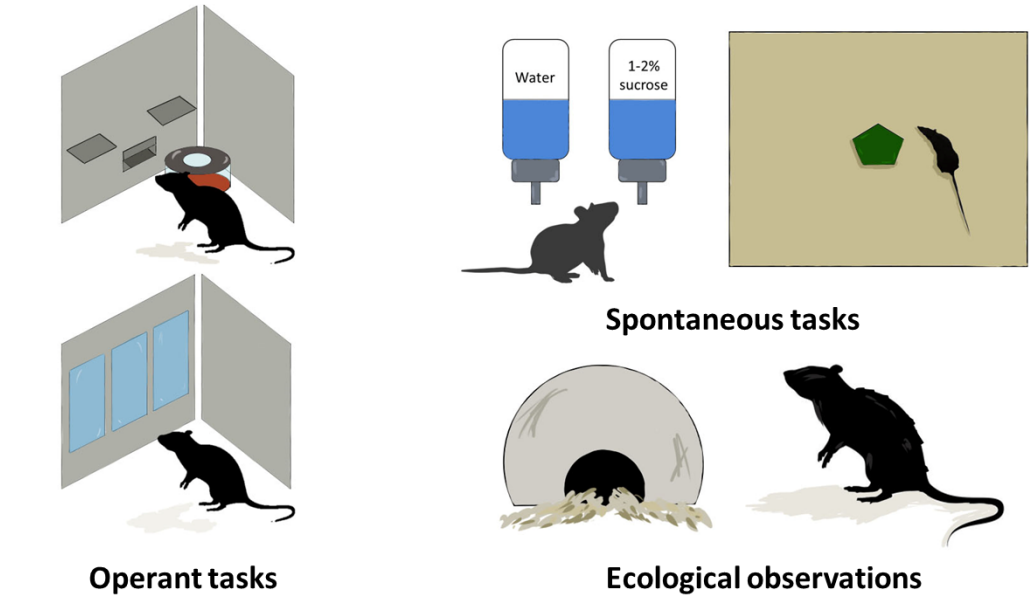


***Fig.2. Current approaches to testing apathy-related behaviour.*** *Motivated behaviour can be tested using operant EBDM tasks such as the PR and EfR tasks. Alternatively, spontaneous tasks which do not require extensive pretraining and rely on natural rodent behaviour can be used such as object exploration and the sucrose preference test (SPT). Home cage observations such as nest quality and coat state can be used as a behavioural read out of self-care.*

## *1.8 Assessment of the emotional blunting domain of apathy*

Emotional blunting, defined as a diminished response to an emotionally salient stimulus, is a core domain of apathy-related behaviour. However, pre-clinical apathy research is focussed almost exclusively on effort-based paradigms. This is a crucial issue in preclinical apathy research for two reasons; firstly, as outlined in section 1.2, for a human patient to be diagnosed with apathy, their symptoms must span two domains. Testing animals in a single behavioural task that only pertains to a single domain limits interpretation of findings. Secondly, as outlined in section 1.4, depression and apathy are dissociated from each other in the domain of affect. The HAMD and AES did not correlate in measures relating to negative state and anxiety. To meaningfully dissociate them in pre-clinical research, the emotional-affective domain of apathy must therefore be considered.

### *1.8.1 Testing anxiety-related behaviour*

There are various ways to assess stress-induced anxiety-related behaviour in rodents, including the novelty supressed feeding test (NSFT). In this test, the rodent is food restricted for 24 hours, and then placed in a novel arena with a bowl of food placed in the centre. This task challenges the rodent

to overcome its natural aversion to a novel environment and approach the bowl and eat from it. The longer the rodent takes to eat from the bowl, the more anxious it is. This task was initially developed to test the efficacy of a range of anxiolytic treatments, including diazepam and amphetamine (Shephard and Broadhurst, 1982). It has also more recently been used in the study of various models of depression, where increased levels of anxiety are observed (Blasco-Serra et al., 2017).

#### *1.8.2 Sucrose/saccharin preference test*

Reduced reward sensitivity has been shown in PD patients with clinical apathy (Muhammed et al., 2016). Reward sensitivity in rodents, particularly in the context of depression and anhedonia, can be measured using the sucrose/saccharin preference test (SPT). In this test, the rodent is presented with a bottle containing normal tap water, and a bottle containing a low concentration (usually ranging from 1-2 %) of a sweet solution. Total liquid consumption is recorded, and the preference for the sweet solution over normal water is expressed as a percentage of total liquid consumed. A behaviourally normal rodent should prefer the sweet solution. Sucrose/saccharin preference has been shown to be reduced in models of depression, anxiety and dopamine antagonism (Szczypka et al., 2001, Planchez et al., 2019).

## *1.9 Using physiological changes as a measure for apathy*

There is evidence in the literature to suggest that physiological changes that occur in anticipation or response to a salient stimulus can be successfully used to study apathy.

#### *1.9.1 Aversion*

On encountering or experiencing an aversive stimulus, a number of physiological changes take place, including an increase in heart rate, blood pressure, breathing rate and the release of hormones including adrenaline and cortisol (Purves D, 2001). These physiological changes could be used as a measure of apathy, where emotional blunting may be related to low emotional arousal, characterised by low resting state of the autonomic nervous system. Perhaps the most widely used physiological measure in human participants is the use of skin conductance response (SCR). Enhanced SCRs have been shown to be associated with a variety of aversive contexts, including emotionally negative images, pain and psychological stress (Le et al., 2019). Though this approach is not yet widely used in studying apathy directly, this concept has been used to assess emotional blunting. For example, in a clinical population with frontotemporal dementia, in which apathy is a core part of its pathology (Rascovsky et al., 2011). The study used SCR to an unwarned auditory startle stimulus. It was shown that the frontotemporal dementia group had lower skin conductance levels (SCLs) compared to patients with AD and healthy controls, pre-, during and post-auditory startle, and that SCLs correlated negatively with scores on the Scale of Emotional Blunting, suggesting they are an appropriate measure of blunted emotional arousal (Joshi et al., 2014). SCLs

have been used in other studies investigating emotional regulation, including a population of patients suffering from post-traumatic stress disorder (PTSD) (Shepherd and Wild, 2014).

### *1.9.2 Reward*

A number of physiological changes in anticipation to reward occur, including changes in heart rate, pupil dilation and skin conductance response. These changes in the context of reward are termed motivational arousal. The use of these physiological changes in the context of apathy is limited, however one study demonstrated that pupil diameter increases with reward salience and using this parameter showed that elderly people have reduced reward sensitivity compared to younger healthy volunteers. Within this same study, it was shown that in a population of elderly patients with PD, those with apathy showed reduced reward sensitivity compared to patients without apathy. Interestingly this finding was linked to dopaminergic modulation, as when patients were tested on their dopamine medication, this difference in reward sensitivity disappeared (Muhammed et al., 2016).

Importantly, physiological measures such as these, including hormonal changes, can be translated to animal models. However, as highlighted above, similar physiological changes can occur as a result of both an aversive event and in anticipation of a positive one. As such, it is important to localise a physiological change to a specific behaviour.

## *1.10 Models of apathy*

An animal model of disease replicates the pathophysiological conditions found in the clinical population. Apathy is a difficult model to conceptualise because it often occurs alongside disease or with otherwise healthy ageing but does not represent a disease state itself. There is some debate over whether apathy is a symptom or a syndrome but most experts now consider it a syndrome due to its multidimensional nature (Husain and Roiser, 2018). The literature surrounding preclinical models of apathy is narrow and hindered by different behavioural approaches to testing with some groups favouring the 'ecological approach', conceptualised by (Cathomas et al., 2015) or effort-based operant paradigms described in section 1.7. What appears to be consistent however, is the focus on a single domain of apathy, which limits interpretation of findings and makes it difficult to dissociate from similar constructs such as a depression phenotype.

### *1.10.1 Pharmacological or stress-induced approach*

Chronic unpredictable mild stress (CUMS), the exposure of a rodent to stressful stimuli at unpredictable intervals, is a common way to model depression but has been suggested to induce apathy behaviour in rodents. A study showed that CUMS reduced both self-grooming behaviour, (previously suggested to reflect goal-directed behaviour and self-care) and reduced exploration of an open field arena (Cathomas et al., 2015). However, there is little evidence to suggest that apathy

is caused by chronic stress in the human literature. As such, this model has limited pathogenic validity, which refers to the similarity in processes that lead to a disease between human and animal (for more information see (Belzung and Lemoine, 2011)). In addition, without the inclusion of additional domains of apathy, it is unclear whether this model just picks up the motivational components of MDD, where the two constructs converge.

Dopamine has been widely cited as a crucial modulator of many aspects of motivated behaviour (see section 1.5). Though not explicitly described as an apathy model, goal-directed behaviour quantified by EBDM tasks can be pharmacologically reduced using dopamine depletion or dopamine receptor antagonists (Salamone et al., 2018) and these findings are often related back to clinical apathy due to similarities in resulting behaviours (Le Heron et al., 2018a). While to some extent, this mirrors conditions in which dopaminergic signalling is altered such as PD, this may not be relevant to ageing and other health impairments and, depending on route of administration (systemic vs targeted brain region), may have a blanket effect on overall motivation, making it difficult to unpick the dimensions of apathy-related behaviour relevant to a particular disease. Even if pharmacological manipulation is targeted to specific regions, the experimenter may be biasing the aspect of motivated behaviour that is affected, requiring expansive pre-existing literature on disease aetiology, which is not always available. As such, this approach, while useful in aiding understanding of normal motivated behaviour, has limited translatability.

#### *1.10.2 Neurodegenerative disease models*

The emerging consensus that neurological disease should not be treated as a whole, but rather as distinct symptoms, particularly those of a trans-nosological nature, has led to a rise in apathy research in the context of a wider disease. As such, a pre-clinical apathy model is most often a model of a neurological disease where apathy is known to occur in humans. This certainly has advantages over the pharmacological or stressor approach, as probing for apathy behaviour in a multi-faceted disease is more reflective of the human condition. A study by (Baumann et al., 2016) provides an excellent example of probing for apathy in a genetic mouse model of PD. A reduction in grooming and nesting behaviour alongside a reduction in reward sensitivity measured using the SPT indicated apathy behaviour across multiple domains. Measures of depressive behaviour were used to rule out a depression phenotype. The importance of a multidimensional approach is highlighted in other studies probing for apathy behaviour in rodent models of PD, where motivational deficits were found alongside increased anxiety and depression behaviours, suggesting a depression phenotype instead (Carnicella et al., 2014, Drui et al., 2014). Apathy-related behaviour has also been studied in a mouse model of HD, where apathy was concluded from a lower breakpoint in the progressive ratio task (Heath et al., 2019, Oakeshott et al., 2012). Studies using AD mice models report a reduction of interest in novelty and an increase in perseverative behaviour,

in line with apathy behaviour. Changes in anxiety behaviour however, are bidirectional, with some work showing an increase in AD models and some a decrease (Lalonde et al., 2012).

There is very little work on whether healthy aged rodents could be used as a model for apathy. As a result, is unclear if the components of apathy present in the same way in ageing as they would alongside neurological disease. One study provides convincing evidence for a motivational deficit in aged mice (Bordner et al., 2011), however only a single dimension of apathy is explored in this study.

### *1.10.3 Additional potential mechanisms: the role of neuroinflammation*

Chronic low-grade inflammation, characterised by elevated levels of C-reactive protein (CRP) is a pervasive feature of ageing (Sanada et al., 2018). There is some evidence to suggest that age-related inflammation is associated with apathy. A population cohort study found that increased CRP levels were associated with apathy symptoms but not depression in older individuals (Eurelings et al., 2015). A potential mechanism for this has been suggested, whereby inflammation induces deep white matter lesions, which in turn lead to the development of apathy (Yao et al., 2019). Therefore, induction of chronic low-grade inflammation may also serve as a preclinical model for apathy.

## *1.11 Section summary*

Apathy is a multidimensional construct that is common in neurodegenerative disease as well as healthy ageing. While there are some studies investigating apathy behaviour in models of neurodegenerative disease or pharmacological manipulation of the dopaminergic system, testing for apathy behaviour in healthy aged rodents has not been investigated. Most studies investigate apathy by quantifying motivated behaviour using EBDM tasks, explorative tasks or home cage observations and fail to consider other dimensions of apathy such as the emotional-affective component. A multidimensional approach to the preclinical study of apathy is important as it allows for dissociation from similar constructs such as depression.

# *2. Biological rhythms*

With each rotation of the earth, humans and other surface-dwelling organisms are exposed to a daily rhythm of light and dark. This daily cycle drives a temporal organisation of behaviour, where most of the physical activity and feeding behaviour are confined to a certain phase of the 24-hour period depending on whether the organism is diurnal or nocturnal. This in turn imposes specific demands on the physiological system, where surges in energy requirement must be anticipated. As such, many biological processes, including hormone release, core temperature regulation and intracellular protein synthesis, occur in temporal oscillations of approximately 24hrs, termed circadian rhythms. Circadian clocks are characterised by their self-sustaining rhythms, which are temperature-

compensated and are entrainable to periodic external stimuli (Astiz et al., 2019). As individuals age, their biological rhythms become shifted or dampened. Though some changes in circadian rhythms represent an inevitable and natural part of ageing, some may reflect a pathological process that correlates with age (Hood and Amir, 2017).

## *2.1 The mammalian circadian clock system*

### *2.1.1 Overview*

At its broadest level, the mammalian clock system consists of three core parts: an input pathway, the core circadian clock, and an output pathway (**figure 3**). The input pathway refers to an external signal that is communicated to the core circadian clock. The change from light to dark over the 24hr period is the most potent external signal. The core circadian clock or 'central pacemaker' refers to the suprachiasmatic nucleus (SCN), a tiny region of the hypothalamus that undergoes endogenous oscillations entrained to the light-dark cycle. The output pathway refers to the nature in which the SCN communicates time of day information to almost every cell and tissue in the body. This is achieved through a variety of neuronal and humoral mechanisms. The three components of the mammalian clock system are described in more detail below.

**Figure 3**

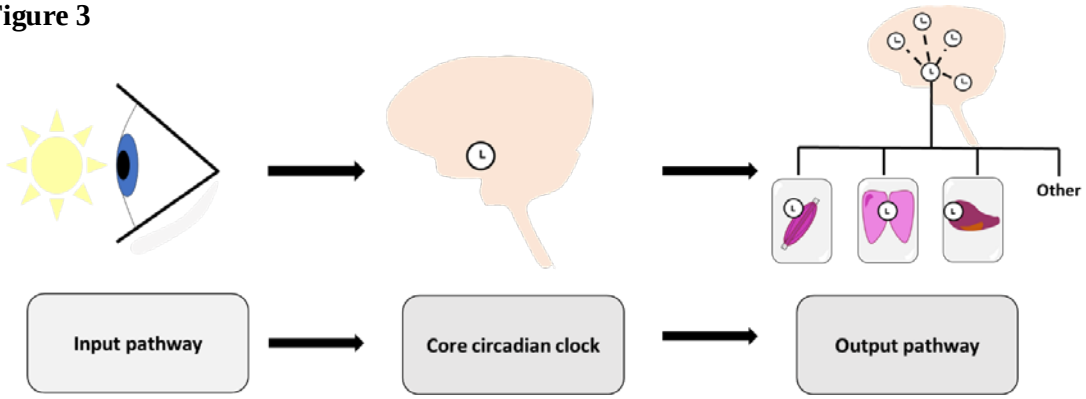


***Fig.3. The basic structure of the mammalian clock system.*** *A circadian clock system requires an input pathway (the retina), a core circadian clock (the suprachiasmatic nucleus) and an output pathway (a variety of humoural and neural signals).*

### *2.1.2 The input pathway*

The SCN receives a range of external stimuli capable of entraining the circadian clock. These external stimuli are referred to as zeitgebers, or 'time-givers'. The most potent of these external stimuli is light. Light information travels through the retina and is received by photosensitive

retinoganglionic cells (pRGCs). This induces expression of melanopsin on pRGCs which drives a series of molecular processes (for full review see (Xie et al., 2019)) including the release of glutamate, which is the main signal for photic entrainment. This information is communicated directly through the retino-hypothalmic tract to the SCN, which in turn drives the transcription of two of the core molecular clock components (see section 2.2). Non-photic stimuli is received by the SCN via the intergeniculate leaflet and geniculohypothalamic tract projections (Astiz et al., 2019).

### *2.1.3 The suprachiasmatic nucleus*

In a series of early studies, the importance of the SCN as the central pacemaker was demonstrated. Bilateral lesions of the SCN to the rodent brain resulted in complete loss of rhythmicity of physiological processes, including locomotor activity, temperature, hormone release and consumption of food/drink. The concept that the SCN is the only endogenous autonomous oscillator and is thus the 'central clock' has been revised over the years, when new evidence emerged that some peripheral tissues, including the liver and pineal gland showed the ability to entrain to light-dark cycle in the absence of the SCN (Cailotto et al., 2009). Additionally, peripheral tissues have been shown to remain entrained to the light-dark cycle following disruption to the SCN (Husse et al., 2011). Non-photic zeitgebers, such as feeding time, also entrain peripheral oscillators independently of the SCN (Sujino et al., 2012, Webb et al., 2009). As such, the SCN is now viewed more as a central synchroniser than a central clock.

### *2.1.4 The output pathways*

It is essential that the SCN synchronises the peripheral tissues and cells so that the entire physiology is synchronised to the light-dark cycle. This includes preparing the body both for releasing hormonal signals in a periodic manner, but also ensuring that tissues are sensitive to these signals at the appropriate times. This occurs first at a local level, where the SCN projects to areas of the dorsal medial hypothalamus (DMH) (primarily the paraventricular nucleus of the hypothalamus (PVN)), where specific cell groups mediate both the release of CRH (corticotrophin releasing hormone) to activate the hypothalamus-pituitary-adrenal (HPA) axis and thus the release of corticosterone (CORT) (cortisol in humans, corticosterone in rodents), and autonomous control (Buijs et al., 2006). Tracing studies have shown that the hypothalamic SCN has parasympathetic and sympathetic autonomic outputs to a variety of organs in the body. It has been proposed that it is through these autonomic neural pathways that the SCN sensitises the organs to hormonal release. Perhaps the best example of circadian coordination of both hormonal and autonomic signals is the release of CORT. CORT is released in a circadian pattern via the HPA axis, where it peaks in nocturnal animals just before onset of the dark phase and reaches its nadir in the light phase. In

diurnal animals it peaks just before onset of the light phase (see **figure 4**). The SCN controls the rhythmic release of CORT via release of adrenocorticotrophic hormone (ACTH) from the pituitary, induced by the rhythmic release of corticotrophin releasing hormone (CRH). ACTH induces release of glucocorticoids from the adrenal cortex. In parallel, via the autonomic nervous system, the SCN enhances sensitivity to ACTH at the adrenal cortex by synchronising the adrenal clocks (Oster et al., 2017).

**Figure 4**

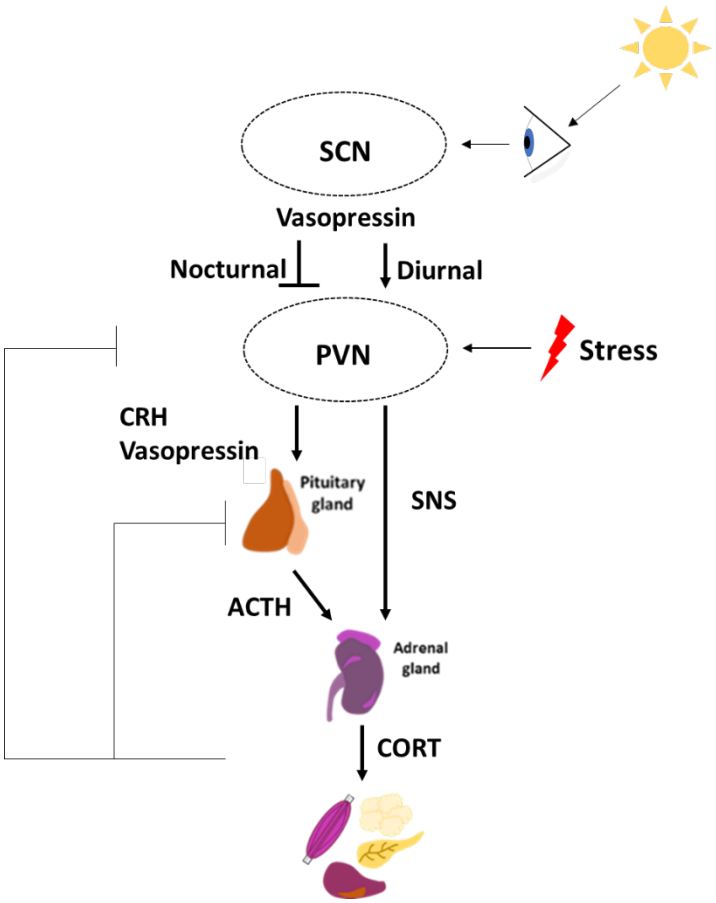


***Fig.4. Circadian release of CORT.*** *The rhythmic release of CORT is under control of the SCN, which is entrained by external light. Vasopressin in the SCN mediates PVN output via a GABAergic synaptic route. In nocturnal animals, vasopressin has an inhibitory effect on the PVN, resulting in reduced CORT release in the daytime. In contrast vasopressin stimulates the PVN in diurnal animals, increasing CORT release during the daytime. Once stimulated, either by the SCN or stress, CRH and vasopressin are released from neurons in the PVN and binds to receptors in the pituitary gland. ACTH is then released from the pituitary gland and binds to receptors in the adrenal gland. Cortisol/corticosterone is released from the adrenal glands and binds to receptors in the periphery where it exerts metabolic effects, as well as the pituitary and PVN where it inhibits its own release. ACTH- adrenocorticotrophic hormone. CORT-corticosterone (rodent), cortisol (human). CRH-*

*corticotrophin releasing hormone. PVN-paraventricular nucleus. SNS-sympathetic nervous system. SCN-suprachiasmatic nucleus of the hypothalamus.*

## *2.2 The molecular clock*

The entrainment of the SCN to the light/dark cycle occurs at the molecular level, where the circadian clock is driven by a transcriptional-translational feedback loop (TTL) (see **figure 5**). Described at its most basic level, the mammalian TTL consists of a positive arm and a negative arm. The positive arm consists of two key transcription factors: brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK). These transcription factors dimerise and initiate the expression of the negative arm genes, period (*per)-1/2* and cryptochrome (*cry)1/2*. PER and CRY dimerise and translocate to the nucleus from the cytoplasm, where they supress their own transcription by inhibiting the transcriptional activity of CLOCK:BMAL1. This dimer also induces the expression of many other genes by binding to the E-box of the target gene promoter region, including *Reverse-erythroblastosis virus (RevErbα/β)* (part of an 'accessory' loop). Or, indirectly through the oscillation of clock controlled output genes (Astiz et al., 2019). It is through this TTL mechanism that thousands of genes show rhythmic expression. It should be noted that the molecular clock is far more elaborate than described here. For a more in-depth review of the molecular clock see (Buhr and Takahashi, 2013). It is the stability of the negative arm that governs the phase of the clock. In particular, the PERs, as they are responsive to the induction of light (Albrecht et al., 1997). Using mice, it was shown that light induces the transcription of *per1/*2 and, depending on when light is induced in the night when light levels should be low, onset of behaviour can be delayed or advanced. It is this change in behaviour onset that demonstrates that the molecular clock entrains to the light-dark cycle (Buhr and Takahashi, 2013).

**Figure 5**

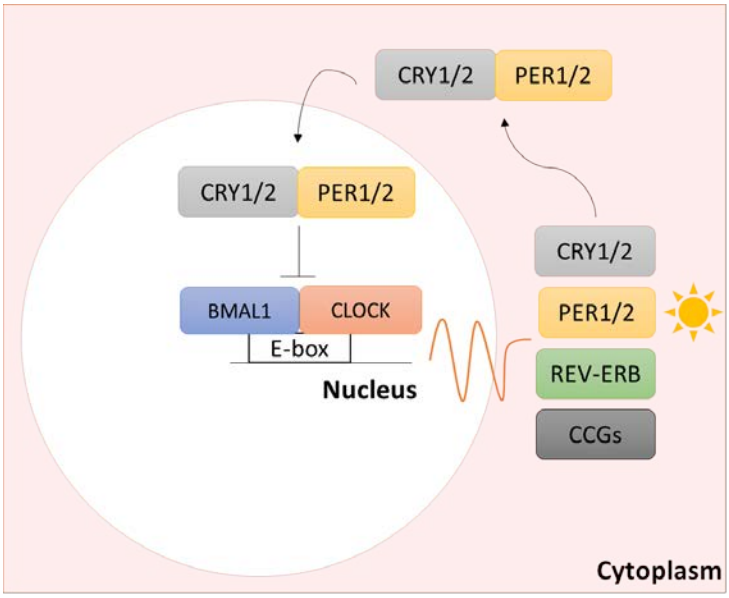


***Fig.5. The mammalian molecular clock.*** *BMAL1 and CLOCK heterodimerise and bind to the E-box in the promoter region of target genes, including cry1/2, per1/2, rev-erb and ccgs (among others), initiating their transcription. CRY1/2 and PER1/2 dimerise and translocate to the cell nucleus, inhibiting the transcriptional activity of BMAL1/CLOCK and therefore their own transcription. Thus, the molecular clock is a transcriptional-translational feedback loop. Per1/2 are also responsive to the induction of light. CCG-clock controlled gene.*

## *2.3 Corticosterone*

CORT has wide-ranging physiological functions, in both body homeostasis and stress regulation. CORT is released in a circadian pattern, where it peaks just before onset of the active phase and reaches its nadir in the rest phase. CORT also shows an ultradian (hourly) pattern of release (Kalafatakis et al., 2018). As described above, the rhythmic release of CORT is controlled by the SCN, but also plays an important role in entraining the peripheral clocks.

### *2.3.1 Glucocorticoid regulation of peripheral clocks*

Glucocorticoids have a powerful effect on the phase of peripheral clocks and exert their effects via the glucocorticoid receptor (GR), which is expressed in almost every tissue of the body. Administration of dexamethasone, a GR agonist, shifted the phase of the mouse liver clock *in vivo* but this effect was not seen in mice with a liver-specific disruption to the GRs (Balsalobre et al., 2000). The effect of glucocorticoids on peripheral oscillators may be tissue specific. A study showed that adrenalectomy disrupted the phase of the molecular clock in the liver, kidney and

cornea, but not other tissues such as the lung. This suggests some tissues are more sensitive to glucocorticoids than others (Pezük et al., 2012).

Glucocorticoids can also modulate oscillators in other regions of the brain. *Per2* expression is blunted in the central amygdala and bed nucleus of the stria terminalis in adrenalectomized rats, and this rhythm is restored by reinstating the CORT rhythm (Segall et al., 2006). In contrast, adrenalectomy has no effect on the SCN (Pezük et al., 2012). There are several ways in which glucocorticoids can modulate the molecular clock. They can directly entrain the clock by regulating the expression of core clock genes including *Per1* and *Per2* by binding to glucocorticoid response elements (GREs). Alternatively, glucocorticoids can be gated. For example, the clock gene CLOCK can also negatively regulate glucocorticoid transcriptional activity periodically via acetylation of GR, acting as a 'gate' for glucocorticoid modulation (Nader et al., 2009).

#### *2.3.2 The glucocorticoid and mineralocorticoid receptors*

Glucocorticoids exert their effect via the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These nuclear receptors are ligand-binding transcription factors that reside in the cell cytoplasm until binding, where they translocate to the nucleus to exert effect. Both GR and MR have structural homology but are bound by glucocorticoids under different conditions. While GR is expressed throughout the brain, MR is confined to the limbic structures, including the hippocampus and amygdala (Reul and Kloet, 1985). It is thought that GR mediates response to peak concentrations of glucocorticoids under stressful conditions, while MR, due to its high affinity for glucocorticoids, mediates response to lower levels of glucocorticoids at the nadir of the circadian cycle. In this way, MR occupation sets the threshold for HPA activation. CORT also has a rapid-acting effect that cannot be attributed to genomic MR and GR. It has been suggested that a membrane-bound MR can regulate these rapid-acting effects. Due to this role, membrane-bound MR may play a key role in stress susceptibility and psychiatric disease (ter Heegde et al., 2015). Indeed, loss of MR expression is consistently reported in psychiatric disease and correlates with disease duration (Xing et al., 2004). The MR:GR ratio is thought to be an important regulator of behavioural adaptation and appropriate stress reactivity (de Kloet, 2014). MR is particularly sensitive to the effects of age, where increasing age is associated with reduced MR expression in frontal structures of the brain.

## *2.4 Age-related changes in biological rhythms*

A healthy circadian system is characterised by stability, a strong amplitude of change, and is in phase with other biological processes (Weinert and Waterhouse, 2007). Discussed below are age-related changes to the three of the main circadian rhythms relevant to this thesis, however many

more important age-related disruptions to biological rhythms have been reported. See (Hood and Amir, 2017) for a full review.

### *2.4.1 The sleep-wake cycle*

The circadian control of sleep makes up one half of the sleep-wake regulatory system, with the other being the homeostatic sleep challenge (Duffy et al., 2015). Changes to the circadian sleep-wake cycle in ageing have been well reported (Duffy et al., 2015). The most prominent change in the aged sleep-wake cycle is a shift to an earlier chronotype. One study tracked chronotype longitudinally for 23 years across 567 adult men and found a reliable shift to a morning chronotype (or, 'early bird') with increasing age (Roenneberg et al., 2007). This shift to an earlier chronotype is not explained by a shortening of the circadian period (Duffy et al., 2011). However, elderly people are exposed to different patterns of light compared to younger adults due to changes in lifestyle, general mobility or confinement to a care home (Scheuermaier et al., 2010). The nature of animal work carries an advantage, such that living conditions can be controlled and so any change in response to light is more likely a change to circadian output.

Early animal work has shown that aged hamsters and mice show a decreased response to pulses of light, unrelated to changes in morphology of the lens, RHT or SCN, but could be due to changes in glutamatergic signalling in response to photic stimuli (Zhang et al., 1996, Biello, 2009). Numerous animal studies have also reported a reduced amplitude of change in activity across the activity-rest cycle and there is also evidence for an altered phase of entrainment, where the onset of locomotor activity is altered with respect to lights out (Valentinuzzi et al., 1997, Davis and Viswanathan, 1998, Penev et al., 1997). However, the nature of this change to entrainment differs between studies with one reporting activity onset to occur earlier than light change and another reporting activity onset to be delayed (Zee et al., 1992, Valentinuzzi et al., 1997).

### *2.4.2 Core body temperature*

In young, healthy adults, core body temperature peaks in the early evening and reaches a nadir in the early morning. The SCN controls circadian changes in core body temperature by inducing heat loss and heat gain mechanisms via the hypothalamic thermoregulatory centres (Weinert and Waterhouse, 2007). Core body temperature is an important synchroniser of the peripheral clocks as well as aspects of normal daily functioning, including sleep quality (Dijk and Czeisler, 1995) and mental/physical performance (Waterhouse et al., 2005).

It has been reported in both rodents and humans that ageing is associated with a reduced amplitude of change in core body temperature as well as an advanced phase shift (Halberg et al., 1981, Czeisler et al., 1992). However, some rodent studies have not found a decrease in amplitude and if phase shifts are corrected for in the aged human literature, then change in amplitude disappears (Gubin et

al., 2006). It has also been suggested that these apparent differences are due to changes in motoric ability and lifestyle differences, rather than any changes to circadian output, and when this is accounted for, age-related changes in core body temperature disappear (Weinert and Waterhouse, 2007). It has therefore also been proposed that circadian core body temperature in ageing is relatively stable and highlights the heterogeneity in findings.

### *2.4.3 CORT*

Compared to the sleep-wake cycle and core body temperature, there is relatively little work on changes to the CORT circadian rhythm in ageing, presumably because repeated hormonal sampling is methodologically more difficult. However, given its wide-ranging physiological function and role in entraining peripheral clocks, it is important that age-related changes in CORT rhythm and an underlying molecular mechanism be elucidated.

Early evidence in the human literature suggests a flattening of CORT circadian amplitude in ageing (Van Cauter et al., 1996, Hood and Amir, 2017). One suggested reason for this blunting is that basal cortisol secretion is enhanced in ageing, thought to result from loss of glucocorticoid negative feedback (Herman et al., 2016).

The use of rodents would allow for analysis of molecular changes in the hypothalamus in concordance with changes in HPA activity. There has been some effort to explore the impact of age on CORT circadian rhythm in aged rodents, however the results are heterogenous and the studies limited by a small number of time points and disruptive sample collection which in itself would produce a CORT response (Issa et al., 1990, Herman et al., 2016). The use of automated blood sampling, whereby blood samples are taken automatically from the freely moving animal would provide a more meaningful profile without the confounds of stress induced by sampling (Windle et al., 1998).

### *2.4.4 Changes to the SCN*

An important role for the SCN in age-related decline in circadian rhythmicity was demonstrated when a foetal graft containing SCN was transplanted into the third ventricle of aged rats, improving circadian rhythms in rats with successful grafts (Li and Satinoff, 1998). Rodent studies have reported an age-related reduction in SCN output. It has been shown that day-night variation in neuronal firing in the aged mouse SCN is diminished. This translates to circadian behavioural output, where day-night locomotion amplitude is also weakened. This is supported by evidence that amplitude is similarly reduced in the subparaventricular zone (SPZ), the main nerve projection path from the SCN to the DMH, where circadian behaviour output is regulated. Individual cell analysis of the SCN showed that rhythmicity within the cell was conserved, but were out of rhythm with each other, suggesting that the SCN neural network synchronicity is weakened and in turn weakens

behavioural output (Nakamura et al., 2016). Neuropeptides, including VIP, play an important role in synchronising the SCN neural network. It was shown that aged rats show an overall lower expression of VIP in the SCN, and show no circadian change in VIP, compared to younger rats where VIP was high in the dark phase and low in the light phase (Kawakami et al., 1997). This could contribute to the reduced synchronicity in the aged SCN. As well as electrophysiological changes to the aged SCN, metabolic changes such as differences in glucose utilisation have also been reported (Wise et al., 1988).

#### *2.4.5 Changes to the molecular clock*

While it is clear that ageing impacts the SCN at a neuronal level, ageing appears to have very little effect on the SCN molecular clock (Yamazaki et al., 2002, Asai et al., 2001, Mattis and Sehgal, 2016), aside for evidence for a reduction in expression of *Bmal1* and *Per2* (Chang and Guarente, 2013). Work in aged primates showed no change in core clock gene expression at either the mRNA or protein level (Eghlidi et al., 2018). Evidence for age-related changes in the molecular clock of peripheral tissues is heterogenous. While some studies suggest changes to the amplitude or phase of core clock genes in some tissues such as the liver, others show that clock gene expression and rhythmicity is relatively well conserved in ageing in many tissues including the heart, lungs, pineal gland and PVN (see (Welz and Benitah, 2019) for full review). This heterogeneity likely comes from studies only focusing on one clock gene at a time or focusing exclusively on either the RNA or protein level. It is also likely that some tissues age at different rates to another. While it seems that overall, there is little change in the core clock machinery in most tissues, these experiments are often done in standard, unchanging laboratory conditions. While controlled conditions are often an advantage of pre-clinical work, this undermines the nature of circadian rhythms to enable the organism to flexibly adapt to a changing environment. It may be here that age-related changes emerge. Indeed, there is evidence to suggest that aged peripheral tissues are slower to adapt to changes in the environment. A study showed that phase resetting of *Per1* in the liver of aged rats to a new light cycle was seriously disrupted compared to younger rats, but *Per1* in the SCN reset normally. This suggests that age does not drastically disrupt the clock machinery, and changes to the periphery may be a result of changes to communication between the SCN and periphery, including a weakening of SCN output. It should be noted that majority of work on age-related changes to the molecular clock occur in non-human species, due to relative ease of tissue availability.

## *2.5 The feeding zeitgeber*

#### *2.5.1 Feeding anticipatory activity*

In a rapidly changing environment, organisms must be able to flexibly adapt to changes in availability of a feeding source to increase the likelihood of survival. Food intake is a potent zeitgeber and a potent synchroniser of the peripheral clocks. Interestingly, the system of feeding entrainment is independent of the SCN and the location of the feeding entrainable oscillator (FEO) remains elusive (see f**igure 6**). The presence of the FEO was conceptualised due to a clear behavioural output: presence of feeding anticipatory activity (FAA). When rodents are put on a restricted diet and fed in the same time window each day, rodents increase their level of locomotor activity and body temperature 2-3hrs preceding timed feeding even if the food is presented in their usual resting phase. Locomotor activity refers to wheel running, unreinforced lever pressing and general cage activity (Mistlberger, 1994). There is also an anticipatory peak in circulating hormones, including CORT (Namvar et al., 2016). This is likely an evolutionary adaptation that allows animals to obtain food even at unusual times relative to their normal activity-rest cycle. Like light-entrained activity, FAA persists in constant conditions (i.e., fasting, not *ad libitum* feeding where it promptly disappears, presumably due to a dampening of the oscillator) and occurs over many daily cycles.

**Figure 6**

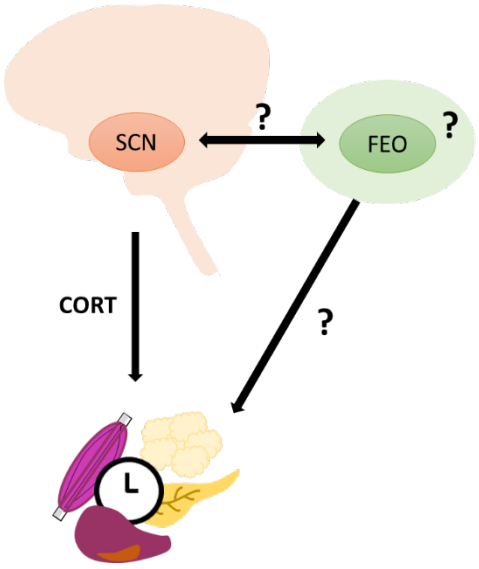


***Fig.6. The interaction between the FEO and peripheral clocks.*** *The FEO has a direct effect on the peripheral clocks, independent of the SCN. The SCN also has a direct effect on the peripheral clocks mediated by the rhythmic release of CORT. The location of the FEO and the mechanism by which it controls the peripheral clocks remains unknown. CORT-corticosterone, FEO-food entrainable oscillator, SCN-suprachiasmatic nucleus.*

### *2.5.2 Is FAA circadian?*

The search for the FEO has persisted over 30 years and still has not yet been elucidated. Bilateral lesioning of the SCN has no effect on FAA and in turn, timed feeding has no effect on SCN

rhythmicity. Therefore, the FEO and the SCN are completely independent systems. In addition, the FEO does not seem to require normal functioning of the known circadian molecular machinery (Storch and Weitz, 2009). One of the hallmarks of circadian rhythms is their persistence in constant conditions. This makes the FEO exceptionally difficult to study because in the context of feeding, this would mean constant food deprived conditions, which clearly aren't sustainable in rodent models. To get around this, studies often shift the timing of feeding or adjust the period of when the food is delivered. In this way it was shown that to generate FAA, food had to be delivered in approximately 24hr intervals. Meals separated by 19hr or 29hr did not generate FAA, showing that FAA is circadian (Bolles and De Lorge, 1962, Bolles and Stokes, 1965). Furthermore, it was shown that rats would exhibit normal levels of FAA in fasting conditions. Together, this evidence points towards the presence of a self-sustaining oscillator rather than an associative learning mechanism, or an internal cue mechanism, in which locomotion is activated when energy levels deplete past a certain threshold.

### *2.5.3 The effect of timed feeding on peripheral clocks*

If a rodent is fed a restricted diet at the same time each day, the SCN eventually becomes uncoupled from the peripheral clocks, but responds normally to light. Restricted feeding profoundly alters the phase of clock genes in the peripheral tissues. Within several days, restricting feeding to the normal rest phase completely inverses the phase of clock gene expression in the liver compared to mice fed *ad libitum* (Damiola et al., 2000). These findings extended to other tissues, including the heart, lungs, kidney and pancreas. It has been suggested that restricted feeding-induced phase shift of peripheral clocks is mediated by glucocorticoids. Glucocorticoids are attractive candidates given a) their previously established role in synchronising the SCN with peripheral clocks (see section 2.3), b) many peripheral tissues express GRs but SCN neurons do not, and c) parallel effects of restricted feeding and dosing with glucocorticoids on peripheral clock gene expression (Balsalobre et al., 2000). However, a direct link has not been established.

### *2.5.4 A role for CORT in FAA*

Given its importance in synchronising the SCN with the peripheral clocks, it stands to reason that CORT would be involved in FAA. CORT has been shown to rise in anticipation of a schedule feeding time (Namvar et al., 2016). However, it is not clear whether this peak in CORT is necessary for normal FAA. The evidence is conflicting, with some studies showing that adrenalectomised (ADX) rats (unable to produce CORT) were not able to adapt to a feeding schedule at both a physiological or behavioural level and had increased mortality (Kaul and Berdanier, 1975). A more recent study also supported this, where it was shown that ADX, food restricted rats did not show FAA, but administration of CORT increased FAA in a dose-dependent manner (Duclos et al., 2009).

In contrast, another study showed that ADX rats could form FAA, however an injection of CORT at a different time point did delay FAA relative to control, suggesting CORT does play a role, but potentially with additional humoural factors (Sujino et al., 2012).

#### *2.5.5 FAA is a motivated behaviour*

This anticipatory activity is an example of motivated behaviour at its most fundamental level and blends the study of reward and motivation, with biological rhythms. As mentioned above, FAA does not require normal functioning of the molecular clock. Mutants lacking several of the core clock genes, including *Clock*, *Cry* and *Per* showed normal FAA (Pendergast and Yamazaki, 2018). However, mice with disrupted dopamine signalling (at the D1 receptor) do not exhibit normal FAA, while other neurotransmitter mutants showed normal FAA. Therefore, FEO may lie somewhere in the dopaminergic circuitry (Pendergast and Yamazaki, 2018). As previously described in section 1.6, dopamine is a key neurotransmitter in motivated behaviour. Mice developed anticipatory activity to timed dosing of a D1 agonist (SKF-81297), timed highly palatable meals (even under *ad libitum* feeding conditions) and stimulant drugs (Gallardo et al., 2014, Mistlberger and Rusak, 1987, Keith et al., 2013). This evidence suggests that mice show anticipatory locomotor activity for rewarding events that are not necessarily needed for survival and suggests that motivated behaviour has a strong circadian component.

#### *2.5.6 FAA can restore normal activity-rest rhythms*

Normal circadian activity-rest behaviour is disrupted in a number of disease states, where locomotor activity shows a blunted amplitude of change in activity across the 24-hr period. There is evidence to suggest that when the SCN is disrupted, the FEO replaces it as the 'central synchroniser' and can restore normal rhythmicity. A study using a mouse model of HD showed this model had a dysrhythmic activity-rest cycle due to disrupted circadian gene expression in the SCN. However, the FEO was found to remain completely intact, and when the mice were put on a restricted feeding diet, normal rhythmicity was restored, where the new peak in activity came just before the onset of feeding (Maywood et al., 2010). This suggests that in disease states where the SCN is weakened, timed feeding could serve as a therapeutic intervention for restoring normal rhythmicity.

## *2.6 Additional zeitgebers*

*Social cues*

Socialising is a key part of survival in many different types of species, with some species developing highly organised social structures that require some form of temporal organisation to efficiently gather food or defend from predators. It is not clear whether social cues are a zeitgeber in themselves, or that they drive other zeitgebers or create a non-specific arousal effect. For example, there is

evidence to suggest that postnatal rat pups entrain to their mother's rhythm even if this rhythm is asynchronous. However, this may be mediated by periodic food intake, rather than an alternative cue, such as an olfactory signal (Mistlberger and Skene, 2004a). In an early study, it was shown that a brief antagonistic encounter between hamsters could induce a phase shift in activity. However, it was later shown that any kind of non-social stimuli, like cage-cleaning, could produce the same effect, indicating adaptation to a non-specific arousal effect (Mrosovsky, 1988). It is not clear from the literature whether organisms would build anticipatory activity to a social cue if it was presented at the same time every day and whether it would persist once this cue was removed, in the same way as the experimental approach to timed feeding. It is also likely that results would be specific to the organism tested, as different laboratory animals have drastically different social hierarchies and social behaviours.

## *2.7 Section summary*

Age-related blunting of circadian rhythms, including the sleep-wake cycle, core body temperature and CORT, have been reported. However, the findings of these studies are heterogenous. These changes do not appear to be caused by overt changes to the molecular clock but may be due to changes in SCN synchronicity. While light is the most potent entrainer of circadian rhythms, feeding time is also important and acts via a central oscillator distinct from the SCN. Ageing does not affect FAA and feeding time has been shown to restore or strengthen circadian rhythms in animal models.

# *3 Circadian variations in behaviours relevant to apathy*

## *3.1 Circadian variation in affective state*

Affective state refers to the underlying emotional state and is distinct from the mood state, which reflects longer term experiences. It can alter the way information is perceived, selected and modulated (Hinchcliffe et al., 2017). There is evidence to suggest that positive affective state shows a diurnal rhythm. Participants were required to complete a mood rating form 7 times a day for a week. It was found that positive affect rose from early morning and peaked around noon and fell towards the evening. Conversely, negative affect remained stable across the day (Clark et al., 2009). Disruption to normal biological rhythms may alter normal affective processing.

## *3.2 Circadian variation in reward behaviour*

Time of day influences the behavioural response to reinforcing stimuli. A study showed that when mice were presented with a palatable substance (peanut butter) or a reinforcing drug

methamphetamine, they showed greater anticipatory activity, general intake and post-ingestion activity when it was presented at ZT4 compared to ZT10 (Keith et al., 2013). ZT refers to 'zeitgeber time' which indicates time in a 24hr entrained cycle. ZT0 refers to start of the light phase. Consumption and seeking of a rewarding stimulus show a clear diurnal rhythm. Most hedonic behaviours peak in the middle of the active phase, where chances of rewarding stimuli being presented are high. It has been shown that mammals self-administer drugs of abuse in a diurnal fashion, with increased administration in the active phase (Webb et al., 2009). It was also shown that electrical brain self-stimulation in rodents peaks mid-late active phase, and this rhythm persists in constant conditions (Terman and Terman, 1970). Sucrose preference also shows circadian variation. Mice consumed more 5% sucrose solution in the active phase compared to the rest phase, but normal water consumption showed no diurnal rhythm. This pattern of sucrose consumption and % sucrose preference persisted in constant dark conditions. However, *Per1/2* mutants did not exhibit a diurnal rhythm in either sucrose consumption or % preference (Bainier et al., 2017). This suggests that reward sensitivity has a circadian component modulated by clock genes and disruption to normal biological rhythms may disrupt normal reward processing.

## *3.3 The role of dopamine in circadian rhythms*

As discussed in previous sections, dopamine is a neurotransmitter that is critically involved in the regulation of motivated behaviour and there is evidence to suggest that its release is under circadian control. Using real-time bioluminescence monitoring, a study showed that the transcription of a key enzyme in dopamine metabolism, monoamine oxidase A (*mMaoa),* shows a circadian rhythm of transcriptional activity. Using mice, it was shown *in vivo* that a core clock gene, BMAL1, directly interacts with this enzyme in a circadian manner, in the ventral tegmental area (VTA). Rhythmic expression of *mMaoa* was also found *in vivo* in the VTA, and this was disrupted in *Per2* mutant mice as well as an overall reduced activity and expression of the enzyme (Hampp et al., 2008). This loss of rhythm reflects loss of sucrose preference in *Per2* mutants described above. As well as dopamine metabolism, other aspects of dopaminergic activity, including synthesis, release and neuronal firing also show diurnal variation (McClung, 2007).

As well as being under circadian modulation, dopamine release has also been described as a core modulator of circadian rhythms. It has also been shown to modulate clock gene expression. When dopamine is released in response to a rewarding stimulus e.g. sucrose intake, or a drug of abuse, its release and subsequent activation of dopamine receptors results in the induction of *Per* transcription (Bainier et al., 2017). Beyond the context of reward, dopamine is an important circadian modulator of other regions of the central nervous system, including the retina. The retina is crucial to the entrainment of the SCN to the environmental light (Korshunov et al., 2017). Therefore, disruption

to dopamine biological rhythms may impact on motivated behaviour or alternatively, disruption to dopaminergic signalling may disrupt normal biological rhythms.

## *3.4 Existing evidence for circadian rhythm disruption in psychiatric disease*

Psychiatric disease and disrupted biological rhythms are closely linked. Much of this research has focussed around MDD. Depressed patients show a reduced amplitude of change in core body temperature and more variability in cortisol secretion and irregular patterns on melatonin release. It has been proposed that the low mood experienced with depression results from a phase shift in the SCN, thus bringing rhythms out of phase with each other (Germain and Kupfer, 2008). While MDD is distinct from apathy as previously described, this literature highlights that disruption to biological rhythms can have a profound impact on psychological health and result in mood disorders. There is comparatively very little work on the relationship between apathy and disrupted biological rhythms. However, it has been reported that apathy scores in patients with dementia correlated with day-night activity amplitude, where higher apathy scores were associated with lower daytime activity (Kuhlmei et al., 2013). In addition, a distinct activity-rest profile was found in apathy patients with AD (Zeitzer et al., 2013) providing some evidence for a link between circadian disruption and apathy.

Modulation of disrupted biological rhythms in depressed patients has been shown to have some therapeutic benefit. For example, light therapy is used as a first-line treatment for seasonal affective disorder (SAD), where patients are exposed to bright white light in the early morning to extend the photoperiod. This method has been shown to improve depression symptoms as measured by the Hamilton Depression Rating Scale (HAM-D) (Campbell et al., 2017). Sleep deprivation has also been shown to have clinical effect in depression. It has been reported that staying awake for 24 hours improves depressive symptoms in 60% of depressed patients the following day (Voderholzer, 2003), showing that non-pharmacological interventions can be used to modulate biological rhythms successfully to clinical benefit.

## *3.5 A potential relationship between apathy and disrupted biological rhythms in ageing*

Considering the evidence that biological rhythms become disrupted in old age, evidence that psychiatric disease can be driven by biological rhythm disruption, and the apparent circadian variation in motivated behaviour and affective state, there may be a relationship between apathy and disrupted biological rhythms in otherwise healthy ageing. This would provide a more specific neurobiological mechanism to explore, beyond global structural changes and changes in functional activity described so far (see section 1.5). This would also provide a target for therapeutic

intervention. At present, existing evidence points towards a relationship between disrupted biological rhythms and apathy in ageing but there is no work exploring a causative mechanism. Potentially, a disrupted activity-rest cycle, driven by a disrupted circadian system at the level of the SCN (asynchrony in neuronal firing, see section 2.4.4) reflects a disruption to the circadian rhythm of motivated behaviour. Indeed, waking activity is driven by motivated behaviours such as socialising, eating and working. Apathy may potentially be a consequence of a chronic disruption to normal circadian variation in reward behaviours and dopaminergic activity, resulting in changes to aspects of emotional and reward processing that result in a blunted behavioural output. By re-entraining or strengthening these circadian rhythms, apathy may be treated. To manage apathy non-pharmacologically would be of huge value to the aged community, where adverse events relating to polypharmacy can negatively impact on health (Charlesworth et al., 2015). As described in section 3.1.2, there are ways in which biological rhythms can be strengthened or restored that has resulted in clinical benefit. Therefore, the exploration of a relationship between disrupted biological rhythms and apathy in ageing would be valuable but has not yet been attempted.

## *3.6 Section summary*

Behaviours relevant to apathy show circadian variation, evidenced at a behavioural and molecular level. Disruption to circadian regulation leads to a disruption in reward behaviour, and dopamine is both under circadian control and acts as a modulator of core circadian clock genes, suggesting a close link between reward, motivated behaviour and biological rhythms. Considering this evidence, there may be a relationship between apathy and disruption to normal biological rhythms in otherwise healthy ageing.

# *4. The use of rodents as a model for ageing*

## *4.1 How translatable is time?*

A common question relating to the use of aged rodents in research is firstly *at what age is a rodent "aged"* and secondly, *how does a rodent's age translate to that of a human*? Despite the widespread use of rodents in biomedical research, very little work has been done to address this question. The lifespan of a human varies widely between country, demographic and sex, with some countries reporting a maximum life expectancy in the late fifties, and others the early eighties (Disabled World, 2020). Lab rats and mice live comparatively accelerated lives of 2-3.5 years, depending on strain and sex (Sengupta, 2013). Some groups use physiological measures such as eye lens weight, tooth growth and musculoskeletal examination to correlate rodent age with that of a human.

However each of these measures are relatively imprecise and are often used in combination to provide a more accurate answer (Sengupta, 2013).

Developmental phases of the rodent show striking temporal differences to that of a human and so, depending on phase of life, a rodent "year" versus a human "year" may differ considerably. Using developmental markers such as age of weaning, sexual maturity, the end of musculoskeletal growth and reproductive senescence, work by (Sengupta, 2013) shows the calculation of a rodents age relative to a humans differs considerably depending on developmental phase. For example, during the weaning phase of a rat, 42.4 rat days = 1 human year. However, during the aged phase, 17.1 rat days = 1 human year. Using this, a broad relationship between rat age and human age is outlined in **table 1**. However, this model does not consider differences in strain and sex and as such assumes a broad maximum lifespan of 2-3.5 years which makes interpretation difficult. Charles River suggest that 19-20 months of age is considered aged due to changes in cognition, biochemistry and brain metabolism. (Charles River Laboratories, 2021).

Jackson Laboratories use sexual maturation and senescence to mark ageing in mice. A mature adult phase, the equivalent to a 20 - 30-year-old human is defined as having passed the developmental stage but not reached senescence: 3 - 6 months for a mouse. 'Middle-aged' is defined as senescence in some but not all biological processes: 6 - 14/15 months old for a mouse, the equivalent of 38-47 years old in a human. Finally, old age is defined as a presence of senescence in almost all biomarkers. 15 - 24 months for a mouse, and 59 - 69 years old in a human. Beyond 24 months, mouse survival drops off markedly, and the mice become susceptible to a range of age-related impairments which would make behavioural interpretation very difficult (The Jackson Laboratory, 2021).

**Table 1**

| Rat years | Human years |
|---|---|
| **0.5** | 18 |
| **1** | 30 |
| **1.5** | 45 |
| **2** | 60 |
| **2.5** | 75 |
| **3** | 90 |
| **3.5** | 105 |

***Table 1 A proposed model of how rat years relate to human years****, taken from (Sengupta, 2013).*

## *4.2 How old is aged?*

An important question perhaps less often asked in rodent ageing research is how old is an aged human? The answer probably changes depending on the person asked and has changed considerably

across time as, in at least some parts of the world, lifespan and quality of life has continued to increase. Indeed, old age is difficult to quantify in chronological years alone, particularly on an individual level and can also be characterised by societal changes, such as retirement. A report by WHO suggests that chronological age is a more relevant indicator of old age in more developed societies and is approximate to retirement age (60-65+ years) (Kowal, 2001). Conversely, in developing countries changes in societal roles are more relevant than age. That is, old age begins when the individual can no longer make an active contribution to society.

The health of the aged rodent must also be taken into consideration when choosing an appropriate age for ageing research. The work in this thesis considers the effects of healthy ageing. As such, it is important that the rodents are tested in the absence of any impairments that go along with advanced rodent age, including tumours, arthritis resulting in reduced motoric capability or infection, that could confound behavioural analysis. As such, it is important to select rodents at an age where they are considered 'ageing/aged' but are still overtly healthy.

## *4.3 The laboratory versus the wild*

### *4.3.1 Rats*

It is important to note there are differences between the laboratory rat and the wild rat (in this case, *Rattus norvegicus*, the species from which the lab rat was originally bred) that may impact on lifespan, and thus on what is considered 'old age' in a rodent. After years of selective breeding and exposure to drastically different environments, we cannot extrapolate the lab rat lifespan to that of a wild rat. Therefore, the question must be considered, is old age in a lab rat the same as that of a wild rat? Unsurprisingly, there is very little data on the lifespan of a wild rat owing to the difficulties in tracking individuals over time. A very old study conducted by (Davis, 1953) suggests *Rattus norvegicus* live little longer than a year. However, this study was limited by the crude estimates of rat age- noted as either 'young' (<100g) or 'adult' (>100g). Furthermore, in typical rat fashion, rats learned to avoid traps making it difficult to estimate survival at an individual level. More recent publications suggest the wild *Rattus norvegicus* lives approximately 2 years but these are assumptions (Jones et al., 1984, Grzimek, 1990). Therefore, it is difficult to make any firm conclusions about lifespan differences, beyond the general theme that lab rats live longer. This is presumably due to lack of predation and in-fighting. This may impact on what is considered 'old age' in the wild rat, but without any firm data it is difficult to speculate.

### *4.3.2 Mice*

Similarly to rats, very little is known about the lifespan of the wild mouse, with estimations varying wildly from as little as 100 days to over 21 months old (Garratt et al., 2011). It has been suggested that inbred laboratory mice have a shortened lifespan in comparison to their wild relatives and one study used wild-derived mice (*mus musculus domesticus)* up to 26 months old (Garratt et al., 2011).

## *4.4 Section summary*

When relating rodent age to human age it is important to take into consideration developmental stage. In mice, sexual maturation and senescence are used as markers of age, with mice ≥15 months considered as onset of old age. In rats, using data from a proposed rat-to-human-years model and Charles River, ≥ 19 months is considered onset of old age. It is important to acknowledge that this applies to laboratory rodents and may differ to the wild strains from which they derived.

# *5 General introduction summary*

We rely on the operational definition of apathy conceptualised by Levy and Dubois to approach the pre-clinical study of apathy-related behaviour. However, this has led to apathy being approached as a unitary construct. In rodents, it is often studied in the context of dopaminergic manipulation or in models of neurodegenerative disease, but human study shows that apathy is also common in otherwise healthy ageing. Beyond gross changes in brain structure, little is known about the underlying neurobiology of apathy in healthy ageing. There may be an interesting potential relationship between apathy and age-related disruption to biological rhythms which could represent a target for further research and therapeutic intervention. However, before this can occur it is important to investigate whether otherwise-healthy aged rodents show apathy-related behaviour and, given the current heterogeneity in the literature, to better understand the nature of disrupted biological rhythms in aged rodents and the potential molecular changes that may underlie this. Therefore, the two overarching aims of this thesis are 1) establish whether aged mice and rats show apathy-related behaviour and 2) characterise age-related changes in biological rhythms in rats and mice at a behavioural and molecular level.

# *6 Chapter aims*

### 6.1 Chapter 2: Testing for an apathy phenotype in aged mice

Given the multi-domain nature of apathy and its similarity to other constructs such as depression, elucidating an apathy phenotype requires a multifactorial approach that considers both emotional and motivational aspects. The aim of this chapter was to use a battery of behavioural tests and

physiological measures designed to quantify emotional behaviour, motivation, reward and cognition to establish a distinct behavioural profile in aged versus young mice.

### 6.2 Chapter 3: Testing for an apathy phenotype in aged rats

Testing apathy-related behaviour in rats could provide valuable insight into species differences in ageing. It is also important to explore other ways of assessing motivation to better understand apathy. The use of rats allows us to go beyond the behavioural battery used in the previous chapter to test cognitive flexibility. Therefore, the aim of this chapter was first to determine whether aged rats show apathy-related behaviour using the battery of behavioural tests used in the previous chapter. The second aim was to assess the impact of age on tasks used to assess cognitive flexibility, using bowl-digging and operant-based methods as a comparison.

### 6.3 Chapter 4: Investigating circadian rhythm changes in aged rats: activity, core body temperature and corticosterone

The investigation of core body temperature, activity and CORT circadian rhythms in combination with molecular analysis of changes in the PVN could provide a valuable insight into age-related changes to circadian rhythms and its underlying neurobiology. Therefore, the first aim of this chapter was to investigate how ageing affects the amplitude of core body temperature, activity and CORT over the 24hr light-dark cycle using rats. The focus of the study was not initially on the impact of food restriction on these outputs, but it became apparent that feeding rats in the light phase had a profound impact on output measures and therefore the second aim of this chapter was to investigate the effects restricted feeding had on the normal circadian pattern of CORT release, activity, core body temperature and the neuronal and receptor expression in the PVN.

### 6.4 Chapter 5: Investigating changes in circadian flexibility in aged rats and mice

Assessing circadian flexibility by analysing changes in activity in the face of competing zeitgebers could provide more information about how the circadian system changes in ageing in a way that is more translationally relevent than constant conditions. The aim of this chapter was to explore the impact of age on circadian flexibility using zeitgebers that are translationally relevant to normal human life: light, feeding, palatable reward and socialisation. Timed feeding was used to investigate (1) the gain and loss of feeding anticipatory activity in rats (2) its impact on light vs dark activity and (3) its impact on activity changes induced by the light zeitgeber. Timed social play was used to investigate whether anticipatory activity would be induced by a social zeitgeber. Mice were used to

explore the impact of age on circadian flexibility by using timed delivery of reward and changes to the light-dark cycle.

# Chapter 2: Testing for an apathy phenotype in aged mice

Data, figures and methods used in this chapter has been published under:

Megan G Jackson, Stafford L Lightman, Gary Gilmour, Hugh Marston and Emma S J Robinson (2021) Evidence for deficits in behavioural and physiological responses in aged mice relevant to the psychiatric symptom of apathy, *Brain and Neuroscience Advances*, 5

(Appendix A1)

and:

Megan G Jackson, Stafford L Lightman, Gary Gilmour, Hugh Marston and Emma S J Robinson (2020) Evidence for an apathy phenotype in aged mice, Biorxiv

**Contribution to work (CRediT author statement):** MJ performed the research, analysed data, wrote and edited the paper.

## 2.1 Introduction

Establishing an apathy phenotype in healthy aged rodents would facilitate a greater understanding of the underlying neurobiology of apathy and could be used to test potential therapeutic interventions. Current approaches to the study of apathy in rodent models often use changes in instrumental responding in effort-based decision making tasks (Salamone et al., 1991) or measures of spontaneous exploration to quantify changes in motivated behaviour (Cathomas et al., 2015). However, apathy is a multidimensional syndrome and contains an emotional-affective component that is consistently overlooked. While there is inevitably some overlap in the behavioural and emotional domains, specifically in areas of reward sensitivity and stress that would affect motivated behaviour, emotional response is rarely analysed in these experiments or outside of the context of effort-based decision making (Magnard et al., 2016). When considering a symptom with multiple domains, a battery of behavioural tests that map onto these domains will provide a more comprehensive analysis of the symptom to the benefit of the overall interpretation of findings, including the dissociation of apathy from depression in the domain of affect. In parallel to a growing clinical effort, there should be more work to dissociate apathy and depression phenotypes in pre-clinical psychiatric models. The use of spontaneous behavioural tasks used to study anxiety or depression-related behaviours may provide an indication of relevant aspects of emotional-affective behaviour in ageing and using a combination of behavioural observation and relevant physiological measurements can provide a more comprehensive overview of the animal's affective state (Paul et al., 2005).

In addition to emotional and motivational changes, age-related degeneration of the dopaminergic system can result in motor performance deficits such as slowing of movement, loss of balance and an increased incidence of falls (Seidler et al., 2010). While this does not pertain directly to apathy behaviour, it can provide insight into how age-related changes to dopaminergic transmission can affect other systems and potentially interact with behavioural tasks.

The aim of this chapter was to use a battery of behavioural tests that map onto aspects of multiple apathy domains to build a behavioural profile in young versus aged mice, with the hypothesis that aged mice would show behaviours that, when taken together, indicate an apathy phenotype. To measure motivational state, tasks with translational analogues were selected where possible; the PR task and EfR task (previously outlined in chapter 1). Both tasks have previously been used in the study of apathy behaviour in both humans and animals (Le Heron et al., 2019; Heath et al., 2019). To rule out potential non-specific age-related confounds that may impact operant task performance such as inflammation of the joints, a non-steroidal anti-inflammatory Rimadyl was administered prior to the EfR task, with the hypothesis that Rimadyl would not affect task performance. To measure behaviours relevant to emotional blunting and often used in the phenotyping of depression

models (Planchez et al., 2019), the NSFT was used to measure stress-induced anxiety and the SPT to measure reward sensitivity. The raised beam test was used to assess the effect of age on different measures of motor performance. To test if any deficits were linked to underlying inflammatory disease animals were tested in the presence and absence of the anti-inflammatory drug Rimadyl on complex motor control. At the end of the behavioural studies, animals underwent an acute restraint stress and both baseline and stress-induced corticosterone and post-mortem c-fos expression were used to investigate the effects of ageing on stress-reactivity at a physiological and cellular level.

## 2.2 Methods

### 2.2.1 Subjects

12 aged male C57bl/6J mice were supplied by Eli Lilly aged from Charles River stock (15 months on arrival, weight 31.6-38.9g and 24 months by experiment conclusion, weights 33.6-40.0g). A cohort of C57bl/6J strain and sex-matched controls were obtained from Charles River (3 months and weighing 21.8-27.6g at experiment start and 12 mo and weighing 30.5-32.4g by end of experimentation). Sample size was based on previous behavioural studies where spontaneous behavioural tasks and operant-based methods were used (Gourley et al., 2016, Rex et al., 1998). Prior to their arrival in Bristol, aged mice were group-housed in enriched caging and fed a daily restricted diet of 3g to promote healthy ageing and prevent obesity. On arrival to avoid in-cage fighting all mice were housed individually in open-top enriched cages with a red plastic house, cardboard tube and wooden chew block. Mice were housed in temperature-controlled conditions (21$^{o}$C) and a 12:12 hour light-dark cycle (reverse lighting, lights OFF at 0815, lights ON at 20:15). Standard lab chow (Purina, UK) was provided *ad libitum* with the exception of operant training where mice were fed a restricted diet of 2 g/mouse. Weights were monitored weekly and maintained to at least 85% of their free feeding weight relative to their normal growth curve. With the exception of the sucrose preference test, water was provided *ad libitum.* Aged mice were checked daily for changes in health and all animals completed the study with no overt signs on illness or motor impairments. At the end of behavioural battery (**fig.1**), groups had aged to approximately 12 and 24 months. Therefore, an additional N = 12 male C57bl/6J mice from Charles River (3 months at experiment onset, weighing 23.2-26.9 g) were brought in as a young group for restraint stress. Mice were singly housed as described above. Food and water were provided *ad libitum.* To prevent potential confounds of stress from handling that may influence stress reactivity, mice spent 1 month in the unit before this experiment and were handled for 3 weeks. All experiments took place during the animals' active phase, and order of testing was counterbalanced by age during each task to account for potential time of day differences. Where videos or pictures were analysed, the experimenter was blind to age. Experiments were performed in accordance with the Animals (Scientific Procedures) Act (UK) 1986 and were approved by the University of Bristol Animal Welfare and Ethical Review Body (AWERB).

**Figure 1**

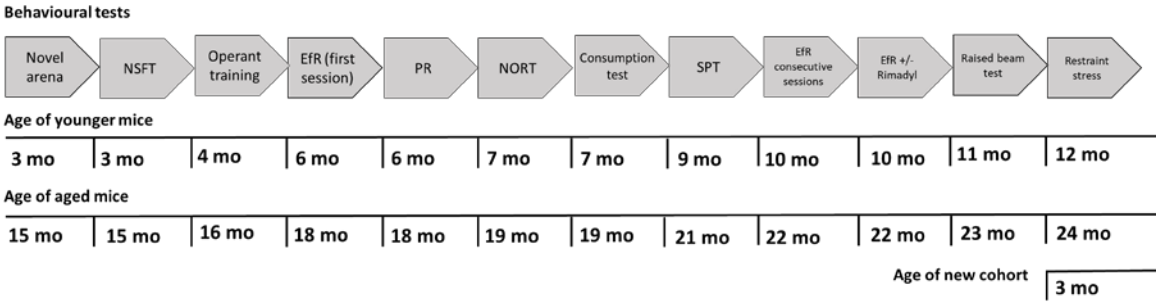


***Fig.1 Timeline for analysis of age-related behaviours.*** *NSFT-novelty supressed feeding test, EfR-effort for reward, PR-progressive ratio, NORT-novel object recognition test, SPT-sucrose preference test.*

### 2.2.2 Novel arena exploration

Mice were placed in a novel circular open field arena (85 cm diameter) with a Perspex floor that was cleaned with 70 % ethanol between mice. The task was completed under red lighting. Movement was captured for 15 min using a Logitech HD Pro Webcam c920 suspended 1 m above the arena. Videos were analysed using Ethovision Xt10 software (Noldus Information Technology, Wageningen). Total distance travelled (cm) and velocity (cm/s) were the measures of interest.

### 2.2.3 Novelty suppressed feeding test

The task was performed using a protocol similar to that previously described by (Shephard and Broadhurst, 1982). Food was taken from the mice 24 h before onset of the task. Mice were placed in the left-hand corner of a novel arena that was made from clear Perspex (40 cm x 40 cm) and lined with sawdust. A ceramic bowl of food was placed in the centre of the arena and latencies for the mice to approach the bowl and to eat from it were recorded using a stopwatch. Between mice, faecal pellets were removed, and the sawdust was shaken to redistribute odour cues. The food bowl was cleaned, and fresh food added. An upper time limit of 15 mins to eat was used but not required.

### 2.2.4 Operant training

Mice were trained in sound-proofed operant boxes (Med Associates Inc), which were run on Klimbic software (Conclusive Solutions Ltd., UK). Unless specified, a session lasted 30 mins. In two sessions pre-training, mice learned to associate the magazine with the delivery of a reward pellet (20 mg rodent tablet, TestDiet, Sandown), where one pellet was dispensed every 40 sec. Mice then underwent continuous reinforcement training (CRF) where a response made in either the left or right aperture resulted in the dispensing of a single reward pellet. The magazine remained illuminated until the mouse collected the pellet. In the next stage of training, mice advanced through ascending fixed ratios (FR) of reinforcement by making responses in either the left or right assigned

aperture, which was counterbalanced across cohorts and remained consistent across the study. The mice progressed through FR stages of training (**fig.2**). Once mice were trained, they completed a minimum of 5 additional FR8 sessions.

**Figure 2**

**A**


**B**

| Stage | Criteria |
|---|---|
| FR1 | 50 + trials, 2 consecutive sessions |
| FR2 | 50 + trials, 2 consecutive sessions |
| FR4 | 50 + trials, 2 consecutive sessions |
| FR8 | 30 + trials, 2 consecutive sessions |

***Fig. 2. Mouse operant box and training stages.*** ***A*** *Operant box.* ***i*** *Nose poke apertures* ***ii*** *Reward magazine* ***iii*** *Reward pellet dispenser.* ***B*** *FR stages of training and criteria for progression.*

### 2.2.5 Effort for reward task

Following FR training mice underwent a single FR8 session with access to powdered standard lab chow presented in a pot, placed in front of the inactive aperture, similar to that previously described by (Salamone et al., 1991). Completing FR8 to obtain a single reward pellet represents the high effort, high reward option while the freely available chow represents the low effort, low value reward option. This is because it is their standard diet, lacks the higher sucrose content of the reward pellets and does not require an operant response to obtain. The chow was accessed via a ½ inch hole in the lid. Chow consumed was measured using change in weight pre and post session, in g. Later in the behavioural battery (**fig.1**) mice were re-tested in the EfR task using the same method but over 5 consecutive sessions.

### 2.2.6 Effort for reward task +/- Rimadyl

Using a within-subject fully counter-balanced design, all animals were re-tested in the effort for reward task following with a clinical dose of the NSAID Rimadyl (2mg/kg) or a saline (0.9 %) vehicle administered subcutaneously. Experimenter was blind to drug dose but not to age group.

### 2.2.7 Progressive ratio task

Mice were tested in a progressive ratio (PR) task, where obtaining each successive reward (*n*) required an increasing number of nose pokes using the algorithm F(*n*)=5 x EXP(0.2*n*)-5 (Roberts and Richardson, 1992). A PR session consisted of a maximum of 100 trials or 120 minutes and included a 1 second intertrial interval. 5 mins of inactivity ended the session. The breakpoint was defined as the last ratio competed before this 5 min period of inactivity. This test was conducted under both *ad libitum* and food restricted conditions. In the *ad libitum* condition, mice were given free access to food for 3 days prior to testing. Mice underwent one session of the PR task under each feeding condition.

### 2.2.8 Consumption test

To test for potential differences in appetite between age groups, mice were food restricted overnight and the next day were presented with free access to either powdered chow or reward pellets for 10 min in the home cage. Amount consumed in g was calculated and normalised to body weight by using g consumed/body weight in kg.

### 2.2.9 Novel object recognition test

The protocol used was similar to that first developed by (Ennaceur and Delacour, 1988). Mice were habituated over 3 days to a Perspex arena (40 cm x 40 cm) for 10 min (day 1) or 5 min (day 2 and 3). It was differentiated from the NSFT arena by changing procedure room and using a paper liner**.** During the sample phase mice were presented with two identical novel objects for 15 min. Following a 4h delay phase in the home cage mice completed a test phase where they were presented with a novel and familiar object for 10 mins. Position of the novel object was counterbalanced across cohorts. Behaviour was captured using a Logitech c920 webcam and exploration, defined here as the animal pointing its nose towards the object at approximately < 2cm, was timed manually using DOSBox 0.74 software. Analysis inclusion criteria was 20+ secs of total exploration in the sample phase. A discrimination ratio was calculated using *(time spent exploring novel object – time spent exploring familiar object)/(time spent exploring novel object + time spent exploring familiar object).* A positive value indicates that the mouse has explored the novel object more than the familiar and has thus successfully discriminated.

### 2.2.10 Sucrose preference test

The protocol used was similar to that previously described by (Willner et al., 1987). Mice had their water removed overnight for ~16h before both the habituation and test sessions which each lasted 4hrs. Habituation lasted 4 days, where mice were placed into test cages containing sawdust and a cardboard tube and were presented with either 1 % sucrose (day 1 & 3) or tap water (day 2 & 4) in two sipper sacks (Edstrom-Avidity Science) with drip-free drinking valves for 4 h. During the test

session mice were presented with one sipper sack containing 1 % sucrose solution and the other containing tap water. Starting position of sucrose was counterbalanced across cohorts. Liquid consumed was weighed at the 1h, 2h and 4h time points and position of sucrose was swapped at these time points to account for potential side bias. % sucrose preference was calculated using (*total amount of sucrose consumed/ (total sucrose + total water consumed)) x 100*.

### 2.2.11 Assessment of complex motor performance and the effects of NSAID pre-treatment

A raised beam test was developed in house (by LJB) to assess complex motor performance. The apparatus consisted of a start podium, a 25cm, 10 mm rod and a goal box (**fig.3**). The mouse is placed on the start podium and must traverse the rod to reach the goal box. Traversal time, foot slips and number of falls are recorded to give a readout of motor performance.

**Figure 3**

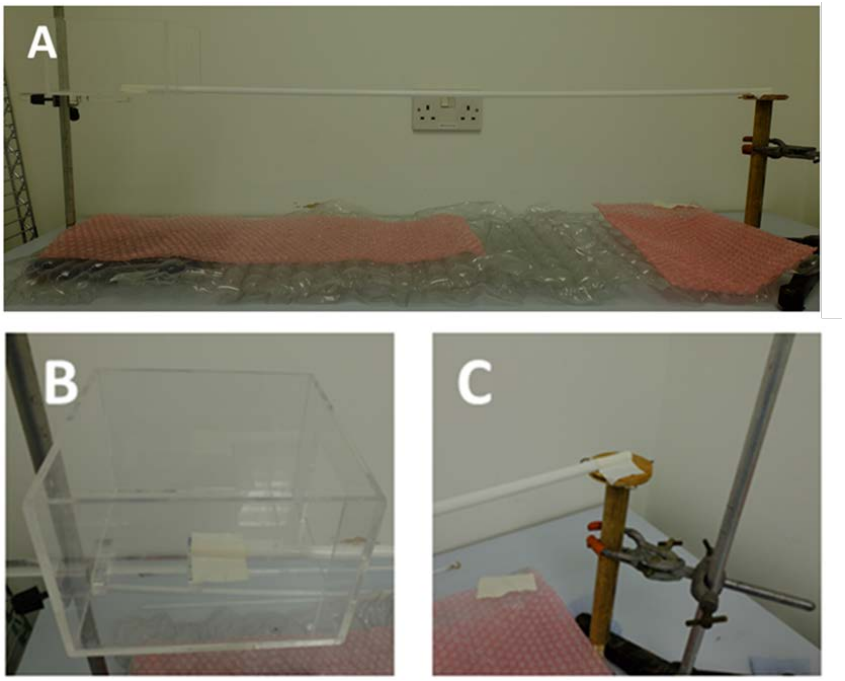


***Fig.3. Raised beam test design*** *(these pictures were taken by Laura Brennan and published in her thesis. Used with permission).* ***A*** *overview of the equipment.* ***B*** *Goal box.* ***C*** *Start podium.*

Mice were pre-trained to traverse the rod by a previous experimenter (LB) approx. 5 months prior as part of a different experiment (see **table 1**). All mice successfully completed training.

**Table 1**

| Stage | Description | Criteria for progression |
|---|---|---|
| **1** | Placed halfway along 10mm rod | 3 half traversals of the rod |
| **2** | Placed halfway along 10mm rod and then placed on start podium | 5 half traversals and 1 full traversal of the rod |
| **3** | Placed on start podium | 5 full traversals of the 10 mm rod |
| **4** | Placed on start podium | 5 full traversals of 8 mm rod |
| **5** | Placed on start podium | 5 full traversals of 6 mm rod |

***Table 1: Training protocol for raised beam test****, developed by Laura Brennan (LJB) and previously published in her thesis.*

Mice were given a refresher training session where they completed 3 half traversals of the 10 mm rod and 3 complete traversals from start platform to goal box. A traversal was complete when all four paws were in the goal box. The goal box contained 20mg rodent tablets (TestDiet, USA). The rod and goal box were cleaned with 70 % ethanol between mice. All mice successfully completed this stage. To study the potential effects of age-related inflammation on motor performance, mice were dosed sub-cut with either a clinical dose of Rimadyl (2 mg/kg) (50 mg/ml, vet formulation) dissolved in saline or saline vehicle 1h before the task. Mice traversed the full length of the rod 3 times. Traversal time, number of foot slips and number of falls was recorded manually and then averaged over the 3 traversals. Mice that fell 3 times from the rod were excluded (n = 1 aged mouse). Traversal time, foot slips and falls were also combined into a composite score developed by LJB (appendix A2). The experiment was a within-subject design and fully counterbalanced. The experimenter was blind to treatment but not to age group.

### 2.2.12 Acute restraint stress

Mice were individually restrained in a restraint tube (Ad Instruments Ltd) and their tail warmed with a heat pad for 3 minutes to promote blood flow to the tail vein. The tail vein was opened with a 25G needle (Sigma-Aldrich, Germany) and blood was collected using a Mitra Microsampling sponge (Neoteryx, USA), with a calculated average blood wicking volume of 10 µl. The mouse was restrained for 30 mins and a second blood sample was taken directly afterwards. Mice were not in olfactory or auditory contact with other mice and the restraint tube was cleaned fully between animals. Sampling time was counter-balanced across the different groups to prevent any circadian differences in baseline CORT. As it has previously been shown psychological stress significantly increases faecal output in rodents, faecal count during testing was used as an additional measure of stress (Mönnikes et al., 1993). Sampling took place between zeitgeber time (ZT) 17-20. Samples

were stored at room temperature with a bag of desiccant until analysis at Eli Lilly. One mouse from the oldest group died before this final experiment.

### 2.2.13 Mass spectrometry analysis of corticosterone (conducted by Dr Sandra Sossick, Eli Lilly)

CORT levels were analysed using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-ES/MS-MS) at Eli Lilly. For full experimental details see (Jackson et al., 2021).

### 2.2.14 Tissue collection

Following restraint stress, mice were returned to the home cage. 60 min later they were killed by cervical dislocation. Brains were immediately extracted and placed in cold 4 % paraformaldehyde (PFA) solution prepared in phosphate buffer saline (PBS). Brains were left in PFA overnight in a 4 °C fridge and then transferred to a 25 % sucrose solution (Sigma Aldrich, UK) prepared in PBS. When brains had sunk to the bottom of the test tube, they were then frozen in OCT (Cryomatrix, Thermofisher) and stored at – 20 ℃.

### 2.2.15 c-fos immunohistochemistry

Whole brains were sliced in 40 µm coronal sections using a freezing microtome (Reichert, Austria). Sections were stored in cryoprotectant (300 g sucrose, 300 ml ethylene glycol, 500 ml 0.1M phosphate buffer topped to 1L with dH2O) at – 20℃ until use.

Sections were washed for 10 mins x 4 in TBS (Trizma buffer saline; 12 g Trizma base, 9 g NaCl, 1 L dH20 pH 7.4) and then incubated in TBS-T (Triton X, 0.1 %) with 3 % normal goat serum (Vector Laboratories) at room temperature for 30 mins. Sections were then labelled with primary antibody rabbit anti c-Fos (ABE457, Merck Millipore) at a dilution of 1:4000 in TBS-T with 3 % normal goat serum and left overnight in the fridge at 4℃. The next day sections were washed again in TBS-T with 3 % normal goat serum and then incubated with secondary antibody goat anti-rabbit (Alexa Fluor 488, Invitrogen) for 3 hours at a dilution of 1:500 in TBS-T with 3 % normal goat serum. The next steps were done in the dark. Sections were washed for 10 mins x 3 in TBS and then then incubated with DAPI (14.3 µM) for 3 minutes. Sections were then washed again in PBS for 5 min x 3 and then float mounted onto subbed slides (VWR). A few drops of Vectashield mounting media (Vector Laboratories) were added before cover slipping.

Images were taken on a Leica widefield microscope with DFC365 FX camera with LasX software. with the gain and exposure changed depending on channel and objective lens (**table 2**) but was kept consistent throughout the experiment.

**Table 2**

| Channel | Exposure (ms), 10x objective | Gain, 10 x objective | Exposure (ms), 5 x objective | Gain, 5x objective |
|---|---|---|---|---|
| **C-fos** | 500 | 2.7 | 999 | 2 |
| **DAPI** | 100 | 2.7 | 500 | 2 |

***Table 2. Microscope settings for analysis of c-fos immunohistochemistry.***

The whole paraventricular nucleus of the hypothalamus (PVN) (bregma level -0.58 - 0.94 mm across 3 sections) was captured using the 10x objective and the central and basolateral amygdala (either the left or right, bregma levels -1.22 – 1.40 mm across 3 sections) were captured using the 5x objective. Some sections were lost due to tissue damage. N = 10 per group amygdala was obtained. N = 10 PVN for aged and young group were obtained, N = 9 for middle aged group. Region of interest was manually drawn around and C-fos was counted manually using the ImageJ cell counter within that area. It was only counted if it colocalised with DAPI to avoid the inclusion of non-specific binding or artefacts. Count was then normalised by dividing by region area. Contrast was adjusted uniformly across all images.

### 2.2.16 Data analysis

Data normality was tested using the Shapiro-Wilk test. Where data was normally distributed or required two-factor repeated measures (RM) analysis then the RM two-way ANOVA, one-way ANOVA or independent t-test were performed where appropriate. Normal data was presented in graphical form using the mean and standard error of the mean (SEM). Where data was not normally distributed, the Mann-Whiney U test or Kruskal-Wallis tests were used and were presented using the median and interquartile range. Significant effects were defined as $p < 0.05$ and trend level effects were defined as $p < 0.1$. Exact p values were reported, with the exception of p values that were $p < 0.0001$, which was reported instead. Significant main effects or interactions were reported in the results section and followed up with appropriate post-hoc analysis and corrected for multiple comparisons. Bonferroni-corrected pairwise comparison was used following RM two-way ANOVA analysis, Tukey's post-hoc comparison was used following one-way ANOVA analysis and Dunn's post-hoc comparison was used following the Kruskal-Wallis test. Trend level effects were reported but not further analysed. Sphericity was checked when using ANOVA tests. If data did not meet the sphericity assumptions, then the Huynh-Feldt corrected values were reported. When data was compared using independent t-tests, the Levene's Test for Equality of Variances was used, and corrections applied if variances were not equal. Outlying data was defined as data points that were 2 standard deviations away from the group mean. Any data points excluded from the study are reported in the results sections. Statistical analysis was performed using IBN SPSS

statistics v.24 and graphs were created using GraphPad Prism v 8.3.0. This statistical approach was used for all chapters of the thesis.

## 2.3 Results

### 2.3.1 Aged mice show reduced exploration of a novel arena but show no deficit in the novel object recognition task.

Analysis of mouse activity in an open field arena showed that in 15 min, younger mice covered a greater distance (cm) than aged mice ($t_{(22)}$ = 2.536, p = 0.019) and had a greater velocity (cm/s) ($t_{(22)}$ = 2.613, p = 0.016) (**fig.4.A&B**). When distance travelled was split into 5 min time bins, there was a main effect of time ($F_{(2,44)}$ = 35.33, p < 0.0001) and age ($F_{(1,22)}$ = 6.434, p = 0.019) but no interaction P > 0.05). Post hoc analysis revealed that a difference in distance travelled between groups only emerged in the second (p = 0.013) and third time bin (p = 0.049) (**fig.4.C**). In the NOR task, there was no difference in discrimination ratio between age groups (p > 0.05) (**fig.4.D**) and no difference in exploration time in either the sample or test phase (p > 0.05) (**fig.4.E&F**).

**Figure 4**

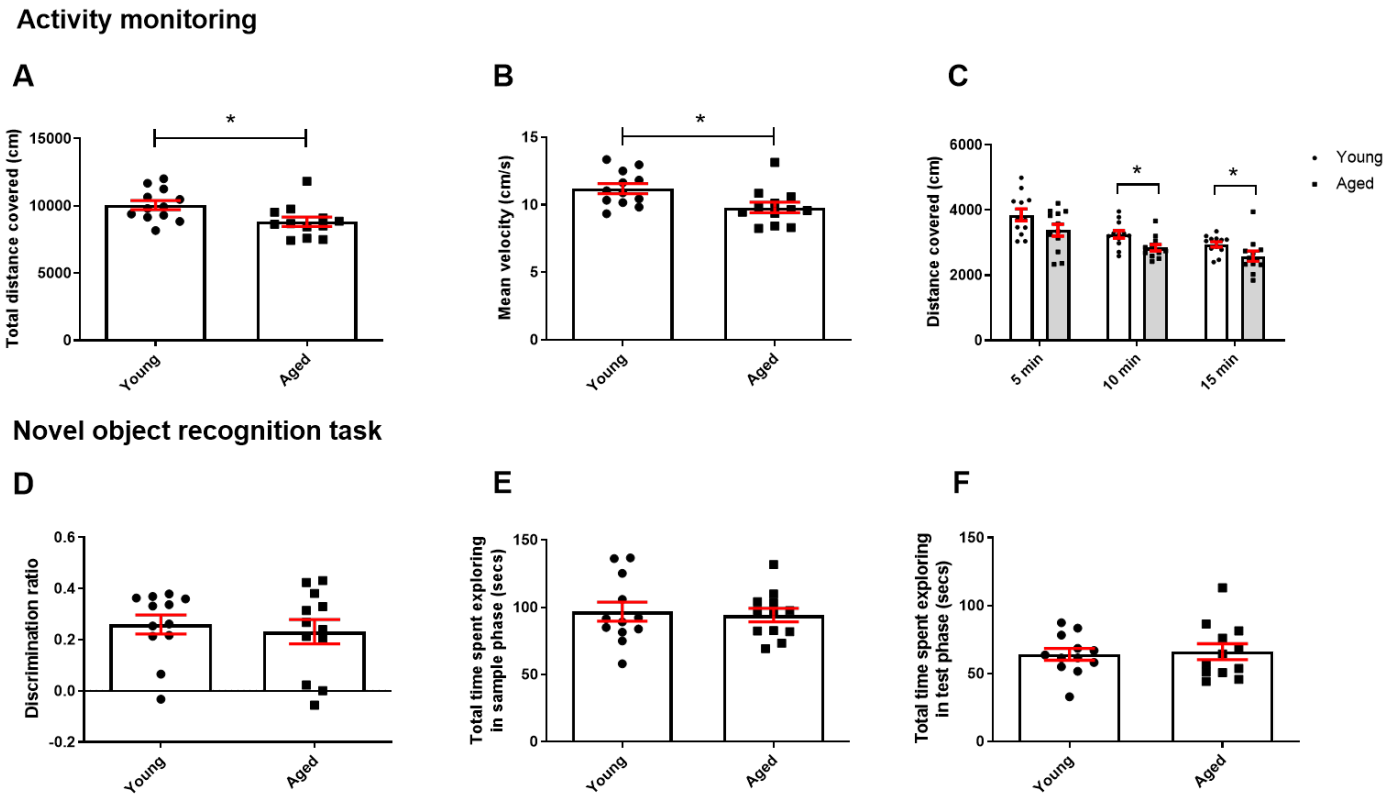


***Fig. 4. Aged mice show reduced exploration of a novel arena but show no deficit in the novel object recognition task.*** *Exploration activity was monitored in an open field arena for 15 min and mice underwent the novel object recognition test with a 4h delay phase.* ***A*** *Aged mice covered less distance than young mice in an open field arena and* ***B*** *had a lower mean velocity ($p < 0.05$, independent t-test).* ***C*** *Aged mice covered less distance in the final two 5 min time bins ($p < 0.05$, RM two-way ANOVA with pairwise comparisons).* ***D*** *There was no difference in discrimination ratio between age groups ($p > 0.05$, independent t-test).* ***E*** *There was no difference in object exploration between groups in the sample phase and the* ***F*** *test phase ($p > 0.05$). Bars are mean ± SEM with data points overlaid. N = 12 per group.*p< 0.05.*

### 2.3.2 Aged mice show changes in motivated behaviour in the progressive ratio and effort for reward tasks.

Following overnight food restriction, there was no difference in consumption of freely available reward pellets or powdered chow between groups, normalised to body weight ($p > 0.05$) **(Fig.5.A&B)**.

Analysis of CRF training performance showed a main effect of session on trials completed ($F_{(3,66)} = 44.471$, $p < 0.0001$) and a main effect of age ($F_{(1,22)} = 8.365$, $p = 0.008$). There was also a session*age group interaction ($F_{(3,66)} = 5.815$, $p = 0.0014$). Post-hoc pairwise comparisons revealed that young mice completed more trials in sessions 4 and 5 ($p \leq 0.003$) (**fig.5.C**). A comparison of the session number at which all fixed ratio training was completed showed no difference between age groups ($p > 0.05$) (**fig.5.D**).

Under food restricted conditions, young mice ended the PR task at a higher fixed ratio than aged mice ($t_{(12.464)} = 4.563$, $p = 0.001$). It should be noted that mice did not reach a true breakpoint, where 5 mins of inactivity ends the session. Instead, they worked until the maximum time limit. FR62 was the highest ratio all mice completed and so was used as a cut off in this following analysis. Analysis of time taken to complete each fixed ratio revealed a main effect of ratio ($F_{(1.403, 30.865)} = 22.812$, $p < 0.0001$), where time taken to complete ratio increased with ratio. There was also a main effect of age ($F_{(1,22)} = 19.960$, $p < 0.0001$) and a ratio*age group interaction ($F_{(1.403, 30.865)} = 13.581$, $p < 0.0001$). Post-hoc pairwise comparisons showed that young mice completed FR6-62 faster than aged mice ($p \leq 0.016$) (**fig.5.E**).

When mice were fed *ad libitum* 3 days before testing, n = 8 young mice and n = 9 aged mice reached a true break point. When only these mice were compared, there was no group difference ($p > 0.05$). However, when all mice were considered, including those that did not reach a true break point, a difference did emerge ($t_{(16.108)} = 2.619$, $p = 0.019$). For the following analysis, a cut off of fixed ratio 40 was used. There was no age-related difference in time taken to complete ratios. In this case, RM ANOVA was not suitable due to the number of missing data points due to animals finishing at varying break points. As the main focus was age-related differences, a series of independent t-tests were completed instead comparing speed between groups at each ratio. There was no age-related difference in speed to ratio completion ($p > 0.05$) (**fig.5.F**).

On first exposure to the EfR task, young mice completed more trials for high value reward pellets than aged mice ($t_{(15.982)} = 5.088$, $p = 0.0001$) but consumed less of the low value chow ($t_{(15.819)} = 2.453$, $p = 0.023$), n = 1 younger mouse excluded as an outlier (**fig.5.G&H**).

When EfR was repeated over 5 consecutive sessions, analysis of number of trials completed showed a main effect of session ($F_{(3.22, 70.833)} = 41.553$, $p < 0.0001$) but no main effect of age, though there

was a trend level effect ($F_{(1,22)} = 3.038$, $p = 0.095$). There was a session*age group interaction ($F_{(3.22, 70.833)} = 2.73$, $p = 0.047$). Post-hoc pairwise comparison revealed that younger mice completed more trials than aged mice in session 4 ($p = 0.046$) and 5 ($p = 0.032$) only (**fig.5.I**). There was no effect of session, age group or session* age group interaction on consumption of chow ($p > 0.05$). However, there was trend towards a session*age group interaction ($F_{(3.59, 75.4)} = 2.237$, $p= 0.08$). N= 1 young mouse was excluded due to being an outlier over multiple sessions (**fig.5.J**). Analysis of average FR8 speed to completion across sessions revealed a main effect of session on speed, where speed increased over sessions in both age groups ($F_{(2.302, 50.636)} = 34.476$, $p < 0.0001$). There was no main effect of age or age*session interaction ($p > 0.05$) (**Fig.5.K**).

**Figure 5**

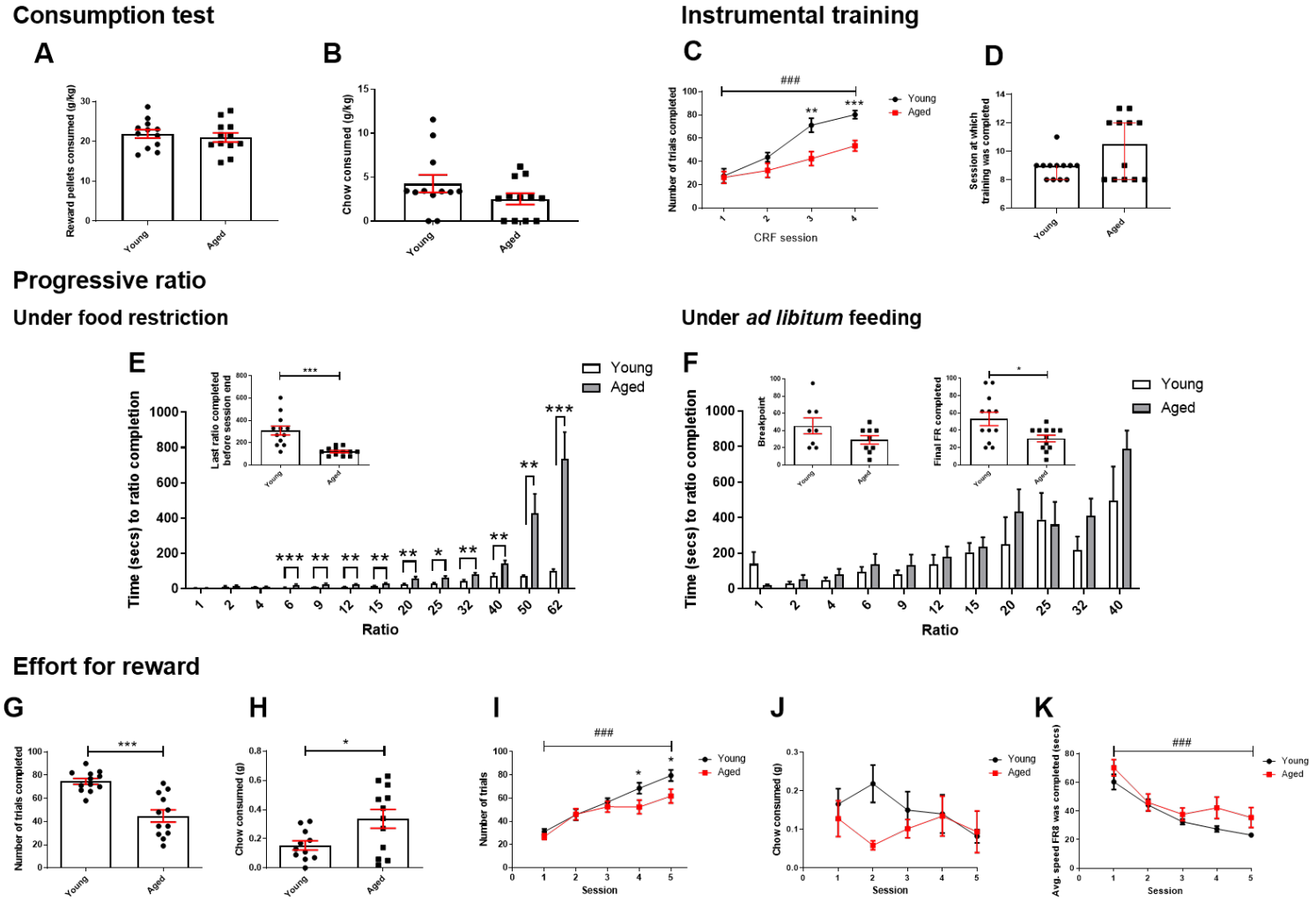
Consumption test
Instrumental training
A
B
C
D
Reward pellets consumed (g/kg)
Chow consumed (g/kg)
Number of trials completed
CRF session
Session at which training was completed
Young
Aged
Progressive ratio
Under food restriction
Under ad libitum feeding
E
F
Time (secs) to ratio completion
Last ratio completed before session end
Breakpoint
Final FR completed
Ratio
Effort for reward
G
H
I
J
K
Number of trials completed
Chow consumed (g)
Number of trials
Session
Avg. speed FR8 was completed (secs)

***Fig.5. Aged mice show changes in motivated behaviour in the progressive ratio and effort for reward tasks.*** *Aged and young mice underwent operant training and completed the progressive ratio and effort for reward tasks.* ***A*** *In a consumption test, there was no difference in reward pellets consumed between age groups ($p < 0.05$, independent t-test)* ***B*** *or freely available chow ($p > 0.05$, Mann-Whitney U test, bars are median ± interquartile range).* ***C*** *Young mice completed more trials on days 3 and 4 under CRF training ($P < 0.01$, RM two-way ANOVA with pairwise comparison).* ***D*** *There was no difference in session at which fixed ratio training was completed between age groups ($p > 0.05$, Mann-Whitney U test, bars are median ± interquartile range).* ***E*** *Under food restriction, young mice completed a higher fixed ratio before session end than aged mice ($p < 0.001$, independent t-test) and had a faster rate of response at FR6-62 (RM two-way ANOVA with pairwise comparison).* ***F*** *Under ad libitum feeding conditions there was no difference in break point between age groups ($p > 0.05$, independent t-test) but young mice completed a higher fixed ratio before session end ($p < 0.05$, independent t-test).* ***G*** *In the EfR test young mice completed more trials than aged mice ($p < 0.001$, independent t-test) and consumed less chow ($p < 0.05$).* ***I*** *Young mice completed more trials than aged mice on day 4 and 5 ($p < 0.05$, RM two-way ANOVA with pairwise comparison).* ***J*** *There was no effect of day or age on chow consumed.* ***K*** *Speed to FR8 completion decreased over session in young and aged mice ($p < 0.001$, RM two-way ANOVA). Unless otherwise stated, bars are mean ± SEM with data points overlaid. N = 12 per group. *p< 0.05, **p < 0.01, ***p< 0.001, ###p < 0.001 (*between-subject, #within-subject).*

### 2.3.3 Rimadyl has no effect on effort for reward performance in both young and aged mice.

A comparison of number of trials completed between young and aged mice under a clinical dose of Rimadyl versus vehicle revealed no main effect of drug, no drug * age group interaction and no effect of age, although there was a trend $F_{(1,22)} = 3.385$, $p = 0.079$ (**fig.6.A**). Similarly, analysis of chow consumed showed no main effect of drug, drug * age interaction or effect of age ($p > 0.05$) (**fig.6.B**).

**Figure 6**

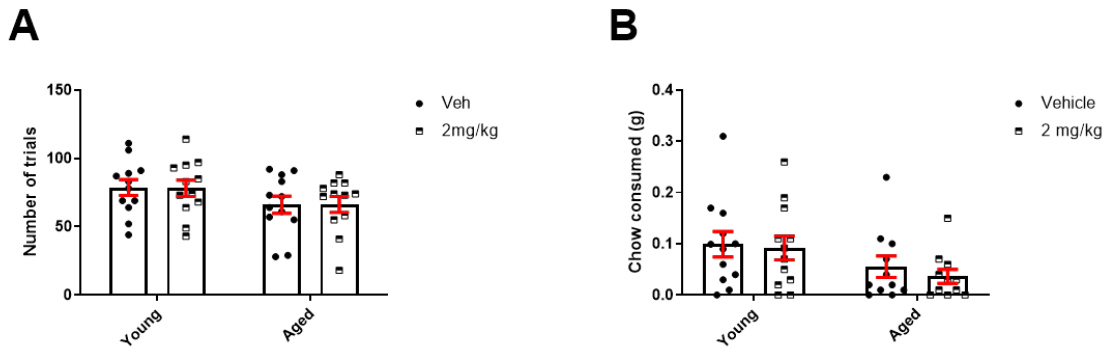


***Fig.6. Rimadyl had no effect on effort for reward performance in both young and aged mice.*** *Young and aged mice underwent the effort for reward task under vehicle or 2mg/kg Rimadyl conditions.* ***A*** *There was no effect of Rimadyl or age on number of trials completed in the EfR task (RM two-way ANOVA, $p > 0.05$).* ***B*** *There was no effect of Rimadyl or age on chow consumed in the EfR task (RM two-way ANOVA, $p > 0.05$). N = 12 per group, bars are mean ± SEM with data points overlaid.*

### 2.3.4 Aged mice show changes in hedonic behaviour, anxiety-like behaviour, and stress-reactivity.

Analysis of % sucrose preference in the SPT revealed a main effect of time on % sucrose preference ($F_{(1.781, 39.191)} = 12.146$, $p < 0.0001$) and age group ($F_{(1,22)} = 16.692$, $p < 0.0001$). There was also a time*age group interaction ($F_{(1.781, 39.191)} = 7.076$, $p = 0.003$). Post-hoc pairwise comparison showed that aged mice had a lower sucrose preference than young mice at all time points ($p \leq 0.043$). During testing there were 3 sipper sack leaks (n = 2 aged and n = 1 young). Where this occurred, the value was replaced with the group mean to permit RM analysis (**fig.7.A**).

Analysis of total liquid consumed during habituation days to the SPT revealed a main effect of day ($F_{(2,44)} = 29.950$, $P < 0.0001$) and age ($F_{(1,22)} = 6.032$, $p = 0.022$). There was also a habituation day*age group interaction ($F_{(2,44)} = 4.44$, $p = 0.018$). Post-hoc pairwise comparison showed that aged mice drank less liquid on the final day of habituation only ($p < 0.0001$) (**fig.7.B**).

In the SPT test, there was a main effect of time on liquid consumed ($F_{(2,44)} = 70.192$, $p < 0.0001$) and of age ($F_{(1,22)} = 26.977$, $p < 0.0001$). There was also a time*age group interaction ($F_{(2,44)} = 14.672$, $p < 0.0001$). Post-hoc pairwise comparison showed that aged mice drank less at hour 2 and 4 ($p < 0.0001$) (**fig.7.C**). However, differences in liquid consumed were accounted for by calculating sucrose preference as a %.

In the NSFT, younger mice took longer to eat from the bowl than aged mice ($t_{(4.208)} = 3.411$, $p = 0.004$) but there was no effect of age on latency to approach the bowl ($p > 0.05$) (**fig.7.D&E**). Aged mice lost a lower % of their body weight than younger mice ($t_{(22)} = 5.654$, $p < 0.0001$) (**fig.7.F**).

Levels of CORT were compared pre- and post- a 30 min restraint stress. There was a main effect of stress ($F_{(1,32)} = 179.608$, $p < 0.0001$) and age ($F_{(1,32)} = 5.772$, $p = 0.007$) and a stress*age group interaction ($F_{(2,32)} = 9.862$, $p = 0.0005$). Post-hoc pairwise comparison revealed that baseline CORT levels did not differ between age groups ($p > 0.05$) but following stress, younger mice had a greater CORT response compared to the middle-aged ($p = 0.0003$) and aged groups ($p = 0.001$) (**fig.7.G**). As an additional measure of stress, faecal count following stress was analysed. There was an effect of age of faecal count ($H_{(2)} = 12.424$, $p = 0.002$). Dunn's post-hoc test showed that younger ($p = 0.004$) and middle-aged mice ($p = 0.011$) had a greater faecal count than aged mice (**fig.7.H**).

Analysis of post-mortem analysis of neuronal activation in the CeA post-stress showed a main effect of age ($F_{(2,29)} = 22.102$, $p < 0.0001$). Tukey's post-hoc analysis revealed that CeA c-Fos count was lower in younger mice compared to middle-aged and aged mice ($p<0.0001$) (**fig.7.I**). Similarly, there was an effect of age on c-Fos count in the BLA ($F_{(2,29)} = 19.915$, $p < 0.0001$), where aged mice had a greater c-Fos count than younger ($p< 0.0001$) and middle-aged mice ($p = 0.029$). Additionally, middle-aged mice had a higher BLA c-Fos count than younger mice ($p = 0.004$)

**(fig.7.J)**. In the PVN, c-Fos count was higher in the PVN in young mice compared to aged mice ($F_{(2,28)}$ = 5.326, p = 0.012, Tukey's post hoc, p = 0.008) **(fig.7.K)**.

**Figure 7**

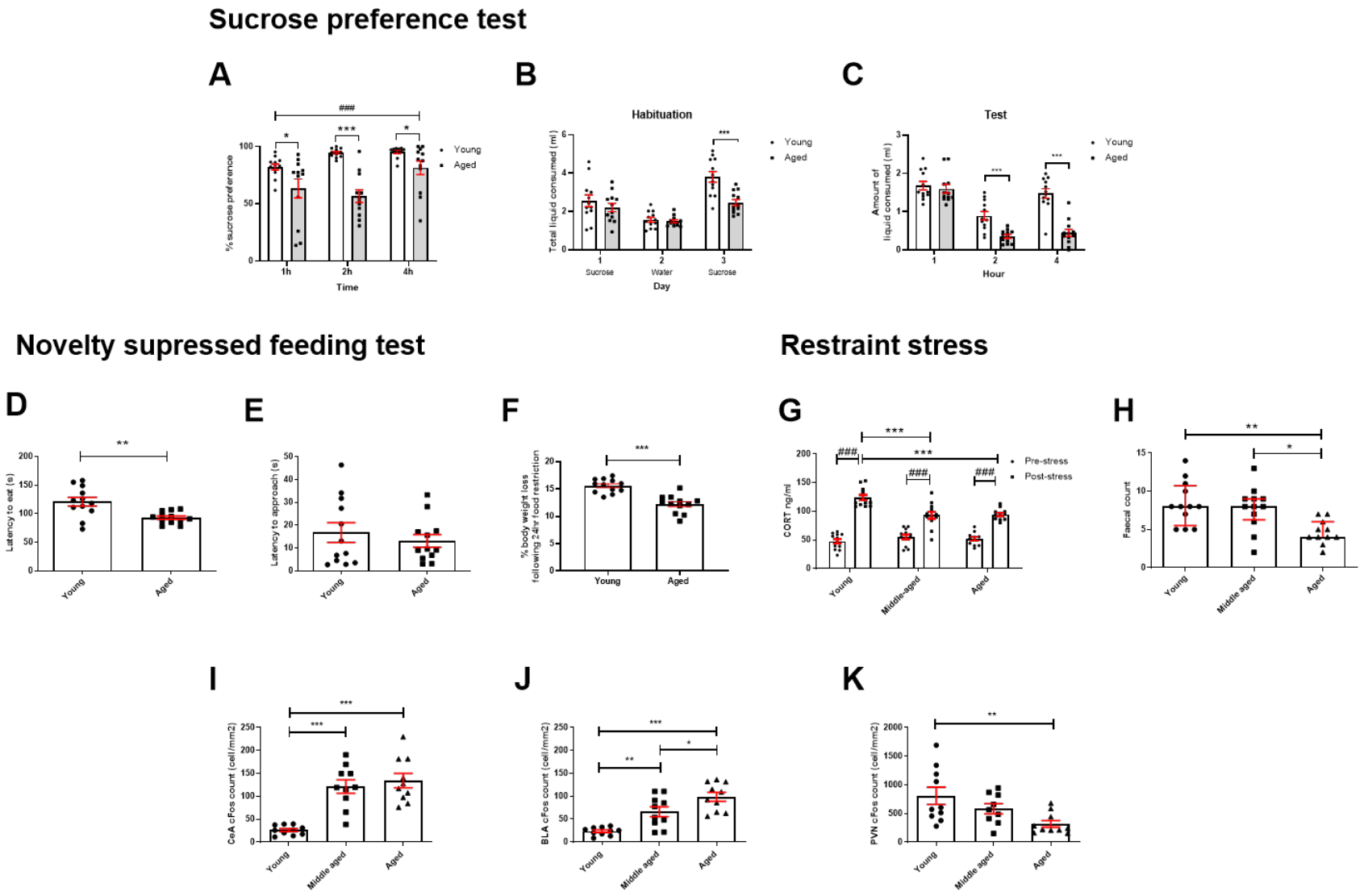
Sucrose preference test
A
B
C
% sucrose preference
Young
Aged
1h
2h
4h
Time
Habituation
Total liquid consumed (ml)
Sucrose
Water
Sucrose
Day
Test
Amount of liquid consumed (ml)
Hour
Novelty supressed feeding test
Restraint stress
D
E
F
G
H
Latency to eat (s)
Latency to approach (s)
% body weight loss following 24hr food restriction
CORT ng/ml
Pre-stress
Post-stress
Fecal count
Young
Middle aged
Aged
I
J
K
CeA cFos count (cell/mm2)
BLA cFos count (cell/mm2)
PVN cFos count (cell/mm2)

***Fig.7. Aged mice show changes in hedonic behaviour, anxiety-like behaviour, and stress-reactivity.*** *Young and aged mice underwent the sucrose preference test, novelty supressed feeding test and acute restraint stress.* ***A*** *At each time point aged mice showed a lower sucrose preference than young mice p < 0.05, RM two-way ANOVA with pairwise comparisons).* ***B*** *Young mice drank more sucrose solution on the final day of habituation compared to aged mice (p< 0.05, RM two-way ANOVA with pairwise comparisons).* ***C*** *Young mice consumed more liquid overall during the test at 2 and 4h (p< 0.05, RM two-way ANOVA with pairwise comparisons).* ***D*** *Aged mice had a shorter latency to eat than young mice (p < 0.01, independent t-test)* ***E*** *but no difference in latency to approach (p > 0.05).* ***F*** *Young mice had higher CORT levels post stress than older mice (p< 0.001, RM two-way ANOVA).* ***G*** *The oldest group showed a reduced faecal count compared to the younger groups (p < 0.05, Kruskal-Wallis test with Dunn's post-hoc, bars are median with interquartile range).* ***H*** *The older groups showed greater CeA activation than younger mice (p < 0.001, one-way ANOVA with Tukey's post-hoc analysis).* ***I*** *and greater BLA activation (p < 0.05)* ***J*** *but reduced PVN activation (p < 0.01). Unless otherwise stated, bars are mean ± SEM with data points overlaid. N = 9-12 per group. *p < 0.05, **p < 0.01, ***p< 0.001, ###p< 0.001(*between-subject, #within-subject).*

**2.3.5 Rimadyl reduces foot slips in aged but not young mice in a rod traversal task.**

In a rod test pilot where traversal time over a 10 mm rod was measured, aged mice took longer to traverse the rod and reach the goal box than younger mice ($t_{(22)}$ = 2.6, p = 0.0164) (**fig.8.A**). In the main task mice traversed across the 10 mm three times and average performance was calculated. Mice were dosed with either a clinical dose of Rimadyl or vehicle. There was no effect of drug on traversal time (p > 0.05) but there was no drug*age group interaction, though there was trend ($F_{(1,21)}$ = 3.040, p = 0.096). There was a main effect of age ($F_{(1,21)}$ = 78.650, p < 0.0001). Post-hoc pairwise comparison showed that aged mice took longer to traverse the rod than younger mice under both drug and vehicle (p < 0.0001) (**fig.8.B**). There was a main effect of drug on foot slips during traversal ($F_{(1,21)}$ = 5.542, p = 0.028). There was also a drug * age group interaction ($F_{(1,21)}$ = 7.251, p = 0.014) and a main effect of group ($F_{(1,21)}$ = 4.581, p = 0.044). Pairwise comparison revealed that old mice made more foot slips than young mice under vehicle conditions (p = 0.007) but under Rimadyl this difference disappeared (p > 0.05). Rimadyl reduced foot slips in aged (p = 0.002) but not young mice (p > 0.05) (**fig.8.C**). Given the limited distribution of data for falls during traversal average falls are reported without statistical analysis. Young mice fell an average of 0.03 times under vehicle and an average of 0.08 times under Rimadyl. Aged mice fell an average of 0.151 times under vehicle and an average of 0.006 times under Rimadyl (**fig.8.D**). Analysis of overall composite score revealed no main effect of drug or drug * age group interaction, though there was a trend ($F_{(1,21)}$ = 3.073, p = 0.094. There was a main effect of age group ($F_{(1,21)}$ = 23.845, p < 0.0001)

and pairwise post hoc comparison revealed that aged mice had a greater composite score than younger mice under vehicle and Rimadyl ($p < 0.0001$ and $p = 0.007$) (**fig.8.E**).

**Figure 8**

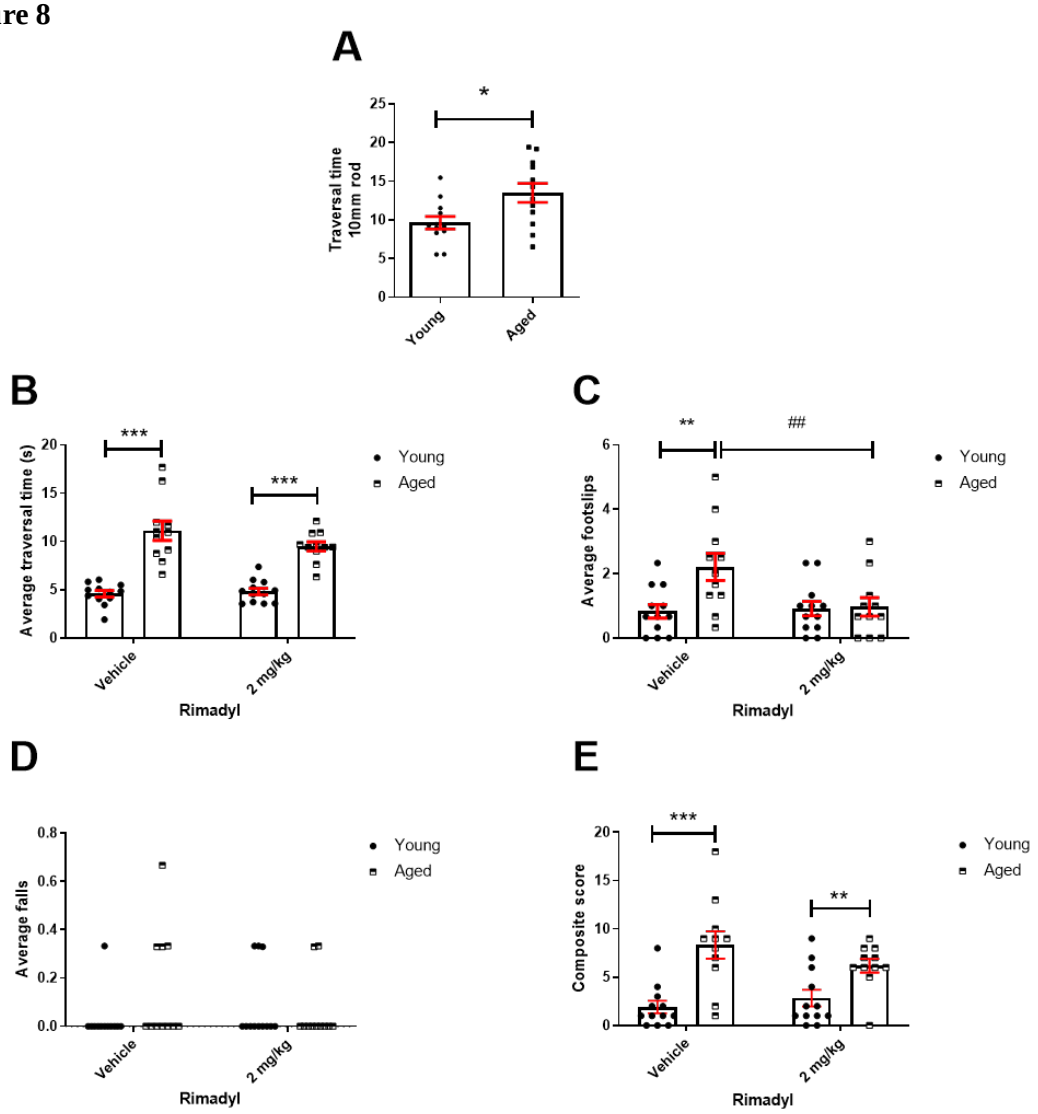


***Fig.8. Rimadyl reduces foot slips in aged but not young mice in a rod traversal task.*** *Young and aged mice traversed a 10 mm rod 3 times and traversal time, foot slips and falls were measured.* ***A*** *Young mice traversed the rod more quickly than aged mice ($P < 0.05$, independent t-test).* ***B*** *Rimadyl had no effect on traversal time in either age group, (RM two-way ANOVA, $p > 0.05$).* ***C*** *Rimadyl reduced foot slips in aged but not young mice (RM two-way ANOVA with pairwise comparisons $p < 0.01$).* ***D*** *Average number of falls across three traversals in aged and young mice.* ***E*** *There was no effect of drug on composite score but aged mice had a greater composite score than younger mice under both vehicle and Rimadyl (RM two-way ANOVA with pairwise comparisons $p < 0.001$ and $P < 0.01$ respectively). N= 12 young mice and N = 11 aged mice. Bars are mean ± SEM with data points overlaid. *p<0.05, **p<0.01, ***p<0.0001, ##p< 0.01 (*between-subject, #within-subject).*

## 2.4 Discussion

This study showed aged male mice show a reduction in novel environment exploration, reduced response vigour in the PR task and preference for the low value/low effort option in the EfR task under conditions of food restriction, suggesting a motivational deficit. These deficits appear unrelated to changes in appetite, general behavioural output or overt motoric deficits, however the effects of age-related inflammation cannot fully be ruled out. Aged mice were quicker to eat in the NSFT, showed a reduced reward sensitivity in the SPT and a blunted response to stress measured by changes in CORT and PVN c-fos activation. Together this indicates emotional blunting and is distinct from a profile of negative affective state seen in depression models (Planchez et al., 2019). In a separate study, aged mice showed a deficit in complex motor control which was partially sensitive to anti-inflammatory treatment. The following discussion considers how these behavioural and physiological deficits are relevant to multiple domains of human apathy.

### 2.4.1 Aged mice show evidence of a reduction in motivated behaviour.

Aged mice were slower and covered less distance in a novel, open field arena. This reduction in locomotion may reflect a reduction in explorative behaviour, which has been shown to have a strong motivational component and is sensitive to manipulation of dopaminergic transmission in the striatum (Patel et al., 2012). In human studies, it has been shown that ageing is associated with psychomotor slowing, independent of other confounding age-related effects and it has been suggested this slowing is due to a reduction in motivation and emotional arousal, which can be independent of confounding age-related effects (Seidler et al., 2010, Tombaugh, 2004). In addition, psychomotor deficits have also been observed in patients with apathy (Feil et al., 2003) and depression (Sawa et al., 2012). It is important to acknowledge that age-related deterioration in motoric capability may play a role in the reduction of locomotion. Findings from the raised beam test suggest some evidence of inflammation in aged mice that may inhibit movement. However, this test was conducted at the end of the behavioural battery, 9 months after novel environment exploration and these deficits may have appeared later in the timeline. In addition, aged and young mice covered equivalent distances in the first 5 mins and a difference only emerged in the two final time bins which suggests a declining motivation to explore rather than an overt motoric deficit. Although it should be noted that these effects were marginal.

During operant conditioning both young and aged mice performed an increasing number of trials over sessions of CRF indicating normal learning in the aged mice. However, by the final two sessions aged mice completed less trials than the younger mice, indicating reduced vigour in response. Importantly, there was no difference in session at which FR training was completed, so any further deficit measured in the PR and EfR tasks were not down to differences in initial learning.

A food restriction protocol was used to induce a higher motivational state. Under these conditions, aged mice finished on a lower ratio in the progressive ratio test and, in line with this, showed a slower rate of responding when completing higher ratios. Of note, mice did not reach a true breakpoint, where mice stopped responding before the session end. Instead, mice continued to respond until the maximum time limit. To induce a true breakpoint, mice were put in a low motivational state by providing food *ad libitum*. Under these conditions, there was no difference in breakpoint, and there was no difference in speed at which each ratio was completed. This suggests rate of response is a driving force in the apparent motivational deficit in aged mice and is only evident when the mice are in a higher motivational state. This is pertinent to the instrumental phase of motivated behaviour, in which the individual moves away or toward the stimulus. This phase is characterised by high levels of activity, vigour and persistence (Le Heron et al., 2018a). In addition to breakpoint, response rate is also used as a measure of motivational state (Tanaka and Hamaguchi, 2019). A slower response rate in the aged mice may be reflective of a reduced vigour to obtain the rewarding stimulus. Slower rates of response have been found in other studies investigating apathy-related behaviour. A reduced rate of response in the PR task was found in a study investigating apathy-related behaviour in a HD mouse model (Oakeshott et al., 2012, Heath et al., 2019) and were consistent with findings in a HD clinical population, where apathy questionnaire scores negatively correlated with breakpoint (Heath et al., 2019). This deficit is likely not due to a reduced valuation of the rewarding stimulus, as the consumption test revealed that aged mice eat an equivalent amount of reward pellets in 10 min to younger mice when they are freely available. There is the potential for increased activity costs experienced by the aged mice to play a role in this deficit, which would increase the perceived level of effort despite the actual operant requirements being equivalent between young and aged mice. This is addressed to some extent by demonstrating that young and aged mice can perform to an equivalent level in devalued conditions. However, it does not fully rule out this effect and may be relevant to human apathy in ageing, where the perceived effort cost of a once-enjoyed activity becomes too high to make it worthwhile.

An alternative way to look at motivated behaviour is the EfR task. The EfR is an effort-based decision-making task that offers the choice between a higher value but higher effort to obtain reward, or a lower value but lower effort to obtain reward. The EfR was run here in two different formats. First, in a single session directly following training, and again over 5 consecutive days later on in the behavioural battery. On first exposure to the EfR task, aged mice completed less trials than younger mice and consumed more chow, indicating aged mice are less willing to put in high effort for reward when an easier to obtain but lower value reward is available, which indicates a reduction in goal-directed behaviour. When the task was presented consecutively later in the behavioural battery, after animals had undergone multiple sessions under FR8 and PR schedules, a group difference emerged only in the final two sessions. This may be driven by changes in appetite due

to the feeding protocol, where mice are given food *ad libitum* over the weekend and food restricted across the week. This design confound is an additional example of food restriction being a potent motivator and links with the previous PR data. Concordantly, speed to FR8 completion increased over the 5 sessions in both groups and may additionally reflect an increase in motivation over sessions. However, as the effect of feeding was not tested directly in the EfR, a firm conclusion cannot be made. Changes in chow consumption were however not observed and may be due to variability resulting from excessive digging/wastage from the bowl. Alternatively, chow consumption may be a less sensitive measure of performance in the task due to the relatively small amounts consumed. Changes in effort-based decision making have also been shown in patients with apathy. PD patients with apathy were less likely to exert effort for reward, specific to when the reward value and effort level were low (Le Heron et al., 2018b). Small vessel cerebrovascular disease patients with apathy were less likely to exert effort for low reward and were less willing to exert high effort for reward (Saleh et al., 2021).

While both the EfR and PR are measures of goal-directed behaviour, the integration of findings from both tasks were used to account for any limitations associated with any one task. For example, the PR task has been criticised due to the potential for the task to become a measure of physical ability rather than motivation. The task is also sensitive to changes in hedonic processing and appetite changes, which makes interpretation difficult. By using the EfR it is easier to account for these confounds. While a shift from the high reward to low reward option would indicate a shift in motivational state, an overall change in consumption would indicate a change in general appetite.

To test whether differences in EfR performance were driven by potential age-related inflammation affecting the joints, mice were given a clinical dose of the non-steroidal anti-inflammatory drug (NSAID) Rimadyl before completing the EfR task. There was no difference in performance in either age group +/- the drug suggesting that either the aged animals were not suffering from joint inflammation or that inflammation does not confound performance in the task. Findings from the raised beam test where Rimadyl reduced the number of foot slips during rod traversal suggests aged mice do show some evidence of inflammation but does not appear to impact this task. This is likely because nose poking does not require complex motor control. While the primary purpose of administering Rimadyl was to reduce potential joint inflammation and thus uncover any potential motoric deficits, it is also important to consider that reduction of systemic inflammation may have had an impact on motivated behaviour. As described in chapter 1, chronic low grade inflammation is characteristic of ageing, and has previously been linked to apathy (Yao et al., 2019). Inflammation has been shown to reduce motivated behaviour, and studies suggest this may be driven by disruption of the reward circuitry and dopaminergic activity (Felger and Treadway, 2017). This may in turn impact performance in the EfR task. Therefore, Rimadyl had the potential to increase performance of the aged mice in the task via a reduction in neuroinflammation. Of note, the difference in trial

performance between age groups disappeared under both drug and vehicle conditions, suggesting that repeat testing over a prolonged period of time could potentially reduce sensitivity of the task to age-related motivational deficit and drug effects. Future work should assess the potential therapeutic impact of anti-inflammatory drugs on age-related motivational deficit.

Mice were also run in the novel object recognition task (NORT), which tests recognition memory by using the rodent's innate exploration preference for novelty over familiar. The NORT serves as a good behavioural control in this context as the task contains no external motivation, reward or punishment and can therefore be used to test for a general age-related blunting in behavioural output. There was no age-related impairment in ability to discriminate novel objects from familiar after a 4h delay phase. These results are consistent with previous work, which shows no impact of age after a 24h delay phase (Wimmer et al., 2012).

Together, these data provide evidence that aged mice show a deficit in the activational phase of motivated behaviour, driven by reduced rate of response. This behaviour appears not to be explained by age-related differences in appetite or reward valuation as measured by consumption tests or general blunting in behavioural output as demonstrated in the NORT. There is the potential for age-related inflammation of the joints to impact on performance in the tasks that cannot be fully ruled out, however it does not appear to impact on EfR performance. It should be noted that the same cohorts of animals were used for all studies in this chapter. As the tests were performed sequentially over a period of 9 months increasing age may have affected some measures. To mitigate this, the order of tasks was randomised between measures of motivation-related and emotional behaviour. However, this design does not fully mitigate a testing order or increasing age effect. It is also difficult in this context to rule out the effects of fatigue, a construct conceptually distinct from apathy but results in a similar phenotypic loss of motivated behaviour (Jurgelis et al., 2021). However, fatigue does not have an emotional/social component while apathy does, which further highlights the importance of assessing apathy behaviour over multiple domains, rather than motivation alone.

### 2.4.2 Aged mice show evidence of emotional blunting.

The NSFT is a measure of stress-induced anxiety behaviour in rodents. It challenges the rodent to overcome its aversion to a novel, open arena and eat from a bowl placed in the centre. In the NSFT, aged mice were quicker to consume food in a novel environment which indicates lower anxiety behaviour. This finding contrasts with studies in rodent models of depression where increased feeding latencies are consistently observed (Planchez et al., 2019). In contrast, aged mice showed reduced reward sensitivity in the SPT, consistent with reward deficits seen in rodent models of depression (Willner, 2016). These behavioural findings provide an interesting contrast to depression-like phenotypes and suggest an emotional blunting rather than negative affective state.

In line with these findings, a study found a reduction in exploratory behaviour and reward sensitivity using 1% SPT in aged (18 months) versus young (3 months) mice but no effect of age in the forced swim test (FST) and tail suspension test (TST), which have traditionally been used as tests of depressive-like behaviour (Malatynska et al., 2012), though there is debate (Commons et al., 2017). While emotional blunting was examined as a separate domain to motivated behaviour, it is likely this finding of blunted anxiety in aged mice impacts on measures of motivation such as exploration of a novel environment, where the open field design and lack of habitation adds an additional anxiety-inducing component.

To explore the apparent reduction in stress-reactivity seen in the NSF test at a physiological and cellular level, mice underwent an acute restraint stress at the end of the behavioural battery. Acute restraint stress reliably increases the stress hormone, corticosterone (CORT) in rodents (Harizi et al., 2007, Rademacher et al., 2008, Nohara et al., 2016) and therefore provides a robust readout of stress-reactivity. Pre-stress, all age groups showed similar CORT levels which suggests no underlying metabolic differences that would make interpretation of stress-induced changes difficult. All groups showed an increase post-stress, but the 12 and 24 mo age groups showed a blunted CORT level compared to the youngest group, indicating a reduction in stress-reactivity. In line with this, the oldest group showed a decrease in faecal output during stress compared to the youngest group. Stress reactivity and active coping in response to aversive experiences was shown to be reduced in other studies using aged mice (Oh et al., 2018) and extends across species to rats and humans (Brugnera et al., 2017, Buechel et al., 2014). However, it is important to acknowledge that there is heterogeneity in findings in the literature and even within this thesis (see chapters 3 and 4), with some finding an exaggerated stress response in the older groups. This variability is likely due to a lack of standardisation across studies including the nature and intensity of the stressor (Novais et al., 2017, Segar et al., 2009).

Neuronal activation in the paraventricular nucleus of the hypothalamus (PVN) was reduced in the oldest versus the youngest group following restraint stress. The PVN is central to the stress response. It contains a large population of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) neurons, which when activated, result in the release of CORT into the circulation. Therefore, the reduction in stress-induced CORT is likely related to this reduction in PVN activation. Neuronal activation in the amygdala was investigated due to its role in integrating the behavioural and the autonomic response to aversive stimuli. Compared to the PVN, there is relatively little activation in this area in response to restraint stress and other psychological stressors and is more sensitive to systemic stress or homeostatic disruption (Day et al., 2005). Interestingly, the older groups show a greater activation of both the central and basolateral amygdala compared to the younger group. The reason for this and its functional significance is unclear. The addition of a non-stressed group could provide additional information about whether the amygdala is inappropriately activated in the older

group. This could potentially be due to a homeostatic challenge such as chronic, low-grade inflammation characteristic of ageing (Gabuzda and Yankner, 2013). Given that PVN activation is low and amygdala activation high in the aged group, it is tempting to speculate a relationship between the two. Indeed, changes to the functional gating of limbic information by local PVN projections have been suggested as a potential mechanism to explain age-related changes to the HPA axis (Herman et al., 2002).

This finding of blunted stress reactivity in aged mice has relevance beyond the field of apathy. It is a potential confound for tasks such as the Morris Water Maze (MWM), a widely used assay to study cognitive impairments in aged animals (Morris, 1981). The MWM is motivated by the intrinsic stress-inducing effects of the animal being placed into water and therefore changes in stress reactivity could influence learning independent of any specific cognitive impairment. It has been shown that MWM performance is closely linked to stress intensity. Rats trained in 19 °C water performed better than rats trained in 16 °C water (higher stress) and 25 °C water (lower stress). A long-term recall probe test showed rats trained in 25 °C water performed worse than rats trained in 16 °C and 19 °C water, and performance was improved if CORT was administered just before task onset, which shows that lower stress conditions can negatively impact performance in the task and a sufficient CORT response is necessary for optimal learning (Sandi et al., 1997).

### 2.4.3 Ageing impacts on performance in the raised beam test.

The raised beam test, where the rodent must traverse rods of various widths to reach a goal box, is a measure of complex motor control, which requires an effective interplay of motor control, cognition, and attention. It is sensitive to pharmacological and lesioning manipulations (Luong et al., 2011). In an initial pilot test, use of the 10 mm rod revealed an increase in traversal time in aged versus young mice and therefore was chosen for the main study. This reduction in gait speed is consistent with other work that demonstrated aged C57bl/6 mice show a reduced traversal time on a balance rod compared to younger mice (Ingram et al., 1981). There are several potential explanations for this; slower traversal time in aged mice may reflect a reduced motivation to reach the goal box or reflects an increased attentional load where the mouse favours accuracy over speed. Age-related motor performance deficits, induced by reduced dopaminergic transmission result in an increased attentional load, such that motor tasks become more cognitively demanding and are less 'automatic'. Slowing can occur because more emphasis is placed on movement accuracy rather than speed (Seidler et al., 2010). However, aged mice also showed an increased number of foot slips, suggesting a reduction in accuracy. There was a potential for general age-related effects such as inflammation or pain to confound results. Indeed, ageing is associated with joint pain and inflammation in both humans and rodents (Loeser, 2013). The anti-inflammatory drug Rimadyl had

no effect on traversal time in either young or aged mice, however it reduced the number of foot slips in aged but not young mice and brought them down to an equivalent level to the younger group. This suggests inflammation/pain may affect the ability of the back foot to grip the rod, resulting in a greater number of foot slips in the aged mice that is improved when symptoms are eased.

When traversal time, foot slips and falls are considered together by using a composite score, Rimadyl does not improve performance in the task. Therefore, only foot slips are sensitive to inflammation or pain related effects and reducing foot slips does not improve traversal time.

### 2.4.4 Conclusion

Aged mice show behavioural and physiological deficits relevant to multiple domains of apathy. Deficits measured by novel arena exploration, PR and EfR under food restriction suggest aged mice show a reduction in motivated behaviour. These behavioural changes were not explained by other age-related confounds such as changes in appetite, overt motoric changes, or general decline in behavioural output. A reduction in reward sensitivity, anxiety behaviour and stress reactivity demonstrated at a behavioural, physiological, and cellular level together indicate emotional blunting rather than a negative affective state. Together, these data suggest that aged mice can be used to study the underlying neurobiology of apathy in otherwise healthy ageing. The finding of reduced stress-reactivity has further relevance to studies of cognition where the task relies on intrinsic stress such as the MWM. Any change in normal stress response could confound interpretation of cognitive ability. The use of the raised beam test provides some evidence that aged mice show some motor impairments which are sensitive to anti-inflammatories and that when assessing age-related complex motoric deficits, foot slips may be sensitive to this non-specific effect.

# Chapter 3: Testing for an apathy phenotype in aged rats

Thank you to Julia Bartlett who completed the effort for reward and progressive ratio experiments on my behalf at the start of the COVID-19 pandemic.

### 3.1 Introduction

There is a tendency in the scientific literature to group mice and rats together, and to assume what is true of a rat must also be true of a mouse. While rats and mice belong to the same order of Rodentia, mice are not just 'tiny rats.' *Rattus norvegicus* (rat) and *Mus musculus* (mice), the species from which lab strains are derived, diverged approximately 20 million years ago. This, for context, is a similar range to the divergence between macaque monkeys and humans (Ellenbroek and Youn, 2016). It follows then that the genetic landscape and functionality of the two species differ quite considerably. While the use of mice has typically been favoured in the past due to its richer genetic toolbox compared to rats, the gap is rapidly closing and forcing scientists to decide which is the better model for their research, considering a number of biochemical, pharmacological and behavioural differences. Using two of the most used strains in neuroscience research, the C57bl/6 mouse and Sprague-Dawley (SD) rat, a microarray analysis showed that approx. 43% of analysed genes were differentially expressed between rats and mice in hippocampal dendritic neurons, compared to approx. 0.5% between two strains of mice (Francis et al., 2014). Given the importance of the hippocampus in behavioural neuroscience, this is particularly pertinent. Even at the level of laboratory housing, rats and mice show striking differences. The social behaviours of the rat and mouse differ considerably, where mice typically show much higher levels of aggression towards cage mates, and death due to cage aggression is not uncommon. In contrast rats show very little aggression but show higher levels of playfighting during the juvenile stage (Kondrakiewicz et al., 2019). Therefore, it is clear that mice and rats show fundamental differences in behaviour that may affect how behaviour changes with age.

Choice of appropriate species may also depend on their ability to perform the task of interest. In addition to a reduction in motoric activity that is observed in human apathy patients, which was tested in the previous chapter with physical effort-based tasks, human patients experience a reduction in cognitive flexibility, planning and decision making (Malpetti et al., 2021). In species capable of more complex cognitive processing, tests of these behaviours may also be relevant to the study of apathy behaviour. The PRLT (described in chapter 1, section 1.7.3) is a translational measure of cognitive flexibility. The subject must flexibly adjust their behaviour to changing contingencies, unlearning a previously rewarded option and learning a previously unrewarded option. There is evidence to suggest that mice show limited behavioural flexibility compared to rats, suggesting that rats are a more suitable model to test more complex cognitive behaviours (Metha et al., 2020). Therefore, the use of rats could allow for cognitive aspects of apathy-related behaviour to be tested in a way that was not possible in the previous chapter.

The rodent PRLT is often conducted in an operant box context, which is characterised by large numbers of trials and repeated sessions which may result in performance in the box becoming procedural and lacking in sensitivity. This is concerning as the PRLT depends on attention to specific types of feedback. Bowl digging assays could be used to translate operant-based tasks into a more ecologically relevant setting and have been used previously to assess affective state, attention and cognitive flexibility (Stuart et al., 2013, Birrell and Brown, 2000, McAlonan and Brown, 2003). Comparing PRL performance in and out of the operant box context could provide important information about how well operant tasks align with more ethologically relevant methods.

It is important to address whether aged rats show apathy-related behaviour as previously shown in aged mice. This could provide important insight into species differences in ageing and apathy-related behaviour, allowing researchers to make a more informed decision on choice of model. Furthermore, the next chapter investigates age-related changes in biological rhythms using aged rats, and it was important that apathy behaviour in rats also be characterised. The use of rats also allows us to go beyond the behavioural battery used in the previous chapter to test cognitive flexibility, which may help to better understand the symptom of apathy. Therefore, the aim of this chapter was first to determine whether aged rats show apathy-related behaviour using the battery of behavioural tests used in the previous chapter. The second aim was to assess the impact of age on tasks used to assess cognitive flexibility, using bowl-digging and operant-based methods as a comparison. The third aim was to investigate a potential molecular mechanism for age-related deficits in cognitive flexibility by assessing MR and GR expression in the dorsal hippocampus.

## 3.2 Methods

### 3.2.1 Subjects

#### Cohort 1

10 aged, male Sprague-Dawley rats (aged 21 months old at start of experiments, weighing 467-530g and 25 months, weighing 480-555g by end) and 10 sex and strain-matched younger controls (4 months old and weighing 310-320 g at start of experiments, 8 months, weighing 470-545g by end) were kindly supplied by Eli Lilly. The aged rats had spent a period of approx. 5 months on a restricted diet (16g fed between ZT2-3) at Eli Lilly. This was continued for a period of 5 days at Bristol to aid adjustment before being put on *ad libitum* feeding for the beginning of experiments.

#### Cohort 2

12 aged male Sprague-Dawley rats (aged 23 months at experiment onset, weighing 440-525g and aged 27 months and weighing 401-560g by experiment end) were kindly supplied by Eli Lilly. Rats arrived at the Bristol unit aged 21 months but due to COVID restrictions experiments did not start until 2 months later. During this delay period aged rats were fed a restricted diet of 18g/rat. One week before experimentation started aged rats were put onto *ad libitum* feeding to match young controls. 2 aged rats died before onset of experimentation (one was S1d due to loss of motor function of the back legs, the other died of a random cardiac event). 10 strain and sex-matched controls were obtained from Envigo at a weight between 300-324g, aged approx. 3 months and aged 7 months and weighing between 403-460g by experiment end.

Rats were put in enriched housing, consisting of a red shelf, wooden chew block and cardboard tube in groups of either threes or pairs (counterbalanced across age groups). Water and food were given *ad libitum*, unless undergoing operant training. They were kept in 12:12 reverse lighting, with lights OFF at 8.15am and ON at 8.15pm. Behavioural testing was done under red lighting and within the animal's active phase unless stated otherwise. Rats underwent a behavioural battery, outlined in **figure 1**.

**Figure 1**

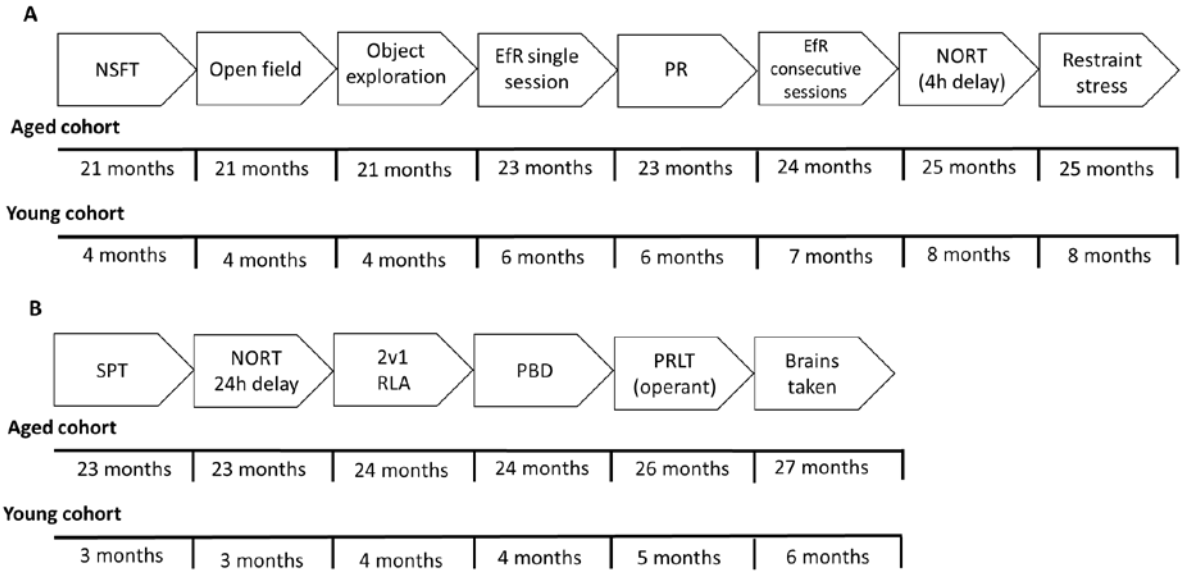


***Fig.1. Timeline for analysis of age-related behaviours.*** *Two cohorts of rats underwent a series of behavioural tasks.* ***A*** *Cohort 1.* ***B*** *Cohort 2. NSFT-novelty supressed feeding test, EfR- effort for reward, PR-progressive ratio, NORT-novel object recognition test, SPT- sucrose preference test, RLA-reward learning assay, PBD-probabilistic bowl digging, PRLT-probabilistic reversal learning.*

### 3.2.2 Exploration of a novel arena

Rats were placed in a square arena (1 m x 1 m) lined with sawdust. Rats were placed in a corner and could explore the arena for a period of 15 min. Sawdust was redistributed between rats. Their movement was recorded using a Logitech webcam and total distance covered (cm) and velocity (cm/s) were analysed using Ethovision xt10 software.

### 3.2.3 Object exploration

Rats were placed in the same square arena as the previous task. A novel object (either a pinecone or a scrubbing brush) was placed in either the centre or to the side of the arena. The rat was placed in the arena and could explore the arena and novel object for a period of 10 mins. Sessions took place over two days, and position and object were counterbalanced over these two sessions and across age group (**table 1**). Their movement was recorded using a Logitech webcam, and the videos were subsequently coded so that the experimenter was blind to age group. Time spent exploring the object and bouts of exploration were manually recorded.

**Table 1**

| Rat ID | Session 1 item | Session 1 position | Session 2 item | Session 2 position |
|---|---|---|---|---|
| **1** | Pinecone | Middle | Scrubbing brush | Side |
| **2** | Scrubbing brush | Side | Pinecone | Middle |
| **3** | Pinecone | Side | Scrubbing brush | Middle |
| **4** | Scrubbing brush | Middle | Pinecone | Side |

***Table 1 Example counterbalancing of object exploration experiment.***

### 3.2.4 Novelty supressed feeding test

Rats were food restricted for 24 hrs before onset of the test. During testing, rats were placed in a circular arena (70 cm in diameter), lined with sawdust. The test was conducted under white lighting, but during the animals' active phase. A bowl was placed in the centre of arena containing standard lab chow. Time taken for the rat to approach the bowl and to eat from it was manually recorded using a stopwatch. Sawdust was redistributed between rats and the bowl was washed and fresh food pellets were used each time.

### 3.2.5 Consumption test

To test for potential appetite confounds that may drive results observed in the novelty supressed feeding test, rats were food restricted for 24hrs. They were then placed in individual test cages they had previously been habituated to, with a known weight of food. After a period of 10 mins, total amount eaten in g was measured. Rats were then returned to *ad libitum* feeding.

### 3.2.6 Operant training

Rats were trained in sound-proofed operant boxes (Med Associates Inc) which were run on Klimbic software (Conclusive Solutions Ltd., UK) similar to the protocol previously described by (Griesius et al., 2020) and to the previous chapter. Each box consisted of two levers positioned either side of a reward magazine which was connected to a reward pellet dispenser (45 mg rodent tablet, TestDiet, Sandown) (see chapter 2, fig 2A). Only one lever was active during training/testing, and positioning was counterbalanced across cohorts. Unless otherwise stated, a session lasted 30 mins. Rats first learned to associate the magazine with the delivery of reward where pellets were dispensed automatically every 40 s for two sessions. Rats then underwent three sessions of continuous reinforcement learning, in which they learned to associate a lever press with the delivery of a reward. They then progressed on to FR training. Rats progressed through FR1, 2, 4 and 8 after two consecutive sessions of 100+ trials, and then FR16 after two consecutive sessions of 70+ trials. All rats then underwent at least 5 additional FR16 sessions.

### 3.2.7 Effort for reward task

Directly after training completion rats underwent a single session of the effort for reward task, as previously described in chapter 2, with the exception that a FR16 rather than FR8 session was used. Further along the experiment timeline rats completed 5 consecutive EfR sessions under both food restriction and *ad libitum* (**fig.1**).

Where performance over multiple sessions were analysed, if a rat had outlying data > 2 sessions it was excluded from analysis. If the rat had outlying data within one session it was replaced with the group mean.

### 3.2.8 Progressive ratio task

Rats were tested in the progressive ratio task, using the same conditions as the previous chapter, with the exception that session lasted 60 min rather than 120 mins. A single PR session was conducted under food restriction conditions and *ad libitum* feeding.

### 3.2.9 Novel object recognition test

Rats were habituated and tested under the protocol used in the previous chapter but with some differences described. Rats completed NORT in a square arena (1 m x 1 m) lined with sawdust and their activity videoed using a Logitech webcam. Animals that did not reach 20 s of exploration in the sample phase were excluded from discrimination ratio analysis (n = 2 aged rats). The sample phase lasted a total of 5 mins and the test phase a total of 3 mins. The delay phase was either 4 h or 24 h.

### 3.2.10 Restraint stress

Rats were restrained for 30 mins using a restraint tube under the same protocol as the previous chapter, where samples were collected immediately preceding and post-stress. Blood was collected by inserting a butterfly needle into the tail vein. Blood was dripped into an Eppendorf containing 50 µL EDTA. Blood samples were then centrifuged at 8000 rpm for 10 mins. Plasma was collected and stored at -20 °c until analysis. A faecal count was not conducted due to lack of faecal response to restraint stress.

### 3.2.11 Corticosterone analysis using radioimmunoassay.

A radioimmunoassay (RIA) was performed in-house to measure total corticosterone from restraint stress blood samples as previously described (Windle et al., 1998). Samples were analysed by RIA in triplicate to account for variation in assay results and the median value was used in analysis.

To denature corticosteroid binding globulin, plasma was diluted 1:50 with citrate buffer (25 mM tri-sodium citrate, 50 mM sodium dihydrogen orthophosphate and 1g/L bovine serum albumin

(BSA) (Sigma-Aldrich, UK), pH 3.0). 100 ng/ml of CORT was serially diluted 1:2 in citrate buffer to create an 11-point standard curve. Known concentrations 20 ng/ml and 100 ng/ml CORT were used as quality controls (QCs). Rabbit anti-rat corticosterone primary antibody (a kind gift from G. Makara, Institute of Experimental Medicine, Budapest, Hungary) was diluted 1:50 in 0.2 ml distilled H2O, 9.8 ml 0.9 % saline solution, 500 ml citrate buffer and added to each sample, the QCs and the standard curve. Iodine corticosterone tracer ([$^{125}$I], Izotop Institute of Isotopes Co., Ltd., Hungary) was then added, diluted to give between 3500-4000 counts per minute (cpm). Samples, QCs and standards were gently shaken and then stored overnight at 4 ℃ to incubate.

Following overnight incubation, samples, QCs and standards were precipitated with charcoal solution (0.05 g Dextran T70 (Pharmacia Biotech, Sweden) and 0.5 g charcoal (Fluka Analytical, Sigma, UK) per 100 ml citrate buffer). They were then centrifuged for 15 min at 4000 rpm at 4 ℃. Supernatant was aspirated, and the remaining charcoal pellet was analysed using a gamma counter (E5010 Cobra II Auto Gamma, Perkin Elmer, Netherlands).

### 3.2.12 Sucrose preference test

During habituation, rats were water restricted for a period of 4 h and were then placed in test cages containing two sipper sacks for 1 h. On the first and third day the sipper sacks contained 2 % sucrose, and on the second day they contained tap water. On the test day, the rat was presented with one sipper sack containing 2 % sucrose and the other containing tap water. Starting position of sucrose was counter balanced and swapped at 30 mins. Amount consumed was measured at each time point.

### 3.2.13 Bowl digging training

Habituation: On the first day, cage mates were placed in a 40 cm$^2$ arena with a tray liner together covering the bottom and two pottery bowls placed at the back of the arena, approx. 1cm apart from each other. Each bowl was filled with reward pellets (LabDiet, 45 mg). Rats explored the arena with the lid fully closed for 10 mins. On the second day, rats were placed individually in the arena for 10 mins.

Pretraining: one reward pellet was placed in either the 10 o'clock, 12 o'clock or 2 o'clock position in one of the bowls. Once the rat had explored the bowls and found the pellet, a pellet was placed in the left-hand corner to encourage the rat to disengage from the bowl and return to the front of the arena. This was repeated until 12 trials were completed. Position of the baited bowl was presented in a pseudorandom order, and this applied to all stages of training. If the rat failed to find the pellet after 30 secs, the trial was scored as an omission. There were two sessions of pretraining.

Digging training: In the first trial, a pellet was placed in one bowl as described in the previous stage. In the second trial, the same bowl was filled with 1 cm sawdust and a pellet was buried in the same

position. This was repeated for 12 trials. On the second and third day, this stage was repeated but with 2 cm sawdust and the empty bowl was removed after the rat started digging in the baited bowl.

Discrimination training: The rat was presented with two bowls filled with 2 cm of a different digging substrate e.g., sponge, perlite, cardboard squares etc. One of these substrates was consistently baited with one reward pellet and the other was mixed with a crushed reward pellet to prevent odour discrimination. In the first trial, the rat explored both bowls until the pellet was found. In the second trial, as soon as the rat chose one bowl, the other was removed. If the rat chose the baited bowl, it was recorded as correct and if the non-baited bowl was chosen it was recorded as incorrect. This was repeated until the rat chose the baited bowl over 6 consecutive trials within a maximum of 20 trials. After this, the rat was considered fully trained and ready for testing.

### 3.2.14 2 vs 1 reward learning assay

The 2 vs 1 reward learning assay (RLA) is a 5 consecutive-day protocol consisting of 4 pairing sessions followed by a testing day as previously described by (Stuart et al., 2013). In the first pairing session, the rat is presented with two bowls containing two different digging substrates. One substrate is baited with either one or two reward pellets and the other was mixed with a crushed reward pellet but is a 'blank'- it contains no reward pellet. Once the rat chooses the baited bowl on 6 consecutive trials the pairing session is completed. On the second pairing session the rat is presented with a new substrate baited with the opposite number of pellets to the day before, along with the blank. On sessions 3 and 4 the first two days are repeated. Therefore, over the course of the 4 pairing sessions, the rat was presented twice with two different substrates baited with either one or two pellets, alongside the same blank. On the testing day, the rat was presented with the two previously baited substrates and no blank (**fig 2**). This time, the substrates were randomly reinforced 20 times with one reward pellet over the course of 30 trials. Each substrate was mixed with a crushed pellet to prevent odour discrimination. Position of the substrates were also counterbalanced pseudo-randomly. Substrate choice over the 30 trials and number of pellets consumed was recorded. A % choice bias for the two-pellet paired substrate was calculated using (number of two-pellet paired choices/30*100)-50. A positive value indicates the rat was biased towards the two-pellet paired substrate. Additional checks for substrate bias, directional bias and number of pellets consumed relative to chance (0 or 10) was performed.

### 3.2.15 Probabilistic reversal learning bowl digging task

In a single session, the rat was presented with two bowls containing two different substrates. One substrate was baited with a single pellet 80 % of the time ('rich' substrate), and the other substrate baited 20 % of the time ('lean' substrate). The rat had to choose the correct (rich) bowl 6 times consecutively within a maximum of 30 trials to successfully 'acquire' the first rule. Once the rat had acquired this initial rule the contingencies were reversed such that the rich substrate became

the lean substrate and *vice versa*. The rule was reversed every time the rat chose the rich bowl 6 times consecutively within 30 trials (**fig 2**). The position of the bowls was swapped pseudo randomly as described in the RLA. The session ended after a maximum of 1hr, or 4 consecutive omissions, or the rat failed to learn a rule within 30 trials.

**Figure 2**

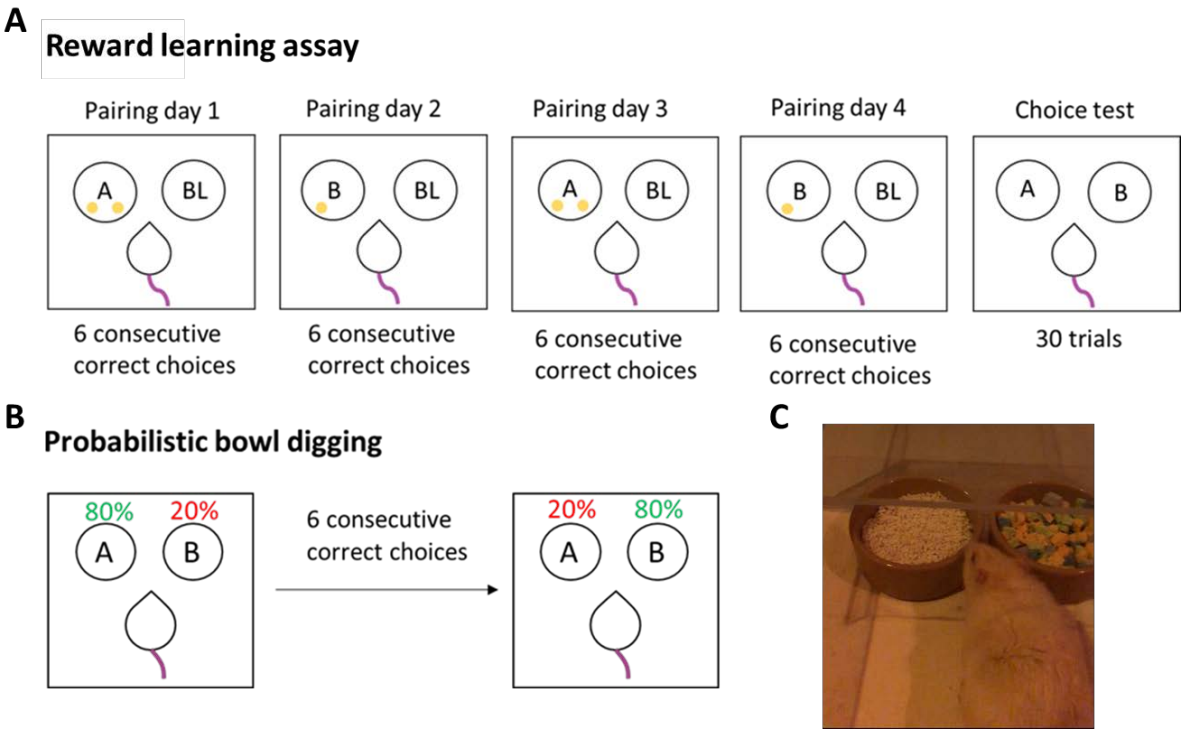


***Fig.2. Bowl digging tasks. A*** *The reward learning assay which consists of 4 pairing sessions followed by a choice test.* ***B*** *The probabilistic bowl digging task.* ***C*** *An aged rat making a digging decision.*

### 3.2.16 Probabilistic reversal learning operant task

Rats were trained in sound-proofed operant boxes as described above. The operant box consisted of an infrared touchscreen panel with three windows within which the animal could respond by touching them. A magazine was positioned centrally opposite the touchscreen panel where rats could access 45 mg reward pellets (TestDiet, Sandown Scientific, UK). The box also contained a house light and tone generator. The task was set up as previously described in (Wilkinson et al., 2020), which was based in the original design used in (Bari et al., 2010).

**Training**

Rats first underwent a 30 min session where a reward pellet was dispensed into the magazine automatically every 40 s. Rats then underwent CRF1 training where touching the initiation square

in the middle window resulted in the delivery of a reward pellet. This was accompanied by a tone playing and the magazine illuminating. The session finished after a maximum of 30 mins or the rat completed 120 trials. Once rats completed 120 trials over two consecutive days, they progressed to CRF2, where the rat must press the initiation square and then the window either to the left or right of it to receive a reward pellet. The session finished after a maximum of 40 mins or the rat completed 200 trials. If the rat touched the initiation square but took >10s to choose a side, then a 10s timeout occurred where the house light came on and no reward was delivered. Once the rat had completed >120 trials over two consecutive sessions it was deemed trained and ready to undergo the probabilistic reversal learning task.

**Probabilistic reversal learning task**

One window was designated the 'rich' stimulus and was rewarded 80% of the time (delivery of reward pellet) and punished 20% of the time (10s timeout and house light on). The other window was designated the 'lean' stimulus and was punished 80% of the time and rewarded 20% of the time. The starting position of the rich stimulus was counterbalanced across cohort and remained consistent across sessions. When the rat made 8 consecutive rich choices, the contingencies swapped, such that the window that was previously designated rich became lean and vice versa (**fig.3**). The contingencies continued to change each time the new rich stimulus was selected 8 consecutive times up to a maximum of 40 mins or 200 trials. Successful learning of the first rule was termed 'acquisition' and successful learning of the new rule was termed a 'reversal'. Where performance over multiple sessions were analysed, if a rat had outlying data > 2 sessions it was excluded from analysis. If the rat had outlying data within one session it was replaced with the group mean.

**Figure 3**

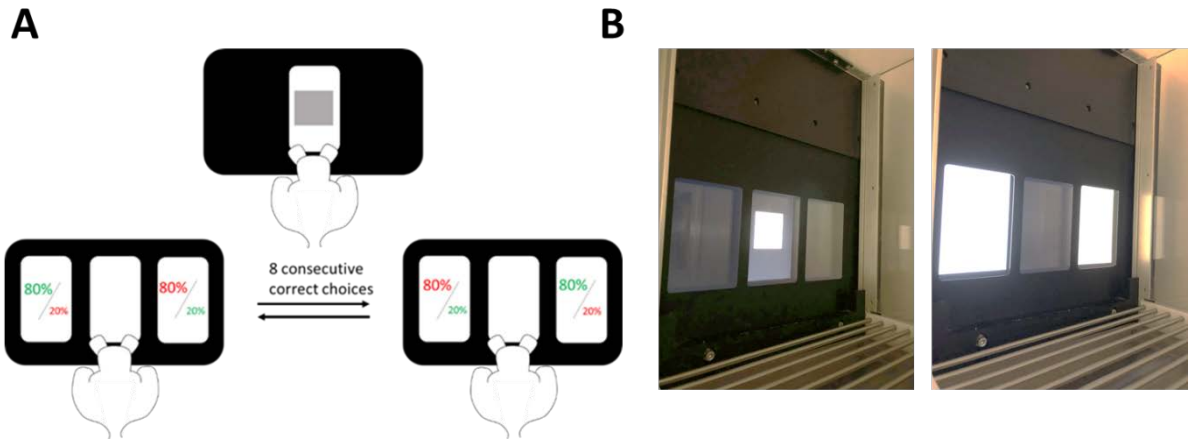


***Fig.3. The operant version of the probabilistic reversal learning task. A*** *The rat presses an initiation square and then must select either the left or right window, which are rewarded either 80% or 20 % of the time.* ***B*** *View from operant box.*

### 3.2.17 Riboprobe in situ hybridisation

#### Tissue collection

Following completion of behaviour rats were S1 with a pentobarbital overdose between ZT15-17. Brains were immediately extracted and frozen on foils on dry ice. Brains were then stored in a -80 °C freezer until use. Brains were sliced in 12 µm coronal sections using a cryostat. Dorsal hippocampal sections were taken at Bregma -2.40 mm. Sections were slide mounted on 5% gelatin coated slides in sets of 6, 3 sections per slide and were stored in the -80 °c freezer until use.

#### Purification of cut DNA and riboprobe synthesis

Cut DNA (previously described by (Seckl et al., 1990)) was purified using the MinElute Reaction Cleanup kit (Qiaquen, cat no 28204). DNA was then run on a 1% agarose gel at 80 µV for approximately 20 min to check that DNA was cut successfully. Presence of additional bands indicated supercoiled uncut DNA present and only DNA with a single band was selected to use in riboprobe synthesis. Radioprobes were then synthesised using the SP6/T7 transcription kit (Roche Applied Sciences, 109 996 44001) and $^{35}$S UTP radiation. RNA was then cleaned with the RNA MinElute RNeasy Cleanup kit (Qiaqen, cat no 74204). 1 µl probe was added to scintillation fluid and radioactivity was counted using a gamma counter (Perkin Elmer Wizard 2). MR sense 9.3 x $10^6$ cpm/µl, MR antisense 9.3 x $10^6$ cpm/µl, GR sense 8.3 x $10^6$ cpm/µl, GR antisense 8 x $10^6$ cpm/µl. These counts were as expected. Probe was then stored at -80 °C before use and used within 3 weeks.

**Riboprobe prehybridisation**

Slides were warmed to room temperature and fixed in cold, 4% paraformaldehyde made up in 1x PBS. Slides were then rinsed twice with 1 X PBS and submerged in a triethanolamine (TEA)/acetic acid (AA) mix (250ml saline, 3.5ml TEA, 0.625ml AA) for 10 mins. Slides were then dehydrated in ascending concentrations of ethanol- 70% (1 min), 80% (1 min), 95% (2 min), 100% (1 min) and then delipidated using chloroform for 5 mins. Slides were then put back in 100% and then 95% ethanol (1 min) before being stood up to dry.

**Hybridisation**

Each slide required 1000,000 cpm/slide, and a total volume of 90 µl/slide. To the probe, 2 µl/slide nucleic acid mix (250 µl single stranded salmon sperm DNA (Sigma D1626) and 250 µl 25mg/ml tRNA (Brewer's yeast, Roche Applied Science, 10109517001, 500 µl sterile water) was added. The mixture was heated at 65 °C for 5 mins and then immediately quenched on ice for 2 mins. 1 µl DTT/slide, 0.5 µl/slide 10 % SDS and 90 µl/slide hybridisation buffer (950 µl 1M Tris, pH 7.4, 190 µl EDTA, pH 8.0, 3570 µl 4M NaCl, 23800 µl deionized formamide (Sigma F9037), 9520 µl 50% dextran sulphate solution (50,000 MWt) (Sigma, D8906), 950 µl 50 X denhardts (Sigma D2532), 1000 µl sterile water) was then added. 90 µl of the final solution was added to scintillation fluid and radioactivity was counted. MR sense 2.2 x $10^6$ cpm/slide, MR antisense 1.5 x $10^6$ cpm/slide, GR sense 890,000 cpm/slide and GR antisense 805,000 cpm/slide. 90 µl of the final solution was added to the slide, covered with parafilm and incubated overnight at 50 °C. $CuSO_4$ was added to the bottom of the tray to prevent bacterial growth. Sense riboprobes were used to demonstrate absence of non-specific binding.

**Riboprobe washing**

The following day parafilm coverslips were floated off in 1x saline sodium citrate (SSC) (20 x stock solution 175.3 g NaCl, 88.2 g trisodium citrate, 800 ml distilled water, pH 7). Slides were then taken through 3 changes of 1 x SSC, dragging the slides back and forth vigorously through the solution to remove background staining. Slides were then washed in 1 x SSC/50 % formamide at 50 °C for 15 mins twice, dipped in 1 x SSC at 37 °C and then placed in a ribonuclease solution (250 µl RNaseA (40 mg/ml) per 500 ml of 1 x SSC) at 37 °C for 30 mins. This occurred a further 3 times. They were then placed back in 1 x SSC at room temp for 5 mins and then dipped in distilled water and dried. Slides were exposed to autoradiography film (FujiFilm Corp, Japan) in complete darkness for 6 days.

**Film development and quantification**

Films were developed using an auto processor (Compact X4 Automatic X-ray film Processor) in complete darkness. Sections were analysed using Scion Image v1.63. Films were placed on a light

box and sections visualised with Leica MZ6 microscope with a CCD camera attachment and were captured and measured in greyscale. The objective lens magnification was set to 1.0 x and light was set to 6. Lighting in the room was kept consistent throughout image capture. The mean optical densities (OD) of the autoradiographs were measured by comparison with simultaneously exposed 14C-labeled microscale samples (Amersham, Bucks, UK). The scale was set to mm using a known distance.

Images were analysed using ImageJ. Area of the region of interest was drawn around using the free draw tool and mean density was outputted in addition to a background value. Background was subtracted from region of interest mean density. A value for the left and right region were obtained and added together. An average value across 3 sections was used for analysis. In the dHPC, GR was measured in CA1 and dentate gyrus regions and MR in CA1, CA2, CA3 and the dentate gyrus.

### 3.2.18 Data analysis

As described in chapter 2, section 2.2.16.

## 3.3 Results

### 3.3.1 Aged rats show a reduction in object exploration without a deficit in overall exploration of a novel arena.

There was no difference in total distance covered (cm) and velocity (cm/s) in aged versus young rats when rats were placed in a novel arena for 15 min ($p > 0.05$, n = 1 young rat and n = 2 aged rats excluded due to recording issue) (**fig.4.A&B**). When distance travelled was split into three 5 minute time bins, there was a main effect of time, where distance travelled decreased over time ($F_{(2,30)} = 122.866$, $p < 0.0001$) (**fig.4.C**). There was no age*time interaction, though there was trend towards a main effect of age ($F_{(1,15)} = 3.501$, $p = 0.081$).

In a separate novel object recognition test, aged rats spent less time exploring objects in the sample phase than younger rats $t_{(12.492)} = 3.775$, $p = 0.002$ and the test phase $t_{(15)} = 3.390$, $p = 0.004$ (**fig.4.D&E**). Both young and aged rats had a positive discrimination ratio ($p < 0.0001$ and $p = 0.0175$ respectively). There was no difference in discrimination ratio between age groups after a 4h delay ($p > 0.05$) (**fig.4.F**). When NORT was repeated in a new cohort of young and aged rats but with a 24h delay phase, aged rats similarly spent less time exploring objects in the sample phase than younger rats ($t_{(18)} = 2.976$, $p = 0.0081$) and test phase ($t_{(17)} = 2.139$, $p = 0.0473$) (**fig.4.G&H**). However, neither young nor aged rats had a significantly positive discrimination ratio ($p > 0.05$), though in young rats there was a trend ($t_{(8)} = 2.203$, $p = 0.0587$) (**fig.4.I**). There was no difference in discrimination ratio between groups ($p > 0.05$).

**Figure 4**

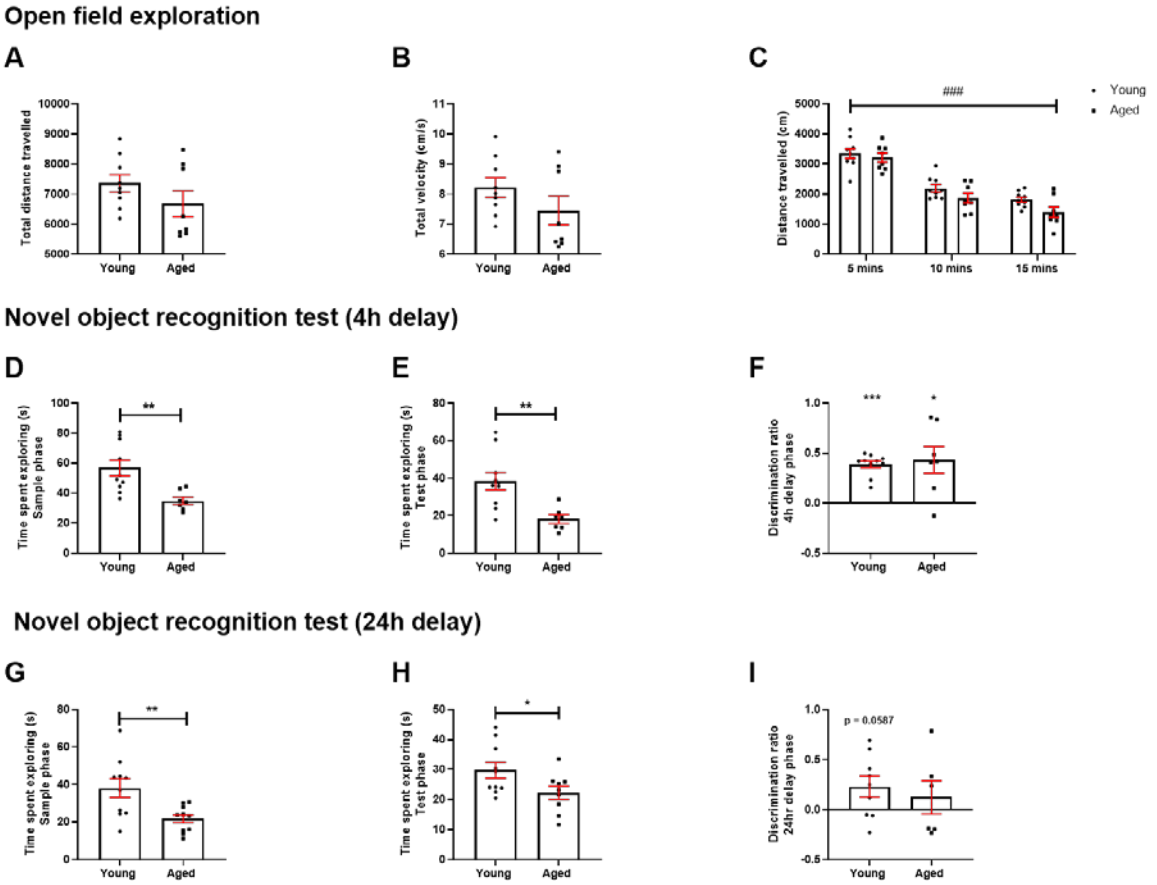


***Fig.4. Aged rats show normal levels of general activity in an open field arena but reduced exploration of novel objects.*** *Rats were placed in a novel arena for 15 min and activity was analysed. Rats also underwent the NORT with a 4h and 24h delay phase.* ***A*** *There was no difference in total distance travelled in the open field arena between age groups* ***B*** *or total velocity ($p > 0.05$, independent t-test).* ***C*** *There was no age difference in distance travelled when broken down into 5 min time bins, but distance travelled decreased over time ($p > 0.05$, RM two-way ANOVA).* ***D*** *Aged rats explored objects for less time in the sample phase ($p < 0.01$, independent t-test)* ***E*** *and the test phase ($p < 0.01$).* ***F*** *There was no age difference in discrimination ratio with a 4hr delay phase ($p > 0.05$, independent t-test).* ***G*** *In a new batch of aged and young rats, aged rats spent less time exploring the objects in the sample phase ($p < 0.01$, independent t-test)* ***H*** *and in the test phase ($p < 0.05$).* ***I*** *There was no age difference in discrimination ratio with a 24hr delay phase ($p < 0.05$, independent t-test). Bars are mean ± SEM with individual data points overlaid. n= 9-10 young rats and n = 6-9 aged rats. *p < 0.05, **p< 0.01, ***p< 0.001, ###p<0.001 (*between-subject, #within-subject).*

### 3.3.2 Aged rats show normal reward sensitivity and elevated levels of anxiety behaviour not characteristic of emotional blunting.

Both young and aged rats showed a significant % preference for 2% sucrose solution against a hypothetical mean of 50 % ($p < 0.0001$). There was no difference in % sucrose preference between age groups ($p > 0.05$) (**fig.5.A**). Two young rats were excluded due to excessive leaking from the nozzle. In the novelty supressed feeding test, aged rats had a greater latency to eat from the bowl compared to younger rats ($t_{(12.409)} = 2.664$, $p = 0.02$) but there was no difference in latency to approach the bowl ($p > 0.05$) (**fig.5.B & C**). Aged rats consumed more chow in a consumption test to assess appetite differences ($t_{(18)} = 2.416$, $p = 0.0266$) (**fig.5.D**).

When rats were given an object to explore placed at either the side or in the middle of the arena, aged rats explored the object for less time than younger rats ($t_{(11.376)} = 6.586$, $p < 0.0001$) (**fig.5.E**) and also showed a reduced number of bouts of exploration ($p < 0.001$) (**fig.5.F**). Aged rats showed a stronger preference for the object placed to the side versus the middle compared to younger rats ($t_{(13.737)} = 3.007$, $p = 0.010$). One young rat was excluded as an outlier (**fig.5.G**).

Rats underwent an acute 30 min restraint stress and plasma corticosterone was measured pre- and post-stress. There was a main effect of stress on CORT levels ($F_{(1,16)} = 55.55$, $p < 0.0001$), where stress increased CORT levels. There was no CORT*age group interaction and there was no main effect of age ($p > 0.05$) (**fig.5.H**) one aged rat died before this final experiment and one young rat was excluded due to incomplete blood sample.

**Figure 5**

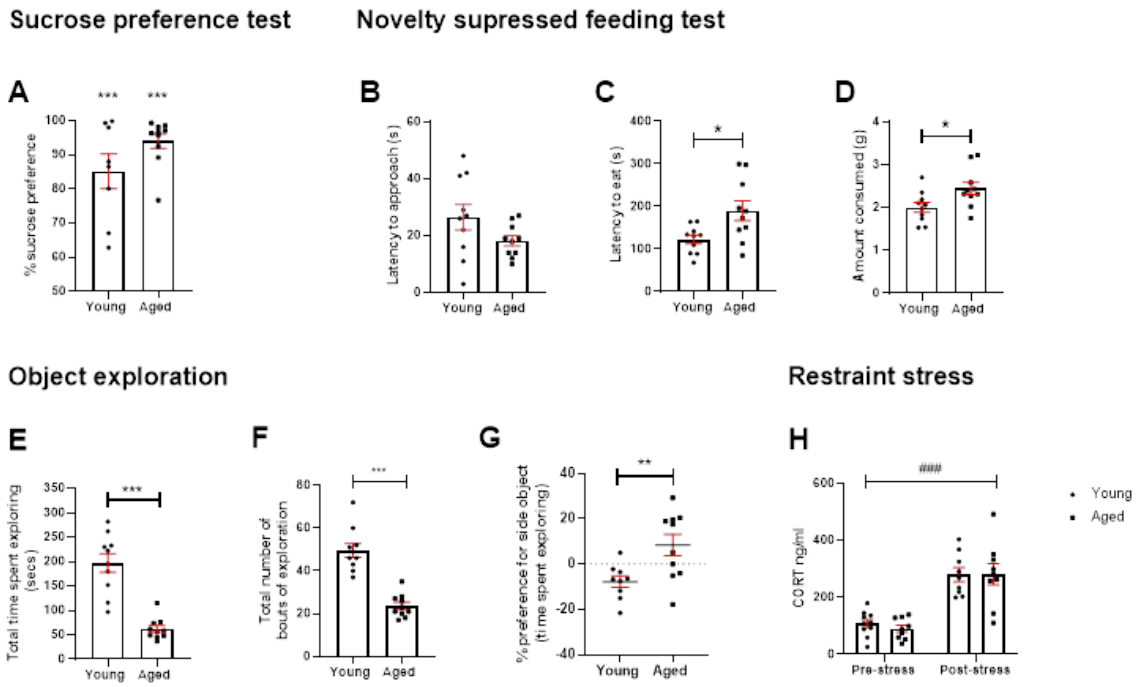


***Figure 5 Aged rats do not show changes in behaviour or physiology characteristic of emotional blunting.*** *Young and aged rats underwent the SPT, NSFT, object placement test and an acute restraint stress to test aspects of emotional blunting behaviour.* ***A*** *Both groups showed a preference for sucrose ($p < 0.001$, one sample t-test) and there was no difference in % sucrose preference between age groups ($p < 0.05$, independent t-test).* ***B*** *There was no difference in latency to approach between age groups ($p > 0.05$)* ***C*** *but aged rats took longer to eat from the bowl than younger rats ($p < 0.05$).* ***D*** *Aged rats consumed more chow in a 10 min consumption test ($p < 0.05$).****E*** *Aged rats spent less time exploring objects in an open field arena ($p < 0.001$, independent t-test)* ***F*** *and had less bouts of exploration than younger rats ($p < 0.001$).* ***G*** *Aged rats had a greater % preference for an object placed to the side versus the middle compared to young rats ($p < 0.01$, independent t-test).* ***H*** *Acute restraint stress increased plasma CORT levels ($p < 0.001$, RM two-way ANOVA) but there was no difference between age groups ($p > 0.05$). Bars are mean ± SEM with data points overlaid (*between-subject, #within-subject). Young rats n= 9-10, aged rats n = 9-10. *$p < 0.05$, **$p< 0.01$, ***$p<0.001$, ### $p< 0.001$.*

### 3.3.3 Aged rats do not show a motivational deficit in operant measures of goal-directed behaviour.

Analysis of number of trials completed under a continuous reinforcement schedule in young and aged rats showed a main effect of session ($F_{(2,36)} = 75.321$, $p < 0.0001$) where performance improved over time. There was no session * age interaction and no effect of age on performance ($p > 0.05$) (**fig.6.A**). There was also no difference in session at which fixed ratio training was completed ($p > 0.05$) (**fig.6.B**).

Analysis of performance in the PR task under food restriction revealed no difference in final ratio completed or break point between age groups ($p > 0.05$) (**fig.6.C&D**). There was also no difference in breakpoint between age groups under *ad libitum* feeding, although there was a trend towards younger rats showing a greater breakpoint ($t_{(18)} = 1.964$, $p = 0.651$) (**fig.6.E**).

In a single session of EfR, conducted directly after training finished, there was no group difference in number of trials completed or chow consumed ($p > 0.05$). n = 1 younger rat excluded from trials data as an outlier (**fig.6.F&G**). When EfR was conducted over 5 sessions, with rats fed *ad libitum* over the weekend and food restricted across the week to match mouse feeding conditions, there was a main effect of session ($F_{(2.613, 41.815)} = 12.006$, $p < 0.0001$) and a session*age interaction ($F_{(2.613, 41.815)} = 4.07$, $p = 0.016$). There was also a trend level effect of age ($F_{(1,16)} = 3.643$, $p = 0.074$). Post-hoc analysis revealed that aged rats increased number of trials completed over sessions (1 v 5, $p = 0.003$) but younger rats did not ($p < 0.05$). Aged rats completed more trials than younger rats on days 3 and 5 ($p = 0.029$ and $p = 0.044$ respectively). N = 1 aged and young rat excluded from trial data due having outlying data over multiple days. There was a trend towards aged rats completing more trials on session 4 ($p = 0.053$) (**fig.6.H**).

There was also a main effect of session on chow consumed ($F_{(3.228, 58.1)} = 3.0$, $p = 0.034$). There was no session*age interaction ($p > 0.05$) and there was a trend level effect of age group ($F_{(1,16)} = 4.373$, $p = 0.051$). Post hoc pairwise analysis of chow consumed over sessions revealed no change over time ($p > 0.05$, n = 1 aged rat chow data replaced with group mean due to a single outlier) (**fig.6.I**). Average speed to FR16 completion analysis was conducted using a series of independent t-tests rather than RM ANOVA due to missing data points from rats completing no trials. There was no difference in speed to FR completion across any of the sessions ($p > 0.05$, rats that completed 1 or no trials across 5 sessions or were excluded from the trial data were excluded from speed analysis (n = 2 young rats, n = 1 aged rats) (**fig.6.J**).

When EfR was conducted over 5 sessions under *ad libitum* feeding, there was no main effect of session, session*age interaction or effect of age on trials completed ($p > 0.05$) (n= 1 aged rat excluded due to outliers over multiple sessions, n = 1 young rat outlier in single session replaced with group mean)(**fig.6.K**). There was no effect of session of chow consumed and no session*age

interaction. However, there was an effect of age ($F_{(1,18)}$ = 7.178, p = 0.015, n = 1 young rat outlier in single session replaced with group mean). Pairwise post hoc analysis revealed that aged rats ate less chow than younger rats on days 2-5 (p = 0.049, 0.021, 0.008 and p = 0.035 respectively) (**fig.6.L**). There was no difference in speed to FR16 completion between groups (p > 0.05, rats that completed 1 or no trials across 5 sessions or were excluded from trials data were excluded from speed analysis, n = 3 aged rats and n = 1 young rats) (**fig.6.M**).

**Figure 6**

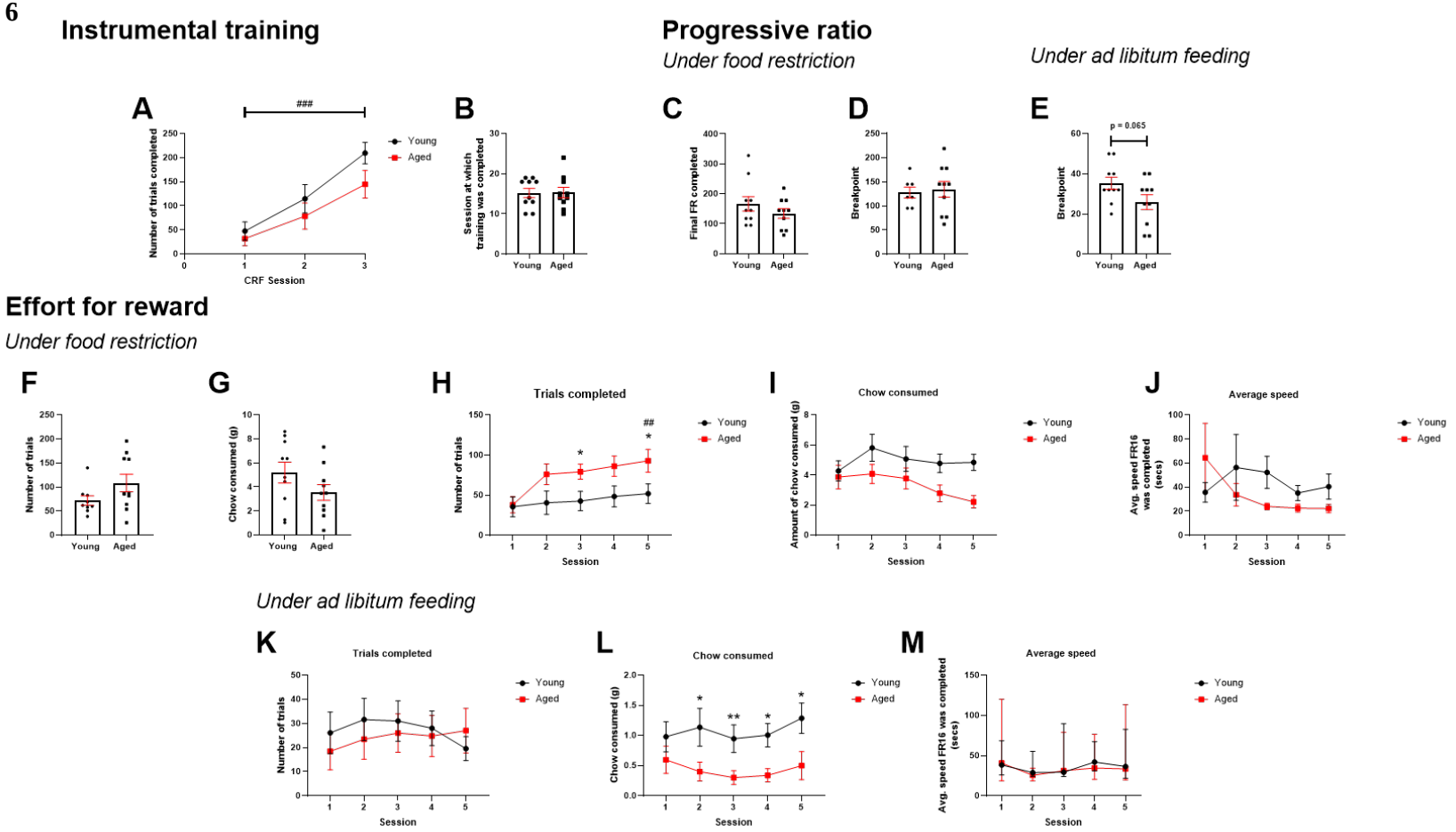


***Fig.6. Aged rats do not show a motivational deficit in operant effort tasks.*** *Aged and young rats underwent the PR and EfR task under food restriction and ad libitum feeding conditions.* ***A*** *CRF performance improved with session (RM two-way ANOVA, P < 0.0001) but there was no effect of age (p > 0.05).* ***B*** *There was no difference in sessions taken to complete fixed ratio training between groups (p > 0.05, independent t-test).* ***C*** *Under food restricted conditions, there was no difference in final fixed ratio completed or* ***D*** *breakpoint in the PR task between age groups (p > 0.05, independent t-test).* ***E*** *Under ad libitum*

*feeding there was no difference in breakpoint in the PR task, though there was a trend towards younger rats having a higher breakpoint ($p > 0.05$, independent t-test).* ***F*** *In a single session of EfR directly after training, there was no difference in trials completed between age groups ($p > 0.05$, independent t-test)* ***G*** *or chow consumed ($p > 0.05$).* ***H*** *Under food restriction, aged rat performance improved over sessions but young rats did not. On days 3 and 5 aged rats completed more trials than younger rats ($p < 0.05$, RM two-way ANOVA with pairwise comparison).* ***I*** *There was no effect of session or age on chow consumed ($p > 0.05$, RM two-way ANOVA)* ***J*** *or speed to FR16 completion.* ***K*** *Under ad libitum feeding conditions there was no effect of age or session of trials completed ($p > 0.05$, RM two-way ANOVA).* ***L*** *Young rats ate more freely available chow on sessions 2-5 compared to aged rats ($p < 0.05$, RM two-way ANOVA with pairwise comparison).* ***M*** *There is no effect of age or session on speed to FR16 completion ($p > 0.05$, RM two-way ANOVA). Bars are mean ± SEM with data points overlaid young n = 8-10 and aged n = 7-10. * $p < 0.05$, ** $p < 0.01$, ## $p < 0.01$, ###$p<0.001$ (*between-subject, #within-subject).*

### 3.3.4 Aged rats show normal reward learning and memory in a bowl digging reward learning assay.

Analysis of trials to criterion over pairing sessions showed a main effect of session on trials to criterion ($F_{(2.617, 44.486)} = 15.764$, $p < 0.0001$) where trials to criterion reduced with session (**fig.7.A**). One aged rat was excluded due to inability to complete pairing sessions. Analysis of latency to choose a bowl showed a main effect of session ($F_{(2.672, 42.758)} = 8.767$, $p < 0.0001$) where latency to choose decreased over time (**fig.7.B**). A further aged rat was excluded due to outlying data over multiple sessions. In the choice test, both young and aged rats showed a significant positive bias for the 2 pellet-paired substrate against a hypothetical mean of 0 ($t_{(8)} = 10.09$, $p < 0.0001$ and $t_{(7)} = 7.222$, $p = 0.0002$ respectively) (**fig.7.C**). One aged rat and one young rat were excluded as outliers. Rats did not eat more pellets than chance (against a hypothetical mean of 10) ($p > 0.05$) and there was no difference in pellets consumed between groups ($p > 0.05$) (**fig.7.D**). There was no substrate bias in either age group (tested against a hypothetical mean of 0) and no difference in substrate bias between age groups ($p > 0.05$) (**fig.7.E**). There was also no directional bias in either age group (tested against a hypothetical mean of 0) or age-related difference ($p > 0.05$) (**fig.7.F**).

**Figure 7**

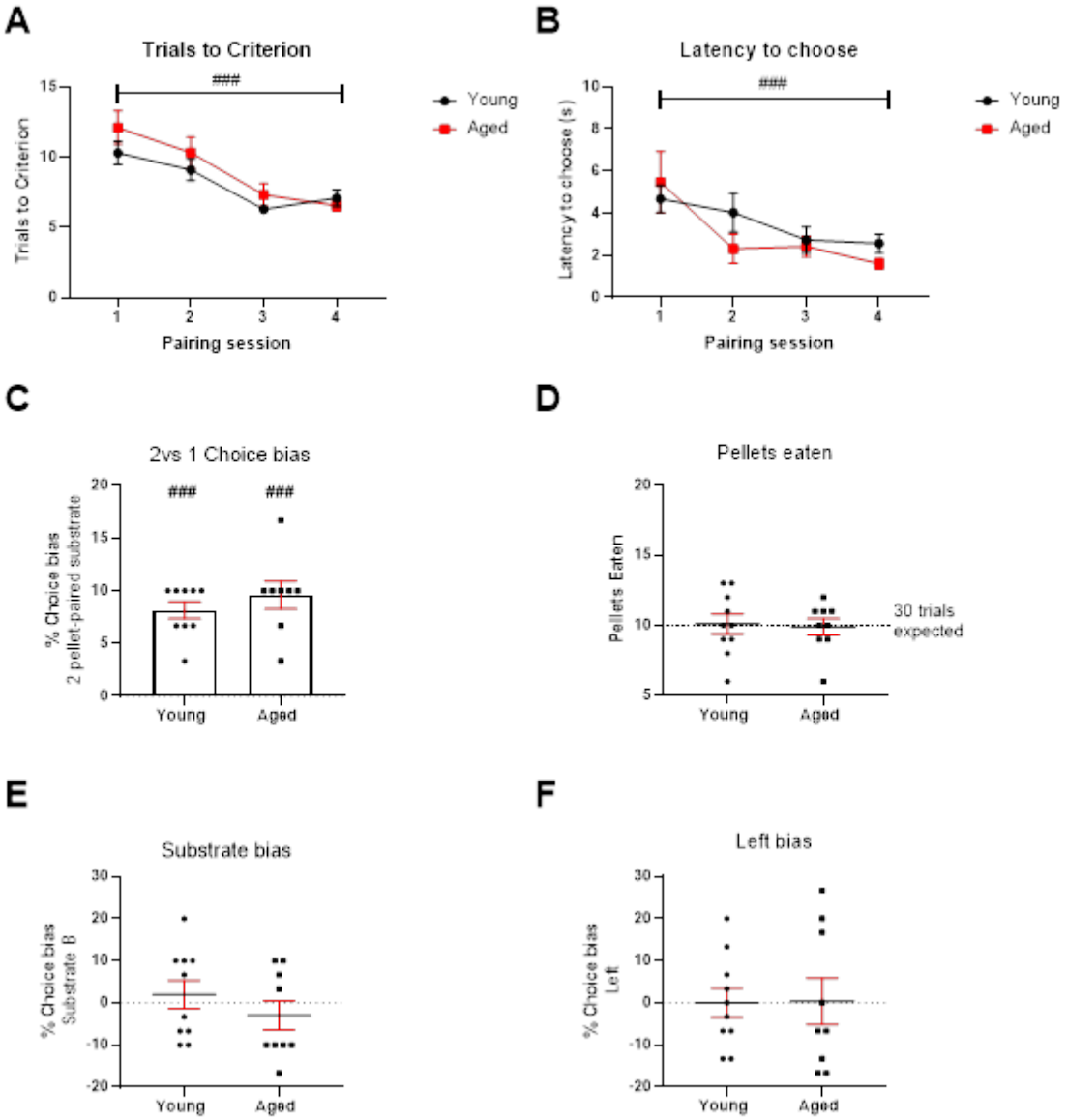


***Fig.7 Aged rats show normal reward learning and memory in a bowl digging reward learning assay.*** *Young and aged rats underwent the 2 vs 1 reward learning assay.* ***A*** *Both young and aged rats show learning over pairing sessions (RM two-way ANOVA, $p < 0.001$). There was no effect of age on learning ($p > 0.05$).* ***B*** *Latency to choose a bowl reduced over pairing sessions (RM two-way ANOVA, $p < 0.001$). There was no effect of age ($p > 0.05$).* ***C*** *Both young and aged rats had a positive bias for the two-pellet paired substrate ($p < 0.001$, one-sample t-test). There was no difference in bias between age groups.* ***D*** *There was no pellet bias ($p > 0.05$, one sample t-test) or difference between age groups ($p > 0.05$, independent t-test).* ***E*** *There was no substrate bias ($p > 0.05$, one sample t-test) or difference between age groups ($p > 0.05$, independent t-test).* ***F*** *There was no directional bias ($p > 0.05$, one sample t-test) or difference between age groups ($p > 0.05$, independent t-test). Bars are mean ± SEM with data points overlaid. Young n = 9-10 and aged n = 8-9 rats. ###$p < 0.001$ (within-subject).*

### 3.3.5 Aged rats can learn but not reverse in a bowl digging version of the probabilistic reversal learning task.

Analysis of trials taken to learn the first rule showed no difference between age groups. Rats that did not learn this first rule were not included in the analysis (n = 1 young rat, n = 3 aged rats) ($p > 0.05$) (**fig.8.A**). Aged rats were unable to reverse and therefore completed less reversals than young rats ($t_{(14)} = 5.001$, $p = 0.0002$) (**fig.8.B**). Analysis of substrate choices showed a main effect of phase (where acquisition and first reversal represent different phases) on win-stay behaviour ($F_{(1,14)} = 30.473$, $p < 0.0001$. There was also a phase*age group interaction ($F_{(1,14)} = 10.640$, $p = 0.006$) and a main effect of group ($F_{(1,14)} = 13.808$, $p = 0.002$). Post hoc pairwise comparison revealed that there was no difference in win-stay behaviour during the acquisition phase, but on the first reversal aged rats showed reduced win-stay behaviour compared to younger rats ($p< 0.0001$). Young rat win-stay behaviour did not change from acquisition to first reversal, however aged rats showed a reduction in win-stay behaviour in the first reversal compared to acquisition phase ($p < 0.0001$) (**fig.8.C**). There was no main effect of phase, phase*age group interaction or effect of age on lose-shift behaviour ($p > 0.05$) (**fig.8.D**). Additionally, there was no effect of phase, phase*age interaction, or age on latency to choose a bowl (**fig.8.E**).

**Figure 8**

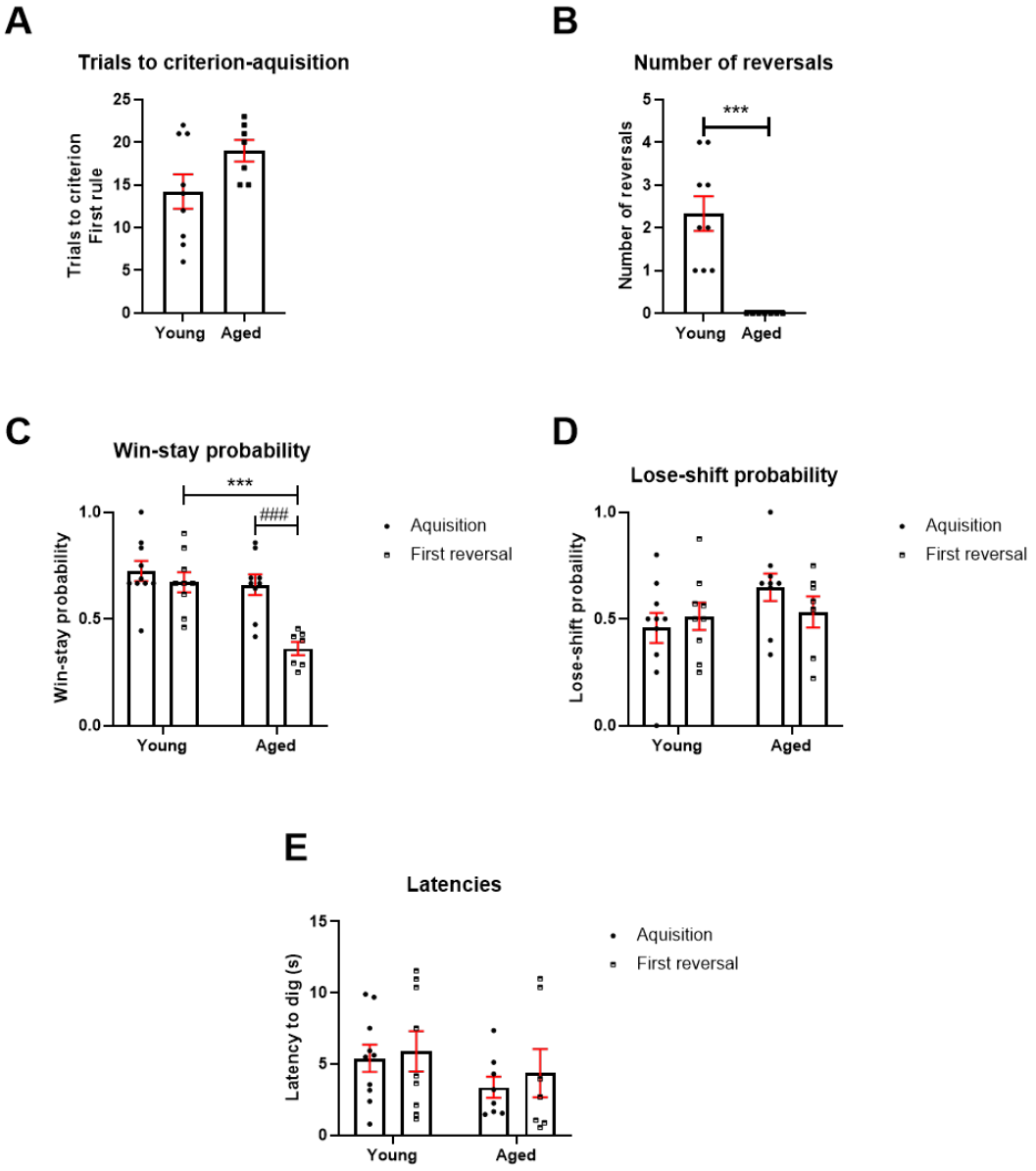


***Fig.8. Aged rats can learn but cannot reverse in a bowl-digging version of the probabilistic reversal learning task.*** *Young and aged rats underwent a single session of the probabilistic reversal learning task.* ***A*** *There was no age-related difference in acquisition of the initial rule ($p > 0.05$, independent t-test).* ***B*** *Young rats completed more reversals than aged rats ($p < 0.001$, independent t-test).* ***C*** *aged rats showed a reduction in win-stay behaviour during the first reversal compared to younger rats ($p < 0.01$, RM two-way ANOVA with pairwise comparison) and showed lower win-stay behaviour during the first reversal versus the acquisition phase ($p < 0.001$).* ***D*** *There was no effect of phase or age on lose-shift behaviour ($p > 0.05$).* ***E*** *There was no effect of phase or age on latency to choose a bowl ($p > 0.05$). Bars are mean ± SEM with data points overlaid. Young rats n = 9, aged rats n = 7. ***p< 0.001, ###p<0.001 (*between-subject, #within-subject).*

### 3.3.6 Aged rats can learn and reverse normally in the operant version of the probabilistic reversal learning task.

Analysis of CRF training performance showed there was no difference in session at which CRF1 and CRF2 training criteria were met between groups ($p > 0.05$) (**fig.9.A**). There also was no difference in average CRF2 performance between groups in the two sessions preceding the task ($p > 0.05$) (**fig.9.B**).

Performance in the PRLT over 7 sessions was next analysed. N = 1 young rat and n = 1 aged rat were excluded due to being run on a separate day where they had to be run early, resulting in a loss of motivation to perform the task. There was no effect of session, session*age interaction or age on trial number at which first rule was learned ($p > 0.05$) (**fig.9.C**). There was a main effect of session on number of reversals ($F_{(4.883, 58.595)} = 6.747$, $p < 0.0001$) where number of reversals increased with session. There was no session*age group interaction or effect of age, though there was a trend level effect ($F_{(1,12)} = 4.395$, $p = 0.058$) (**fig.9.D**). There was also a main effect of session on win-stay behaviour ($F_{(3.887, 54.420)} = 4.129$, $p = 0.006$), where win-stay behaviour increased over sessions. However, there was no session*age group interaction or main effect of age ($p > 0.05$) (**fig.9.E**). Analysis of lose-shift behaviour showed an effect of session ($F_{(2.553, 35.771)} = 4.612$, $p = 0.011$) where lose-shift behaviour increased with session. There was no session*age group interaction or main effect of age ($p > 0.05$) (**fig.9.F**). Analysis of total trials completed across sessions revealed a main effect of session ($F_{(2.954, 41.351)} = 6.565$, $p = 0.01$), where trials completed increased over time in young but not aged rats. There was no session*age interaction, although there was a trend level effect ($F_{(2.954, 41.351)} = 2.777$, $p = 0.054$. There was no effect of group ($p > 0.05$) (**fig.9.G**). There was no effect of session, session*age interaction or age on trial initiation time ($p > 0.05$) (**fig.9.H**).

**Figure 9**

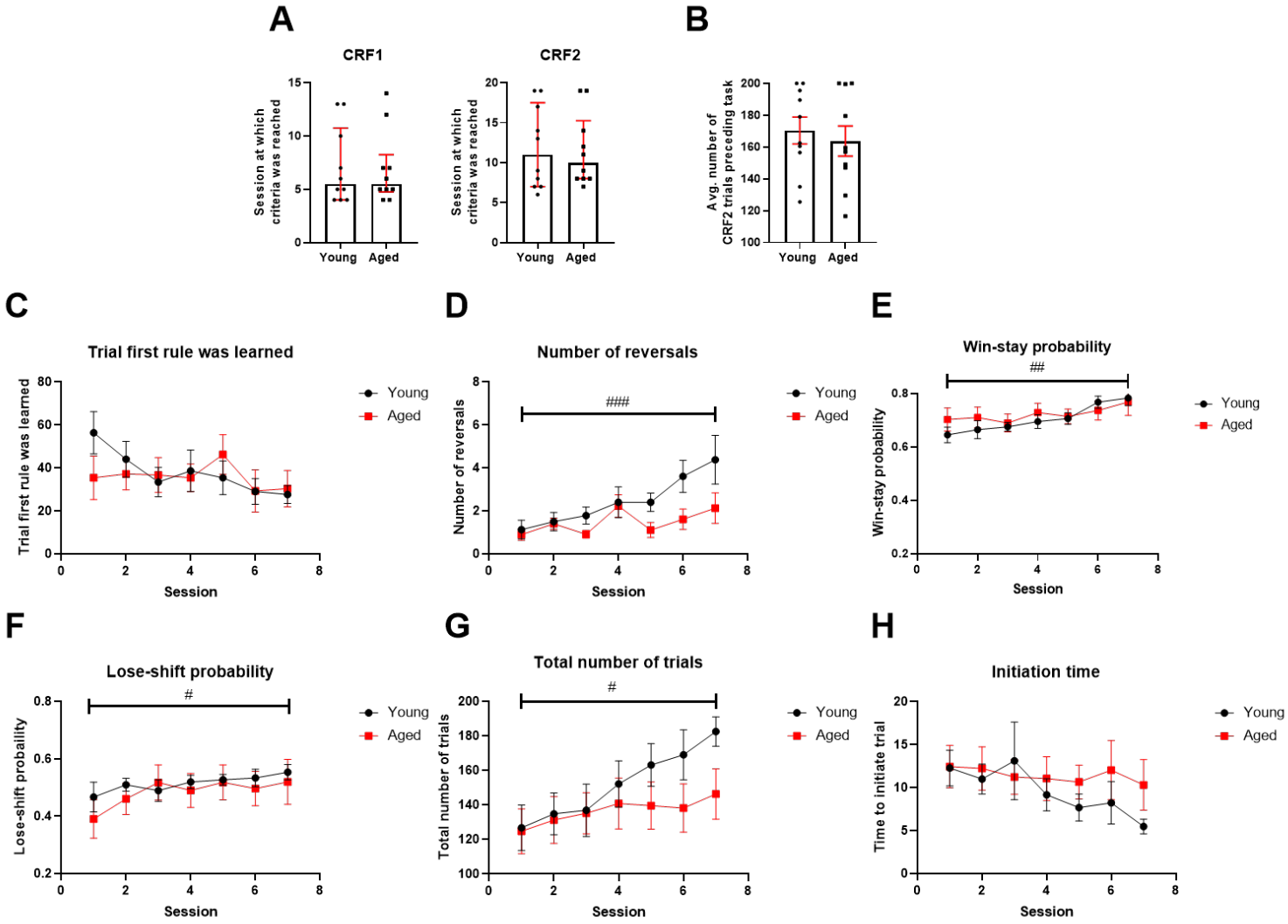
A
CRF1
Session at which criteria was reached
Young
Aged
CRF2
Session at which criteria was reached
Young
Aged
B
Avg. number of CRF2 trials preceding task
Young
Aged
C
Trial first rule was learned
Trial first rule was learned
Session
Young
Aged
D
Number of reversals
###
Number of reversals
Session
Young
Aged
E
Win-stay probability
##
Win-stay probability
Session
Young
Aged
F
Lose-shift probability
#
Lose-shift probability
Session
Young
Aged
G
Total number of trials
#
Total number of trials
Session
Young
Aged
H
Initiation time
Time to initiate trial
Session
Young
Aged

***Fig.9. Aged rats show no impairment in learning or reversals in the operant version of PRL.*** *Young and aged rats underwent 7 sessions of the PRLT.* ***A*** *There was no age-related difference in sessions taken to complete criteria for CRF1 and CRF2 ($p > 0.05$, Mann-Whitney U test). Bars are median ± interquartile range with data points overlaid.* ***B*** *There was no difference in average trials completed in the two CRF2 sessions preceding the PRLT ($p > 0.05$, independent t-test).* ***C*** *There was no effect of session or age on trial at which first rule was learned ($p > 0.05$, RM two-way ANOVA).* ***D*** *Number of reversals completed increased with session ($p < 0.001$, RM two-way ANOVA) but there was no effect of age ($p > 0.05$).* ***E*** *Win-stay probability increased with session ($p < 0.01$, RM two-way ANOVA), but there was no effect of age ($p > 0.05$).* ***F*** *Lose-shift probability increased with session ($p < 0.05$, RM two-way ANOVA) but there was no effect of age ($p > 0.05$).* ***G*** *Total number of trials completed increased with session in young but not aged rats ($p < 0.05$, RM two-way ANOVA) but there was no effect of age ($p > 0.05$).* ***H*** *There was no effect of session or age on trial initiation time ($p > 0.05$, RM two-way ANOVA). Unless otherwise stated, bars are mean ± SEM with individual data points overlaid. N= 8 per group. #p< 0.05, ##p<0.01, ###p<0.001 (within-subject).*

### 3.3.7 Aged and young rats show equivalent levels of GR and MR expression in the dorsal hippocampus.

Analysis of mean density of MR expression in the CA1, CA2, CA3 and dentate gyrus (DG) regions of the dorsal hippocampus of young versus aged rats showed a main effect of region on mean density ($F_{(2.16,38.882)} = 193.652$, $p < 0.0001$). However, there was no main effect of age or age*region interaction ($p > 0.05$). Post-hoc analysis revealed that all regions showed different levels of MR expression to each other ($p \leq 0.002$) with CA2 showing the greatest MR expression and CA3 showing the lowest (**fig.10.B**). Analysis of mean density of GR expression in the CA1 and DG regions of the dorsal hippocampus similarly showed a main effect of region ($F_{(1,18)} = 48.685$, $p < 0.0001$), but no main effect of age or age*region interaction ($p > 0.05$). Post-hoc analysis showed that GR expression in the DG was greater than in CA1 ($p < 0.0001$) (**fig.10.C**).

**Figure 10**

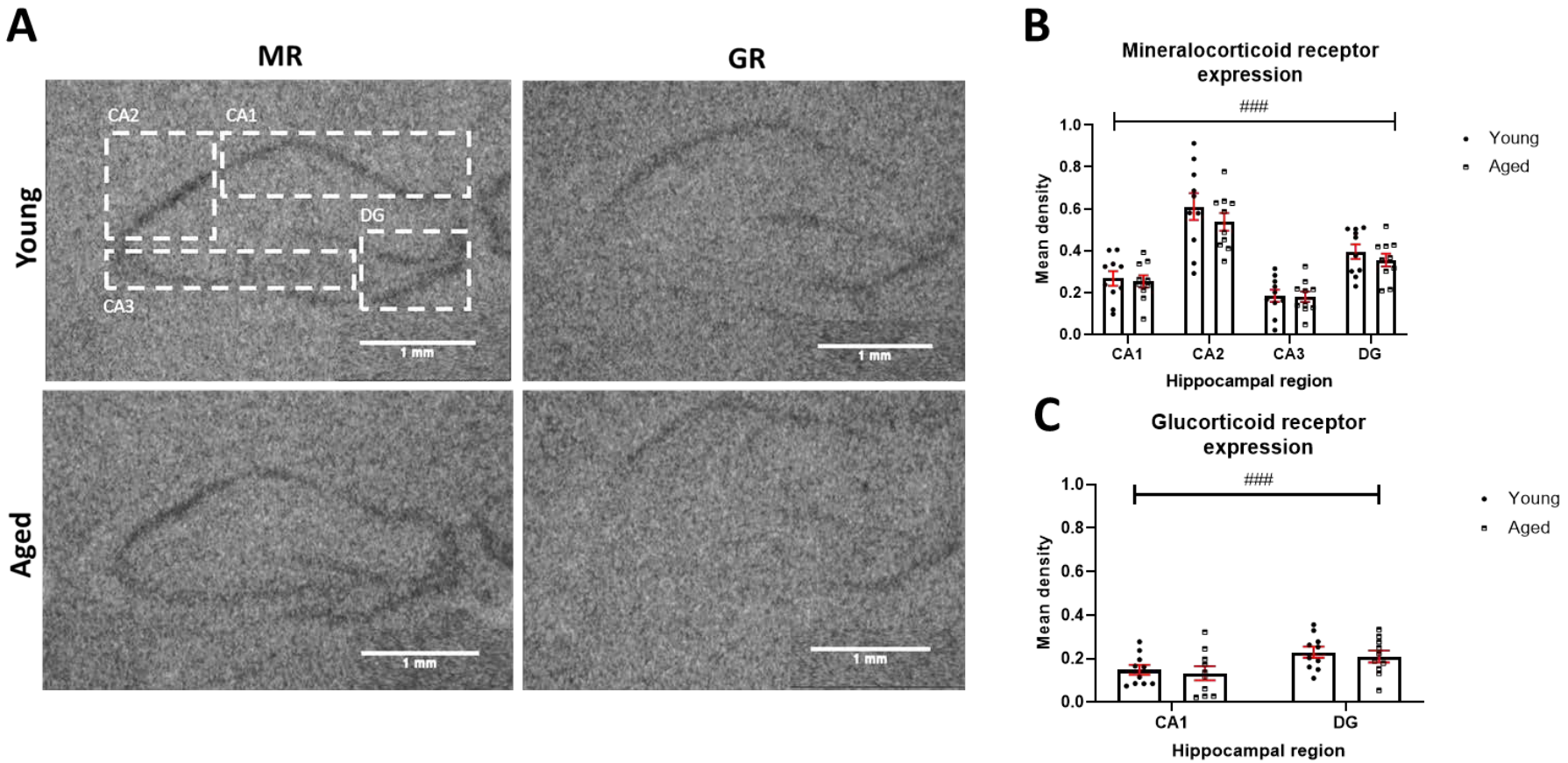


***Fig.10. Aged and young rats show equivalent levels of MR and GR expression in the dorsal hippocampus.*** *Brains were flash frozen on dry ice and sections underwent in situ hybridisation to visualise MR and GR expression.* ***A*** *Representative pictures of the dorsal hippocampus. Scale bars are 1 mm.* ***B*** *There was no age-related difference in MR mean density but there was an effect of region (p< 0.0001, RM two-way ANOVA).* ***B*** *There was no age-related difference in GR density but there was an effect of region (p < 0.0001, RM two-way ANOVA). Bars are mean ± SEM with data points overlaid. n = 10 per group. ###p< 0.001 (within-subject), MR-mineralocorticoid, GR-glucocorticoid, CA-cornu ammonis, DG-dentate gyrus.*

### 3.4 Discussion

This study showed that aged rats showed a reduction in novel object exploration but no deficit in novel arena exploration, EfR or PR task performance suggesting aged rats do not show a deficit in physical measures of motivated behaviour. Aged rats showed an increased latency to eat in the NSFT and preference for the side versus middle object in the object exploration test but equivalent levels of % sucrose preference and stress responsiveness suggesting a lack of emotional blunting. Aged rats showed normal reward learning, memory, and core affective state in the RLA but were unable to reverse in a bowl digging version of the PRLT. The impairment appeared specific to a loss in win-stay but not lose-stay behaviour and suggests perseverative behaviour. MR and GR mRNA expression in the dHPC did not differ between groups and may not represent a mechanism by which perseverative behaviour occurs. The following discussion considers how this behavioural and physiological profile compares to that of aged mice and how it relates to the domains of human apathy.

#### 3.4.1 Aged rats do not show the same pattern on motivational deficits as aged mice.

Aged rats showed a reduction in the exploration of novel objects in both the object exploration test and the NORT but showed normal novel environment exploration. These results were in direct contrast to the mouse data, where a reduction in novel environment exploration but normal object exploration in aged mice was observed. However, consistent with the aged mice, aged rats did not show an impairment in discrimination ratio compared to younger rats in the NORT. These contrasting results highlight that while both tasks measure exploration, the context clearly drives different behaviours. The novelty of a new arena not only induces a drive to explore but is also anxiety-inducing due to its open-field design. As shown in the previous chapter, aged mice show emotional blunting, and this may interact with exploratory drive and result in a reduction in locomotion that is not observed in aged rats. Conversely, during novel object exploration, the animal is previously habituated to the arena, and so this extra dimension is removed. Here, aged rats show a reduction in novel object exploration, indicating a motivational deficit. These results are consistent with other rat studies showing a similar decline in novel object exploration with advancing age (Marshall et al., 2019, Hamezah et al., 2017) and a reduction in interest in novelty has been observed in apathy patients (Yamagata et al., 2004, Kaufman et al., 2016). It is surprising that aged mice do not show a similar deficit, however, it has been suggested that mice experience more stress in an arena task than rats and require longer periods of habituation (Ellenbroek and Youn, 2016). Therefore, engagement with novel objects may not be the priority in this context and is less likely to reflect motivated behaviour.

Aged rats showed equivalent levels of responding to younger rats under a CRF schedule of reinforcement and showed no difference in session at which FR training was completed, indicating

normal learning and equivalent levels of response vigour in the task. There was no age-related difference in breakpoint in the PR task under either food restricted or *ad libitum* feeding conditions, although there was a trend towards the younger rats showing a high breakpoint under *ad libitum* conditions. In the EfR task, a single session directly after training showed no difference between groups, but when repeated over 5 consecutive sessions, aged rats completed more trials than younger rats, and trended towards consuming less chow, in contrast to aged mice where a reduction in number of trials was observed.

Together this data reveals two key findings. Firstly, unlike aged mice where a clear response deficit was observed when animals were put in a higher motivational state using food restriction, aged rats show no such deficit. It has previously been shown that instrumental responding is preserved in aged rats (Samson et al., 2014, Nomura et al., 2004) and it may be that when physical exertion for reward is required, aged rats are unimpaired.

Secondly, when rats are put in in a low motivational state by being fed *ad libitum*, younger rodents show greater consumption of chow in the EfR task but equivalent number of trials and a trend towards a higher breakpoint in the PR task. Taken together, these data indicate that younger rats have a greater appetite than aged rats under *ad libitum* feeding conditions. This has the potential to drive the findings under food restriction, where a priority to consume more food more quickly may drive the younger rats to choose the lower effort option. However, as younger rats showed an equivalent breakpoint to aged rats in the PR under food restriction, it is unlikely that appetite differences under these conditions are driving the results.

Together, these data suggest that in contrast to aged mice, when quantifying motivational state by using measures of physical effort in an operant box context, aged rats do not show an impairment and under certain conditions appear to show greater levels of motivated behaviour than younger rats. This apparent lack of deficit is not consistent with apathy-related behaviour. However, a reduction in object exploration suggests a reduction in motivated behaviour, suggesting spontaneous methods of motivation may be more sensitive in rats. However, it is difficult to conclude from a single test in isolation. Overall, this suggests that age-related changes in motivated behaviour differ between rats and mice. This is unlikely to be driven by differences in chronological age between species at time of testing as both were tested in most motivation-based tasks at similar time periods after they are considered ‘aged’ (4-5 months and 3-4 months in rats and mice respectively). Potential reasons for this species difference are explored in more detail in chapter 6.

### 3.4.2 Aged rats show no evidence of emotional blunting.

In the NSFT, aged rats showed an increased latency to eat, indicating an increase in anxiety-related behaviour compared to younger rats. A consumption test following a 24hr restriction showed that aged rats consumed more chow, indicating an increase in appetite. However, this is unlikely to drive the age-related effect on latency to eat as an increase in appetite would drive the aged rats to consume the food faster. This finding of greater anxiety in the aged rats was surprising as it contrasted with aged mice, where a reduced latency to eat was observed. To further probe this apparent increase in anxiety behaviour and to further demonstrate this effect was not driven by appetite, an object placement exploration test was conducted, where exploration of an object placed in the middle of an arena versus the side was investigated. Higher exploration of the object placed to the side could suggest higher levels of anxiety-like behaviour. This is based on the principle that more anxious rodents tend to exhibit higher levels of thigmotaxis and cross the centre less in an open field arena (Seibenhener and Wooten, 2015). Aged rats showed greater preference for exploration of the side object compared to younger rats, further suggesting elevated anxiety-like behaviour and therefore in agreement with the NSFT results. It should be noted that this task has not yet been validated as a measure of anxiety. However, other studies have also shown elevated anxiety behaviour in aged rats in non-appetitive tasks such as the elevated plus maze (Boguszewski and Zagrodzka, 2002, Frussa-Filho et al., 1992).

To test whether this apparent increase in stress reactivity in rat ageing was consistent at a physiological level, rats underwent a 30 min acute restraint stress. Young and aged rats showed equivalent levels of baseline CORT and showed an increase in CORT post stress. There was no difference in stress-induced CORT between groups, suggesting no difference in stress reactivity at a physiological level. The reason for this may be due to the experimental timeline. The NSFT was tested in the rats at age 4 mo and 21 mo, and the restraint stress was conducted at age 8 mo at 25 mo. There is the potential for the younger group to have become too old to show a robust difference between groups (it was not possible to order new young rats due to this experiment taking place over the pandemic). As highlighted in the previous chapter, reports of age-related changes in stress-reactivity in the literature are heterogenous, even within-species studies and using the same stressor. One study found that aged rats showed increased plasma CORT following a 20 min acute restraint stress compared to younger rats (Garrido et al., 2012), another found equivalent levels following 20 min stress (Issa et al., 1990). A study showed that even within the same animals, two different methods of restraint produced different corticosterone responses (Shoji and Miyakawa, 2020). This heterogeneity highlights the importance of using multiple measures to assess a particular domain.

Aged rats showed equivalent levels of % sucrose preference in the SPT compared to younger rats indicating normal levels of reward sensitivity. This contrasts aged mice which showed a reduction in sucrose preference across the duration of the test.

Together, these data indicate that aged rats do not show emotional blunting consistent with apathy-related behaviour and would be an unsuitable model to test this domain when investigating its underlying neurobiology.

### 3.4.3 Aged rats show evidence of perseverative behaviour but normal reward learning and memory.

In a bowl digging reward learning assay (RLA), aged rats showed no deficit in the initial stages of training or trials to criteria in the pairing sessions, indicating normal reward learning. In addition, there was no difference in latencies to dig, suggesting equivalent levels of motivation to engage with the task and no motoric impairment. This lack of impairment in reward learning is consistent with operant box training results. Both age groups showed a positive bias for the two-pellet reward substrate, showing rats were successfully capable of associating a substrate cue with a higher value reward and showing a preference for this substrate over one paired with a lower value reward as previously demonstrated (Stuart et al., 2019, Giertler et al., 2003, Stuart et al., 2013). There was no difference in % choice bias between groups, suggesting normal reward memory and equivalent levels of core affective state in aged and young rats. Core affective state has not been measured in aged versus young rats in this context before, but this RLA has previously been shown to be sensitive to changes in core affective state, where models of early life adversity (ELA) and chronic administration of pre-depressant drugs are unable to show a positive bias for the higher value reward-paired substrate (Stuart et al., 2019, Stuart et al., 2017).

Rats were then tested in a novel version of the probabilistic reversal learning version of the bowl digging task (PRLBD), which provides a behavioural readout of cognitive flexibility. Most rats successfully learned to select the probabilistically rewarded substrate and while there was no group difference, there was a trend towards aged rats taking more trials to learn in the acquisition phase. Strikingly, when the rule was reversed such that the previously rich substrate became the lean substrate and vice versa, all aged rats were unable to reverse, indicating a reduction in cognitive flexibility. Impairments in cognitive flexibility have been demonstrated in conditions with which apathy commonly occurs, including ageing, PD, AD, MDD and HD (Nilsson et al., 2015). To investigate this deficit further, win-stay and lose-shift behaviours were analysed.

Win-stay and lose-shift are measures of how the rats use positive and negative feedback respectively to guide their next decision. In the acquisition phase, young and aged rats showed equivalent levels of win-stay behaviour, that is, the likelihood they will choose the same substrate in the next trial after it has been rewarded in the previous trial. This suggests aged rats show normal positive

feedback sensitivity. However, in the first reversal phase, aged rats show a reduction in win-stay behaviour compared to younger rats. This deficit was not seen in lose-shift behaviour, the likelihood the rat will choose the other substrate on the next trial after it was unrewarded on the previous trial. Therefore, this reversal learning impairment is likely due to a failure to remove prior associations with a positive cue, known as 'perseverative' behaviour (Nilsson et al., 2015). Perseverative behaviour in old age has been widely reported in other rodent studies. Aged rats could learn normally but showed impaired reversal in a spatial T maze discrimination task, odour discrimination task and visual discrimination task, showing this behaviour is consistent over multiple sensory domains (Stephens et al., 1985, Brushfield et al., 2008). Elderly humans also show impairments in reversal learning and a mild impairment in acquisition learning compared to younger adults (Weiler et al., 2008). This perseverative behaviour may reflect a lack of exerted cognitive effort, and thus a motivational impairment may only emerge in aged rats when the action requires cognitive rather than physical effort. This could be explored further by using a cognitive effort for reward task, where a simple working memory task is required instead of a set number of instrumental responses.

Rats were then tested in an operant box version of the PRLT where the underlying principle of the task is the same, but the rat responds by pressing a touchscreen with his nose. In contrast to the bowl digging version of the task, over seven sessions, aged rats showed no difference in number of reversals, win-stay or lose-shift behaviour compared to younger rats, however consistent with the bowl digging task there was no difference in latency or trial at which the acquisition trial was learned. It has previously been shown that ethological relevance can impact on within-session learning, rats learned an odour discrimination tasks more quickly than a visual discrimination task (Brushfield et al., 2008). Therefore, the operant version of the PRLT may lack the sensitivity to pick out age-related differences in cognitive flexibility. By using more ethologically-relevant cues, the rats may form stronger associations between the cue and reward, which are then more difficult to unlearn and may require greater attention to reward feedback. This would mean an impairment in cognitive flexibility would become more apparent in this version of the task. This may be why the aged rats showed a deficit in the bowl digging but not the operant version of the task. The implications of this are discussed in more detail in chapter 6. It should be noted that often in this type of testing, sessions are repeated until a stable baseline is reached. However, this study was stopped after 7 sessions because over the Christmas period, the health of the aged rats declined to the point where it was decided that general health problems might make meaningful behavioural interpretation difficult, and the study was ended early.

It has previously been shown that a normal GR:MR ratio of function is required for optimal behavioural flexibility. In particular, MR expression predominating over GR in the hippocampus is required for normal cognitive and emotional behaviour (Gomez-Sanchez and Gomez-Sanchez,

2014). An upregulation of GR expression in this region has been linked to behavioural perseverance (Berger et al., 2006). The dorsal hippocampus (dHPC) has been shown to be important in behavioural flexibility (Busse and Schwarting, 2016). Therefore, an age-related disruption to GR and MR expression in the dHPC could represent a molecular mechanism by which perseverative behaviour occurs. In the present study, there was no age-related difference in GR and MR mRNA expression in the CA1, CA2, CA3 or dentate gyrus regions of the dHPC. These results contrast with previous work which has shown that GR and MR mRNA levels are reduced by 30-40 % in the aged rat whole hippocampus (Morano et al., 1994). However, this may be due to their use of the whole hippocampus and may be driven by changes in the ventral rather than dorsal hippocampus. Another study showed no difference in GR and MR mRNA expression in any of the HPC subregions between age groups (Yau et al., 1994). The present study would have benefitted from assessing the ventral hippocampus as well as other brain regions such as the orbito-prefrontal cortex, which has been shown to be required for normal reversal learning in a bowl digging task (Brushfield et al., 2008). There is the potential that dHPC may be required for spatial reversal tasks only.

### 3.4.4 Conclusion

Aged rats do not show a profile of behavioural and physiological deficits consistent with apathy behaviour as described in chapter 1, but there is some evidence to suggest a deficit when higher levels of cognitive effort are required. In contrast to aged mice, a reduction in object exploration in the aged rats indicated a reduction in motivated behaviour, but it was found that when motivational state was measured using changes in physical effort, aged rats showed no deficit. In addition, there was no evidence for emotional blunting at either the behavioural or physiological level with aged rats showing higher levels of anxiety behaviour. Aged rats showed normal reward learning and memory as well as core affective state in a bowl digging task but were unable to reverse a previously learned rule, suggesting a reduction in cognitive flexibility which may also reflect a decline in willingness to exert cognitive effort. This deficit in reversal learning was not seen in the operant version of the task and may be due to a comparative lack of sensitivity in the operant task, highlighting the importance of using ethologically relevant cues. GR and MR mRNA expression in the dHPC was not affected by age and therefore may not represent a molecular mechanism by which perseverative behaviour occurs. However more studies are required to elucidate this. Overall, this work highlights for the first time, the profound age-related behavioural differences between mice and rats and shows that aged rats are not suitable for testing the underlying neurobiology of apathy-related behaviour when considering measures of physical effort or emotional blunting. However, aged rats may show a motivational deficit when cognitive but not physical effort is required. Potential explanations for these species' differences are explored in more detail in chapter 6.

# Chapter 4: Investigating circadian rhythm changes in aged rats: activity, core body temperature and corticosterone

## 4.1 Introduction

Both human and rodent studies suggest that circadian rhythms become blunted or shifted with age, including the activity-rest cycle, core body temperature and release of hormones such as CORT and melatonin (Hood and Amir, 2017). However, the exact nature of these age-related changes reported by studies is heterogenous. As these rhythms are driven by the change in light across the 24hr period, much of the literature has focussed on age-related changes to the light-entrainable oscillator, the SCN, as an explanation for this blunting of circadian rhythms. There is very little change to the core clock machinery, such as the rhythmic expression of *bmal1* and *clock* in the SCN (Yamazaki et al., 2002), but a reduction in synchronicity in neuronal firing in the SCN has been reported as an explanation for blunted behavioural output (Nakamura et al., 2015).

The PVN receives strong circadian input from the SCN and plays a critical role in the release of CORT. It contains a large population of corticotrophin releasing hormone (CRH) neurons and arginine vasopressin (AVP) neurons, and their activation via limbic circuitry, humoural signalling or direct projections via the SCN results in the downstream release of CORT into the circulation. The release of CORT is regulated by a negative feedback loop, where binding of CORT to glucocorticoid (GR) and mineralocorticoid receptors (MR) in the PVN blocks its own release (Oster et al., 2017). CORT is released rhythmically or in response to stress. Investigation of age-related changes to the circadian expression of CRH and AVP neuronal populations and GR in the PVN could provide valuable information about CORT regulation and its potential impacts on circadian regulation. As both a product and driver of circadian output, the disruption of the rhythmic release of CORT could have profound and wide-ranging consequences to normal health and the ability to form an appropriate stress response. There is some evidence to suggest that stress responsiveness is modulated by the time of day, where sensitivity to a physical stressor is normally higher during the active phase, and psychological stress in the inactive phase (Koch et al., 2016). Disruption to the normal CORT rhythm may therefore disrupt normal stress responsiveness. In addition to these roles, the PVN is a significant regulator of energy intake and metabolic processing (Mazier et al., 2019). As food-seeking is a main driver for waking activity in the rodent, the PVN represents an interesting target for research into age-related changes to circadian rhythms at both a behavioural and physiological level.

Studies of age-related changes in the circadian release of CORT are very limited. There are some reports of the rhythm becoming blunted in humans and rats, driven by a rise in nadir CORT (Van Cauter et al., 1996, Herman et al., 2016). However, these studies are limited by a restricted number of time point samples, and the potentially stressful nature in which they are collected.

Obtaining temporally meaningful and non-invasive CORT samples from freely moving rats would provide valuable information about CORT release in a way that previous studies have been unable

to, providing more accurate information about how ageing affects rhythmic CORT release. The assessment of the activity-rest cycle allows for the study of the impact of age on circadian rhythms at a behavioural level. The study of the core body temperature rhythm allows for the study of circadian rhythms at a physiological level and provides an additional readout of metabolic activity. Therefore, the first aim of this chapter was to investigate age-related changes in amplitude in activity, core body temperature and CORT in freely moving rats using an automated blood sampling system. The effects of ageing on stress-reactivity were additionally tested using a loud white noise test. While the focus of this study was not initially on the impact of food restriction on these outputs, it became apparent that the restricted nature of the experimental rats' diet could have a profound impact on both brain and behaviour and so the second aim of this chapter was to investigate the effects restricted feeding had on CORT release, activity, core body temperature and the neuronal and receptor expression in the PVN.

## 4.2 Methods

### 4.2.1 Subjects

A cohort of 8 aged male Sprague-Dawley rats aged 23 months by end of experimentation and a cohort of 8 sex and strain matched younger controls aged 7 months by end of experimentation were obtained from Eli Lilly. While at Eli Lilly rats were fed a restricted diet of 16g per day between ZT1-4. This was continued at Bristol. Water was provided *ad libitum*. Rats were kept in standard 12:12 lighting, with lights ON at 7.00 am and OFF at 19:00 pm. The aged rats weighed between 505-570 g by end of experimentation, and younger rats weighed between 395-450 g. Before experimentation rats were housed in groups of three in open topped cages with a tube and chew bar for enrichment. During experimentation rats were singly housed (**fig.1**).

**Figure 1**

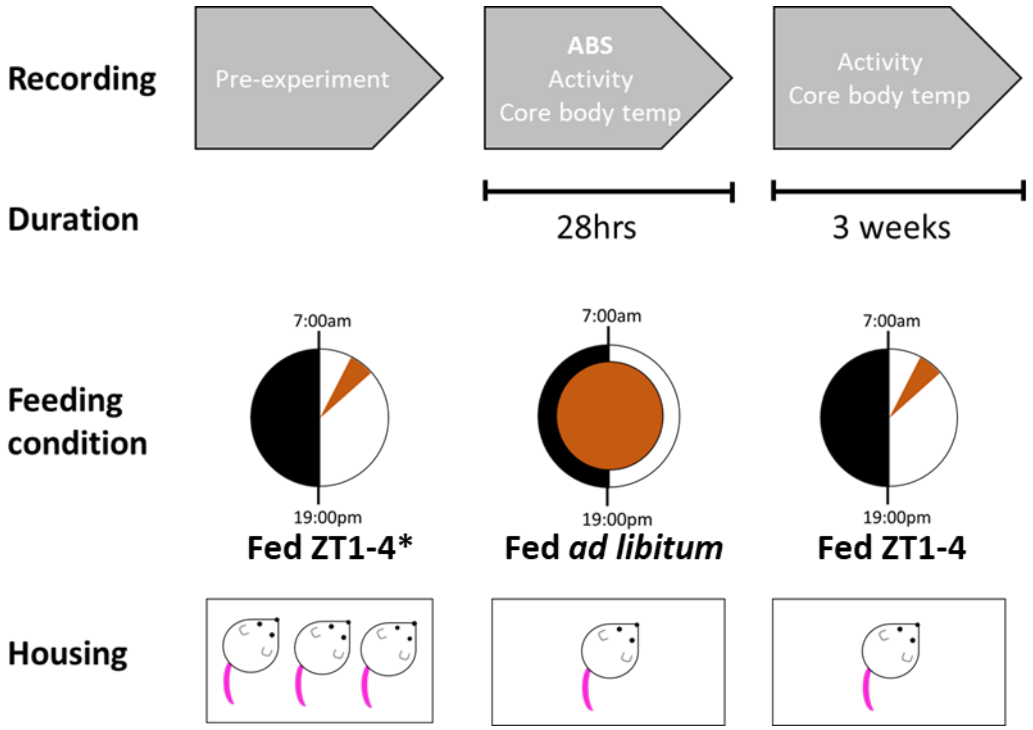


***Fig.1 Physiological/behavioural recordings timeline and experimental conditions.*** *Recording-refers to the physiological recording that occurred at a particular stage of the experiment. Duration-refers to duration of recording stage. Feeding condition-dark portion of circle refers to dark phase, white portion of circle refers to light phase, brown portion of circle refers to timing of feeding. Housing-refers to number of rats housed within a cage at each stage of the experiment. *Fed ZT1-4 with exception of two days post-surgery where rats were fed ad libitum.*

### 4.2.2 Surgery under general anaesthesia

Rats were anaesthetised with gaseous isoflurane (100 % w/w/ liquid vapour, Merial, UK) and medical grade air. Rats were given 1 mg/kg sub-cutaneous injection of Rimadyl (Pfizer, UK) to manage post-operative pain. During the surgical procedure and recovery, rats were placed on heat mats to maintain body temperature. Following surgery an injection of 2.5 ml glucose saline (0.9 % NaCl, 5 % glucose) (Braun, Germany) was administered sc.

### 4.2.3 Automated blood sampling using jugular vein cannulation.

Intravenous cannulation of the right jugular vein was performed as previously described (Windle et al., 1998). The area of the right jugular vein was located by first identifying pulsing of the blood through the bare skin and making an incision in the rostral/caudal plane. It was then exposed by careful blunt dissection. The jugular vein was then cannulated using an inhouse designed cannula. The other end of the cannula was exteriorised via the nape of the neck and enclosed in a metal spring and vascular access button system (INSTECH, USA). The button was secured to the muscle wall using a single stitch. Skin was then sutured to close any gaps around the button. When righting reflex was restored, animals were housed individually in soundproofed rooms. This was to prevent any external noise inducing a CORT response and disrupting the basal profile. The protective spring around the jugular cannular was attached to a mechanical swivel (INSTECH, USA), that rotated 360°in a horizontal plane and 180°through a vertical plane, allowing rats to move freely around the cage during automated blood sampling (ABS).

The cannulae were flushed daily with heparinised saline and a small volume of blood (~ 5 µl) was withdrawn to ensure patency of the jugular cannulation. 200 µl heparinised saline always remained in the cannula to prevent clotting at the site. This occurred for 4 days preceding the ABS day. If blood was unable to be withdrawn for two consecutive days, then the cannula was blocked, and the animal was unhooked from the system and not used for ABS. This occurred in n = 1 young rat and n = 2 aged rats. On the ABS day, 40 µl blood was withdrawn automatically from the animal every 10 mins for 28 hours, starting from 6 am and finishing at 10 am the following day. Blood was replaced with the same volume of heparinised saline. The blood was deposited into tubes containing heparinised saline at a dilution of 1:3. At 8am, a 100 dB noise was automatically played for 10 mins, serving as a stressor to assess the stress response in aged vs young animals. The background noise in the sampling room was measured at approx. 55 dB. The system allowed for 8 rats to be sampled at once so was done in two sets, first aged and then young rats. The cages were adjacent to each other and were open top so that the rats could smell, see and hear each other. Rats were fed *ad libitum* two days post-surgery, in line with license requirements. They were then placed back on food restriction for one day prior to ABS. On the day of ABS, rats were placed on *ad libitum* feeding

to minimise disturbance in the room. They were placed back onto food restriction the following day.

### 4.2.4 Telemetry probe implantation

Implantation of the telemetry probe was conducted at the same time as the automated blood sampling surgeries. An incision was made along the midline rostro-caudally to expose sub-cutaneous tissue and muscle covering the rib cage. A small hole was then made in the muscle, exposing the peritoneal cavity close to the liver. The hole was widened using blunted scissors, and the sterilised telemetry probe (PTD 4000 E-mitter, Starr Life Sciences Corp) was inserted into the abdominal cavity. The wound was then sutured, cleaned and covered with wound powder to promote healing.

### 4.2.5 Radiotelemetry

Animals were placed in individual housing, mounted on signal receivers (ER-4000 receiver, Starr Life Sciences Corp, US). Radiotelemetry data began 1 day following surgery. Data was collected in 10 min epochs and viewed using Respironics VitalView software (Mini Mitter Co. USA). Activity counts were outputted when the telemetry probe changed position relative to the charging signal received from any of 3 concentric charge/receive grids inside the ER-4000 Receiver. These data are not a measure of specific distance, but a relative measure of gross motor activity level over time. Measurements were taken for a period of 3 weeks.

### 4.2.6 Corticosterone radioimmunoassay (RIA)

A radioimmunoassay was performed in-house to measure total corticosterone from ABS blood samples, as described in chapter 3, section 3.2.11. The intra and inter-assay coefficients of variation for the assay have previously been established as 16.7% and 13.3% respectively (Spiga et al., 2011).

### 4.2.7 In situ hybridisation

16 male Sprague Dawley rats aged 18 months at experiment onset and 17 younger controls aged 5 months at experiment onset were supplied by Eli Lilly. Rats were maintained in the same groups of 3-4 that they were housed in at Lilly and on the same feeding schedule; 16g of food at ZT2-3. Water was provided *ad libitum*. Half of the rats (n = 8 aged and n = 9 younger rats) were killed by isoflurane anaesthetisation and subsequent cervical dislocation at ZT2 (n= 4 aged and n = 5 young) and ZT14 (n = 4 aged, n = 4 young) 3 weeks after arrival. The whole brain was flash frozen on dry ice. Tissue was stored in a -80 ◦c freezer until use. The other half of the rats were pair-housed and underwent surgery for nanotag implantation as described in the next chapter.

***In situ hybridisation tissue preparation***

Brains were sliced in 12 µm coronal sections using a cryostat. They were sliced from bregma -1.44 to -2.5 mm, according to the rat brain atlas. Sections were slide mounted on 5% gelatin coated slides in sets of 6, 3 sections per slide and were stored in the -80 °c freezer until use. For this experiment, the PVN was analysed at bregma level -1.8mm and the arcuate nucleus (Arc) at -2.3 mm.

**Oligoprobes**

***Terminal Deoxynucleotide Transferase (TdT) labelling of oligonucleotides***

The corticotrophin releasing hormone (CRH), arginine vasopressin (AVP) and pro-opiomelanocortin (POMC) probes (**table 1**) were labelled at the 3' end with $^{35}$S dATP using the Terminal Deoxynucleotidyl Transferase (TdT) enzyme method. On ice, 1 µl oligonucleotide was added to 28 µl distilled water, 10 µl 5x tailing buffer, 5 µl $CoCl_2$, 5 µl 35 S dATP and 1 µl TdT making a final concentration of 5 µM oligonucleotide. The mixture was then incubated for 1h at 37 °C. The oligonucleotides were then purified using the nucleotide removal kit (QIAGEN, 28304). 1µl labelled probe was added to scintillation fluid and radiation was counted using a gamma counter (Perkin Elmer Wizard 2). The counts for each probe were 102,000 cpm/µl (CRH), 132,000 cpm/µl (c-Fos), 213,000 cpm/µl (POMC) and 106,000 cpm/µl (AVP).

**Table 1**

| Oligoprobe | Company | Sequence (5'-3') |
|---|---|---|
| **CRH** | Sigma-Aldrich | CAGTTTCCTGTTGCTGTGAGCTTGCTGAGC TAACTGCTCTGCCCTGGC |
| **AVP** | Sigma-Aldrich | CAGCTCCCGGGCTGGCCCGTCCAGCT |
| **POMC** | Sigma-Aldrich | CTTCTTGCCCACCGGCTTGCCCCAGCAGAAGTGCTCCA TGGACTAGGA |

***Table 1. Oligoprobe sequences and suppliers.***

***Prehybridisation wash***

Slide-mounted sections were brought to room temperature and submerged in 4 % formaldehyde (made up in phosphate buffer saline (PBS)) for 5 mins at room temp and then washed twice with 1 x PBS. Slides were then submerged in a triethanolamine (TEA)/acetic anhydride (AA) mix (250 ml sterile saline, 3.5 ml TEA, 0.625 ml AA) for 10 mins. Slides were dehydrated in ascending concentrations of ethanol 70 % (1 min), 80 % (1 min), 9 5% (2 min), 100 % (1 min) and then delipidated using chloroform for 5 mins. Slides were then put back in 100 % and then 95 % ethanol (1 min) before being stood up to dry.

***Oligoprobe hybridisation***

Each slide required a count of 200,000 cpm/µl and a final volume of 90 µl. 90 µl hybridisation buffer (HB) (ingredients) was added to the required volume of probe. For every 100 µl of HB 1 µl 1 M dithiothreitol (DTT) (Sigma D9779), dissolved in 0.01M sodium acetate (pH 5.2) was added. 90 µl of the final solution was added to scintillation fluid and counted. CRH and had a count of approximately 140,000 cpm/slide and POMC and AVP had counts of approximately 20,000 cpm/slide (lower than expected). 90 µl final solution was added to each slide and cover slipped with parafilm. Slides were incubated overnight at 37 °C in a humid chamber with $CuSO_4$ solution to prevent bacterial growth.

***Oligoprobe wash***

The following day parafilm coverslips were floated off in 1x saline sodium citrate (SSC) (20 x stock solution 175.3 g NaCl, 88.2 g trisodium citrate, 800 ml distilled water, pH 7). Slides were then taken through 3 changes of 1x SSC, dragging the slides back and forth vigorously through the solution. Slides were then washed 4 times for 15 mins each in 1x SSC at 55°C and then two 30 min washes at room temperature. After 2 brief dips in distilled water, slides were then blow dried until dry and exposed to autoradiography film (FujiFilm Corp, Japan) for 18 days (CRH and POMC) or 6 days (AVP) in complete darkness. Films were developed for longer to account for lower cpm than expected. 14C-labeled microscale sample standards (Amersham, Bucks, UK) were also exposed to the same film to allow calibration before analysis.

**Film development and quantification**

Films were developed using an auto processor (Compact X4 Automatic X-ray film Processor) in complete darkness. Sections were analysed using Scion Image v1.63. Films were placed on a light box and sections visualised with a Leica MZ6 microscope with a CCD camera attachment and were captured and measured in greyscale. The objective lens magnification was set to 1.0 x and light was set to 6. Lighting in the room was kept consistent throughout image capture. When capturing the PVN and the Arc a magnification of 1.25 x was used. The mean optical densities (OD) of the autoradiographs were measured by comparison with simultaneously exposed 14C-labeled microscale samples. The scale was set to mm using a known distance.

Images were subsequently analysed using ImageJ. A background value for each slide was calculated by drawing a circle and obtaining a mean density value. Area of the region of interest was drawn around using the free draw tool. The mean density and area were outputted. Final measurement was calculated using ((density – background) x area). A value for the left and right region was obtained and added together. This was done using 3 sections and an average value was taken. CRH was measured in the parvocellular area of the PVN and AVP was measured in both the magnocellular and parvocellular areas due to its region-specific roles. POMC was measured in the lateral Arc.

**Riboprobes**

The same method was used as outlined in the previous chapter. Counts at probe synthesis were MR sense 13 x $10^6$ cpm, MR antisense 14 x $10^6$ cpm, GR sense 18 x $10^6$ cpm, GR antisense 9 x $10^6$ cpm. These counts were at expected levels. Cpm/slide was: MR sense 400,000 cpm/slide, MR antisense 520,000 cpm/slide, GR sense 1.9 x $10^6$ cpm/slide and GR antisense 700,000 cpm/slide. Sense riboprobes were used to demonstrate absence of non-specific binding.

The region of interest was the PVN at the same bregma level as above and were exposed for 6 days. Images were captured under the same microscope settings as above. There was not sufficient MR expression in the PVN under these experimental conditions to analyse.

### 4.2.8 Data analysis

During analysis of the ABS CORT data, outlying samples were removed and were treated as a missing value. To establish a peak and nadir CORT time range, an averaged 24-hr CORT profile for the young (control) group was plotted, and a time range for the nadir and peak CORT were determined visually and agreed by two different researchers. This range was then applied for group analysis. Time periods of interest were pre-selected prior to analysis and based off the literature. Periods of interest were whole light phase, whole dark phase, ZT11-12 (pre-lights off activity), ZT12-14 (post-lights off activity) and ZT22-24 (control period). Area under the curve (AUC) analysis was applied to all time periods of interest (e.g., activity x time) using GraphPad Prism v7, which uses the trapezoidal method. The AUC values were then used for subsequent analysis.

The statistical approach used was as described in chapter 2.

### 4.3 Results

#### 4.3.1 Aged rats show blunted dark phase activity driven by activity directly after lights off.

Analysis of AUC activity in the light phase versus the dark phase of the automated blood sampling day revealed a main effect of phase on activity ($F_{(1,14)}$ = 41.392, p < 0.0001). There was no phase*age group interaction, although there was a trend level effect ($F_{(1,14)}$ = 3.69, p = 0.075). There was a main effect of age ($F_{(1,14)}$ = 6.396, p = 0.024). Pairwise post hoc analysis showed that both young and aged rats have a greater level of activity in the dark phase compared to the light phase (p < 0.0001 and p = 0.007 respectively). While young and aged rats show similar levels of activity in the light phase (p > 0.05), young rats show greater activity in the dark phase compared to aged rats (p = 0.012) (**fig.2.C**). When AUC activity at the beginning of the dark phase (ZT12-14) and end of the dark phase (ZT22-24) were compared, there was a main effect of ZT ($F_{(1,14)}$ = 42.532, p < 0.0001). There was also a ZT*age group interaction ($F_{(1,14)}$ = 4.734, p = 0.047) and an effect of age ($F_{(1,14)}$ = 4.992, p = 0.047). Post-hoc analysis revealed that younger rats showed greater activity compared to aged rats at ZT12-14 (p = 0.009) but not ZT22-24 (p > 0.05) (**fig.2.D**).

**Figure 2**

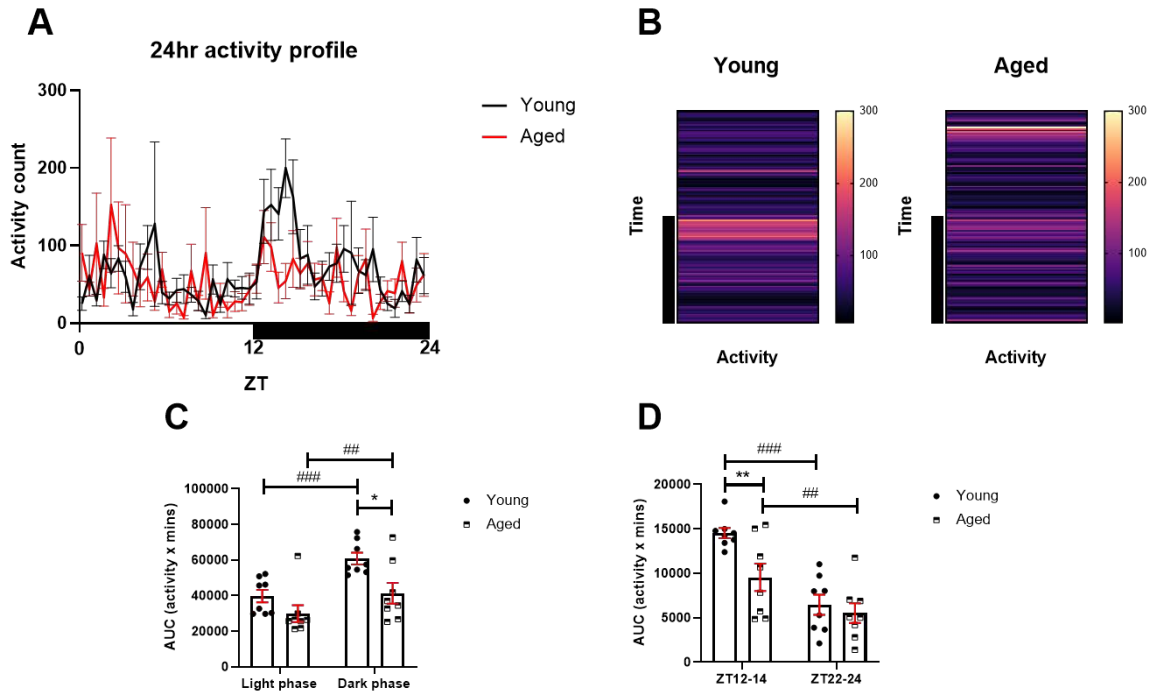


***Fig.2. Aged rats show a reduction in dark but not light phase activity compared to younger rats.*** *Young and aged rats were implanted with a telemetry probe and an activity count was output every 10 mins during the ABS day.* ***A*** *24hr average activity trace in old and young rats, with data plotted every 3 10 min measurements.* ***B*** *Heatmap showing activity over the 24hr period.* ***C*** *AUC activity was higher in the dark phase compared to the light phase in both young and aged rats. Aged rats*

*had lower dark phase activity than young rats (p < 0.05, RM two-way ANOVA with pairwise comparisons).* ***D*** *Aged rats had lower activity AUC compared to young rats at ZT12-14 but not ZT22-24 (p< 0.01, RM two-way ANOVA with pairwise comparisons). Bars are mean ± SEM with data points overlaid. N=8/group. *p< 0.05, **p< 0.01, ##p<0.01, ###p<0.001 (*between-subject, #within-subject).*

### 4.3.2 Aged rats show normal light-dark core body temperature amplitude.

Analysis of AUC core body temperature in the light phase versus the dark phase of the ABS day showed a main effect of phase ($F_{(1,14)}$ = 41.896, p < 0.0001). However, there was no phase*age group interaction or main effect of age (p > 0.05). Pairwise post hoc comparison showed that core body temperature is higher in the dark phase than the light phase in both age groups (p < 0.0001) (**fig.3.C**). When core body temperature was compared at the start of the dark phase (ZT12-14) and end of the dark phase (ZT22-24), there was a main effect of ZT ($F_{(1,14)}$ = 118.908, p < 0.0001). There was no ZT*age group interaction, although there was a trend level effect ($F_{(1,14)}$ = 4.026, p = 0.065). There was no main effect of age. Pairwise post hoc comparison revealed that core body temperature AUC is greater at ZT12-14 than ZT22-24 (p < 0.0001) (**fig.3.D**).

**Figure 3**

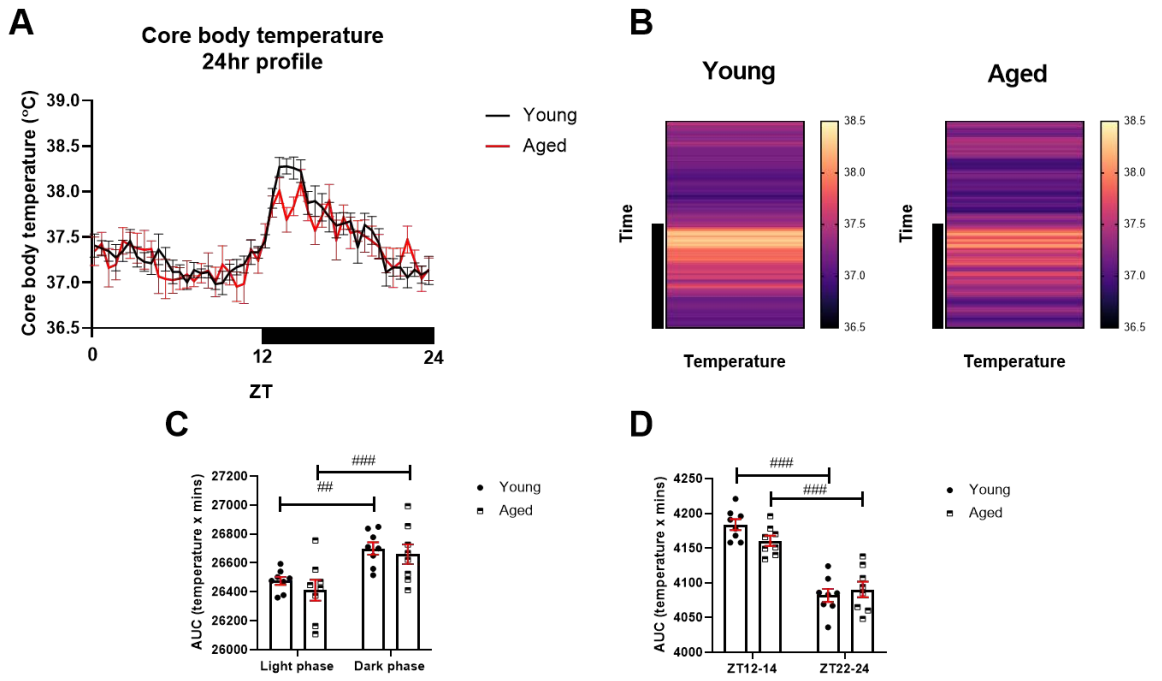


***Figure 3. Aged rats show an equivalent core body amplitude to younger rats.*** *Young and aged rats were implanted with a telemetry probe and core body temperature was output every 10 mins during the ABS day.* ***A*** *24hr trace of average core body temperature in young and aged rats, with data plotted every 3 measurements.* ***B*** *A heat map showing core body temperature over the 24hr*

*period. **C** Core body temperature was greater in the dark phase compared to the light phase in young and aged rats (p < 0.01, RM two-way ANOVA with pairwise comparison). There was no effect of age (p > 0.05). **D** Core body temperature was higher at ZT12-14 compared to ZT22-24 (p < 0.01, RM two-way ANOVA with pairwise comparison). There was no effect of age (p > 0.05). Bars are mean ± SEM with data points overlaid. N = 8 per group. ##p < 0.01, ###p< 0.001(within-subject).*

### 4.3.3 Aged rats show a blunted circadian CORT amplitude of change over the 24hr period.

Analysis of AUC blood plasma CORT levels at the peak and the nadir of the circadian cycle showed a main effect of phase ($F_{(1,11)}$ = 16.251, p = 0.002). There was also a phase*age group interaction ($F_{(1,11)}$ = 5.263, p = 0.042) and a main effect of age ($F_{(1,11)}$ = 7.474, p = 0.019). Pairwise post hoc comparison showed that young rats have a higher AUC CORT at the peak vs the nadir of the circadian cycle (p = 0.001) however aged rats did not (p > 0.05). Aged rats had lower AUC CORT at both the nadir and peak of the circadian cycle than younger rats (p = 0.011 and p = 0.027 respectively) (**fig.4.C**). Analysis of AUC CORT in the two hour time bins preceding the light change showed a main effect of ZT ($F_{(1,11)}$ = 7.147, p = 0.022). There was no ZT * age group interaction, although there was a trend level effect ($F_{(1,11)}$ = 4.089, p = 0.068). There was a main effect of group ($F_{(1,11)}$ = 7.930, p = 0.017). Pairwise post hoc comparison revealed that aged rats had a lower AUC CORT at ZT10-11 and ZT11-12 compared to younger rats, and these levels did not change across time bin (p > 0.05). However, AUC CORT was higher at ZT11-12 compared to ZT10-11 in young rats (p = 0.005) (**fig.4.D**). Analysis of AUC CORT at the start of the dark phase (ZT12-14) and the end of the dark phase (ZT22-24) showed a main effect of ZT ($F_{(1,11)}$ = 45.541, p < 0.0001). There was also a ZT*age group interaction ($F_{(1,11)}$ = 5.179, p = 0.044 and a main effect of age ($F_{(1,11)}$ = 9.198, p = 0.011). Pairwise post hoc comparison showed that AUC CORT was lower in aged rats at both ZT12-14 and ZT22-24 (p = 0.014 and p = 0.036 respectively). In both young and aged rats AUC CORT was lower at ZT22-24 compared to ZT12-14 (p < 0.0001 and p = 0.011 respectively) (**fig.4.E**).

**Figure 4**

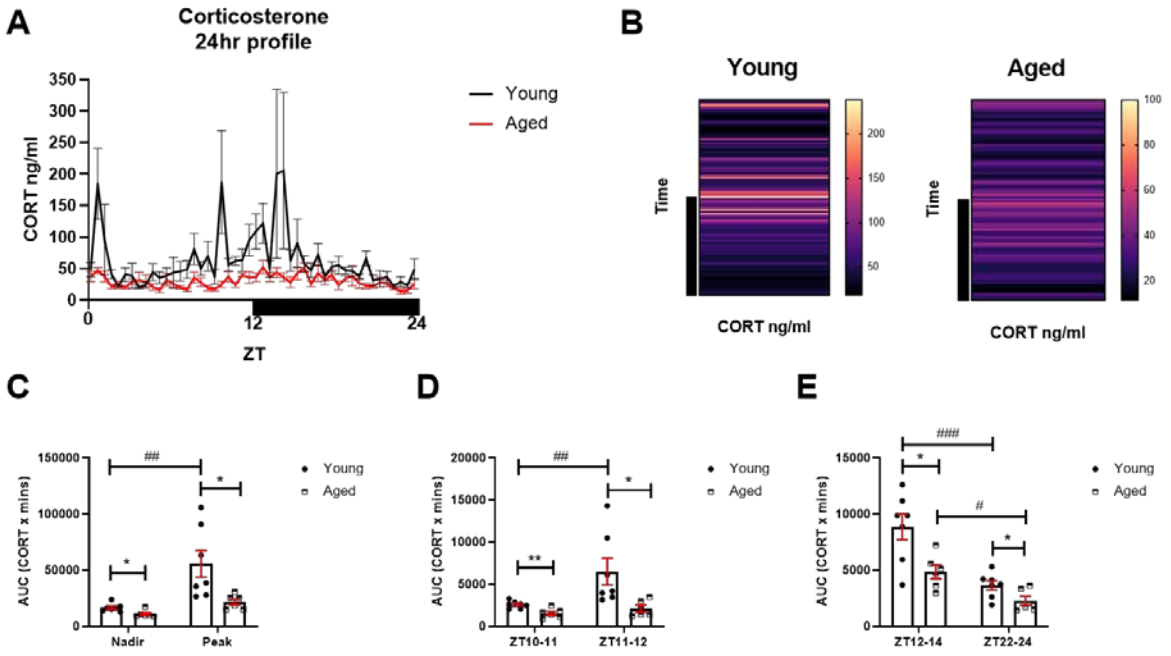


***Figure 4. Aged rats show a blunted CORT circadian rhythm at both the nadir and peak of the cycle.*** *Young and aged rats underwent automated blood sampling for 24hr.* ***A*** *24hr average plasma CORT trace in young and aged rats, with every 3 measurements plotted.* ***B*** *Heat map of CORT levels over the 24hr period.* ***C*** *Young rats have higher AUC CORT at the peak vs the nadir of the circadian cycle ($p < 0.01$, RM two-way ANOVA with pairwise comparison) but aged rats do not ($p > 0.05$). Young rats had higher nadir and peak CORT compared to aged rats ($p < 0.05$).* ***D*** *Young rats showed an increase in AUC CORT from ZT10-11 to ZT11-12 ($p < 0.01$, RM two-way ANOVA with pairwise comparison) but aged rats did not ($p > 0.05$). Young rats had higher AUC CORT at both of these time points compared to aged rats ($p < 0.05$).* ***D*** *Both young and aged rats had higher AUC CORT at ZT12-14 compared to ZT22-24 ($p < 0.05$, RM two-way ANOVA with pairwise comparison). Aged rats had lower AUC CORT than young rats at both timepoints ($p < 0.05$). Bars are mean ± SEM with individual data points overlaid. Young rats n = 7, aged rats n = 6. *p < 0.05 #p< 0.05, ##p<0.01, ###p<0.001 (*between-subject, #within-subject).*

### 4.3.4 Aged rats show a blunted CORT but not activity or temperature response to noise stress.

A comparison of AUC activity 30 mins pre-stress and 30 mins post-stress showed a main effect of stress ($F_{(1,14)} = 51.402$, $p < 0.0001$). There was also a stress*age group interaction ($F_{(1,14)} = 5.722$, $p = 0.031$ but no main effect of age ($p > 0.05$). Pairwise post hoc comparison showed AUC activity increased in both young and aged rats ($p < 0.0001$ and $p = 0.005$ respectively) but there was no age-related difference in activity ($p > 0.05$) (**fig.5.B**). A comparison of AUC core body temperature 30

mins pre-stress and 30 mins post-stress showed a main effect of stress ($F_{(1,14)}$ = 17.610, p = 0.001). There was no stress*age interaction or main effect of age (p > 0.05). Pairwise comparison showed core body temperature only rose with stress in the aged but not young rats (p = 0.001 and p > 0.05 respectively) (**fig.5.D**). Analysis using AUC CORT was not possible due to a larger number of missing samples. Instead, analysis of baseline CORT (an average of the three measurements preceding noise stress) versus peak CORT following noise stress was conducted instead. Analysis revealed a main effect of stress on CORT ($F_{(1,11)}$ = 36.004, p < 0.0001). There was also a stress* age group interaction ($F_{(1,11)}$ = 19.314, p = 0.001) and a main effect of age $F_{(1,11)}$ = 31.326, p < 0.0001). Pairwise post hoc comparison revealed that aged rats had a lower baseline CORT and peak stress CORT than younger rats (p = 0.042) and p < 0.0001 respectively). In young rats, peak CORT following stress was higher than their baseline CORT (p < 0.0001) however in aged rats it did not reach significance (p > 0.05) (**fig.5.F**).

**Figure 5**

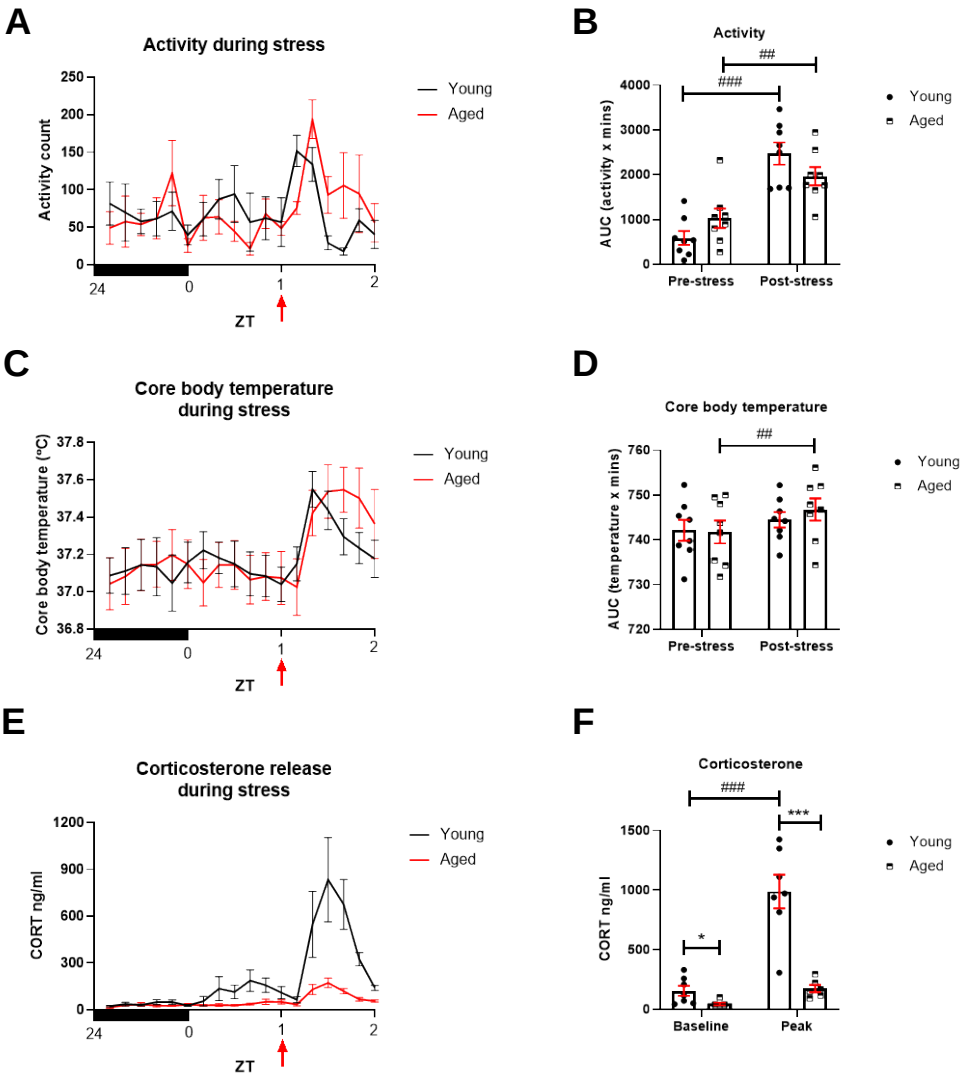


***Figure 5. Aged rats show a blunted CORT but not activity or temperature response to noise stress.*** *At the end of the 24hr ABS, 99db white noise was played for 10 mins. Activity, core body temperature and CORT were analysed pre- and post-stress.* ***A*** *Activity trace in young and aged rats pre and post stress. The red arrow indicates onset of noise stress.* ***B*** *Both young and aged rats showed an increase in activity following noise stress (p< 0.01, RM two-way ANOVA with pairwise comparison).* ***C*** *Core body temperature trace in young and aged rats pre and post-stress.* ***D*** *Aged rats showed an increased in core body temperature after stress but young rats did not (p< 0.01, RM two-way ANOVA with pairwise comparison).* ***E*** *CORT trace in young and aged rats pre and post stress.* ***F*** *CORT increased following stress in young but not aged rats (p< 0.001, RM two-way ANOVA with pairwise comparison). Bars are mean ± SEM with data points overlaid. Young n = 7-8, aged n = 6-8. *p< 0.05, ***p<0.001, ##p<0.01, ###p<0.001 (*between-subject, #within-subject).*

### 4.3.5 Restricted feeding subverts normal light-dark activity in aged but not young rats.

Following the ABS day, rats were put back on food restriction for a further 3 weeks. Light versus dark activity was compared to assess the effect of resuming food restriction over time. Analysis of total AUC activity in the light versus dark phase at day 5, 11 and 18 of recording in young rats showed a main effect of day ($F_{(2,14)} = 5.142$, $p = 0.021$). There was also an effect of phase ($F_{(1,7)} = 22.109$, $p = 0.002$). There was no day*phase interaction, although there was a trend level effect ($F_{(2,14)} = 3.199$, $p = 0.072$). Pairwise post hoc comparison revealed that dark phase activity is reduced at day 18 compared to day 5 ($p = 0.025$). At day 5 and 11 dark phase activity was greater than light phase activity ($p = 0.01$ and $p = 0.005$ respectively) however by day 18 this difference disappeared ($p > 0.05$) (**fig.6.D**). When this same analysis was conducted in aged rats, there was no main effect of day, although there was a trend level effect ($F_{(2,14)} = 2.927$, $p = 0.087$). There was a main effect of phase ($F_{(1,7)} = 15.090$, $p = 0.006$) but no day*phase interaction ($p > 0.05$). Pairwise post hoc comparison showed that light phase activity was higher than dark phase activity at day 11 and day 18 ($p = 0.022$ and $p = 0.009$ respectively) but not day 5 ($p > 0.05$) (**fig.6.E**). Activity during feeding period was also considered to assess whether changes to light phase activity were driven by the act of feeding itself and not the activity preceding or following it. When activity during the feeding period was compared between age groups, there was no effect of day or age ($p > 0.05$) (**fig.6.F**).

**Figure 6**

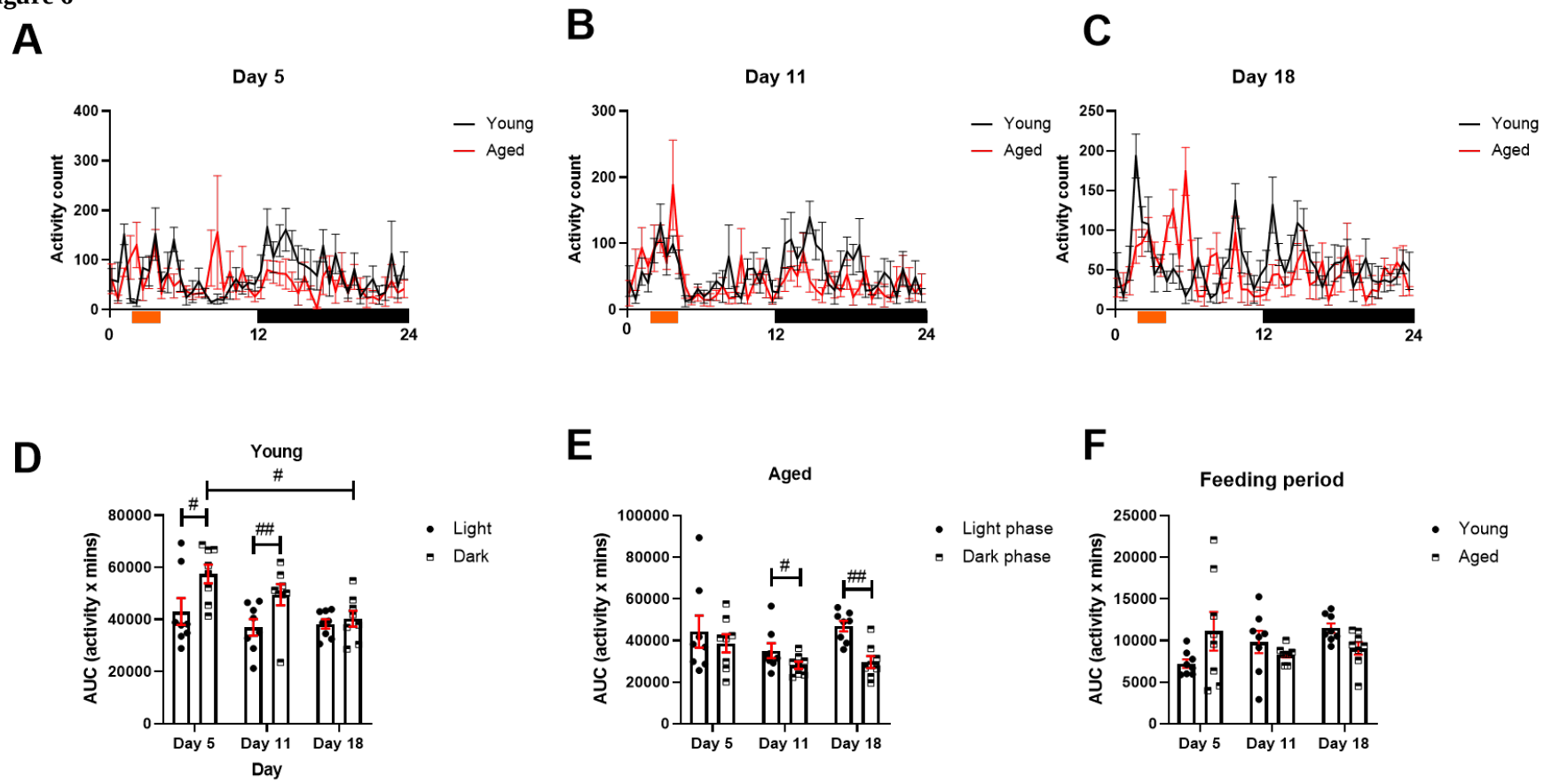


***Figure 6. Restricted feeding subverts LD activity in aged but not young rats.*** *Young and aged rats were fed 16g food between ZT1-4 daily. Activity was output in 10 min bins from implanted telemetry probes.* ***A*** *Activity trace on day 5 of recording. Orange bar indicates feeding time and black bar indicates dark phase.* ***B*** *Day 11.* ***C*** *Day 18.* ***D*** *In young rats, dark phase activity was lower at day 18 compared to day 5 ($p < 0.01$, RM two-way ANOVA with pairwise comparisons). At days 5 and 11 activity was higher in the dark compared to light phase ($p < 0.05$).* ***E*** *In aged rats, activity was higher in the light phase than dark phase at days 11 and 18 ($p<0.05$, RM two-way ANOVA with pairwise comparisons).* ***F*** *There was no difference in activity during feeding period*

*between young and aged rats (p> 0.05, RM two-way ANOVA). Bars are mean ± SEM with data points overlaid. n = 8 per group. #p<0.05, ##p<0.01 (within-subject).*

### 4.3.6 Restricted feeding subverts normal light-dark core body temperature in aged rats and flattens core body temperature rhythms in young rats.

Analysis of AUC core body temperature in the light phase versus the dark phase at day 5, 11 and 18 of recording in young rats showed no main effect of day, phase or day*phase interaction, although there was a trend ($F_{(1.286, 7.716)} = 4.924$, $p = 0.052$) (**fig.7.D**). When this same analysis was done in aged rats, there was a main effect of day ($F_{(2,14)} = 4.171$, $p = 0.038$). There was also a main effect of phase $F_{(1,7)} = 13.407$, $p = 0.008$ and a day*phase interaction ($F_{(2,14)} = 39.669$, $p < 0.0001$). Post hoc pairwise comparison showed that light phase core body temperature was higher at day 18 compared to day 5 and day 11 ($p = 0.017$ and $p < 0.0001$ respectively). Dark phase core body temperature was lower at day 11 and 18 compared to day 5 ($p = 0.02$ and $p < 0.0001$). It was also lower at day 18 compared to day 11 ($p = 0.021$). There was no difference in light versus dark phase core body temperature at day 5, but at day 11 and day 18 light phase core body temperature was higher than dark phase core body temperature ($p = 0.018$ and $p < 0.0001$ respectively) (**fig.7.E**). When core body temperature during feeding time was compared between age groups and across days, there was a main effect of day ($F_{(2,28)} = 21.244$, $p < 0.0001$ and a day*age group interaction $F_{(2,28)} = 16.039$, $p < 0.0001$. There was no main effect of age group though there was a trend level effect ($F_{(1,14)} = 3.558$, $p = 0.08$). Pairwise post hoc comparisons revealed that at day 11 aged rats had a greater core body temperature compared to younger rats ($p = 0.04$) **(fig.7.F)**.

**Figure 7**

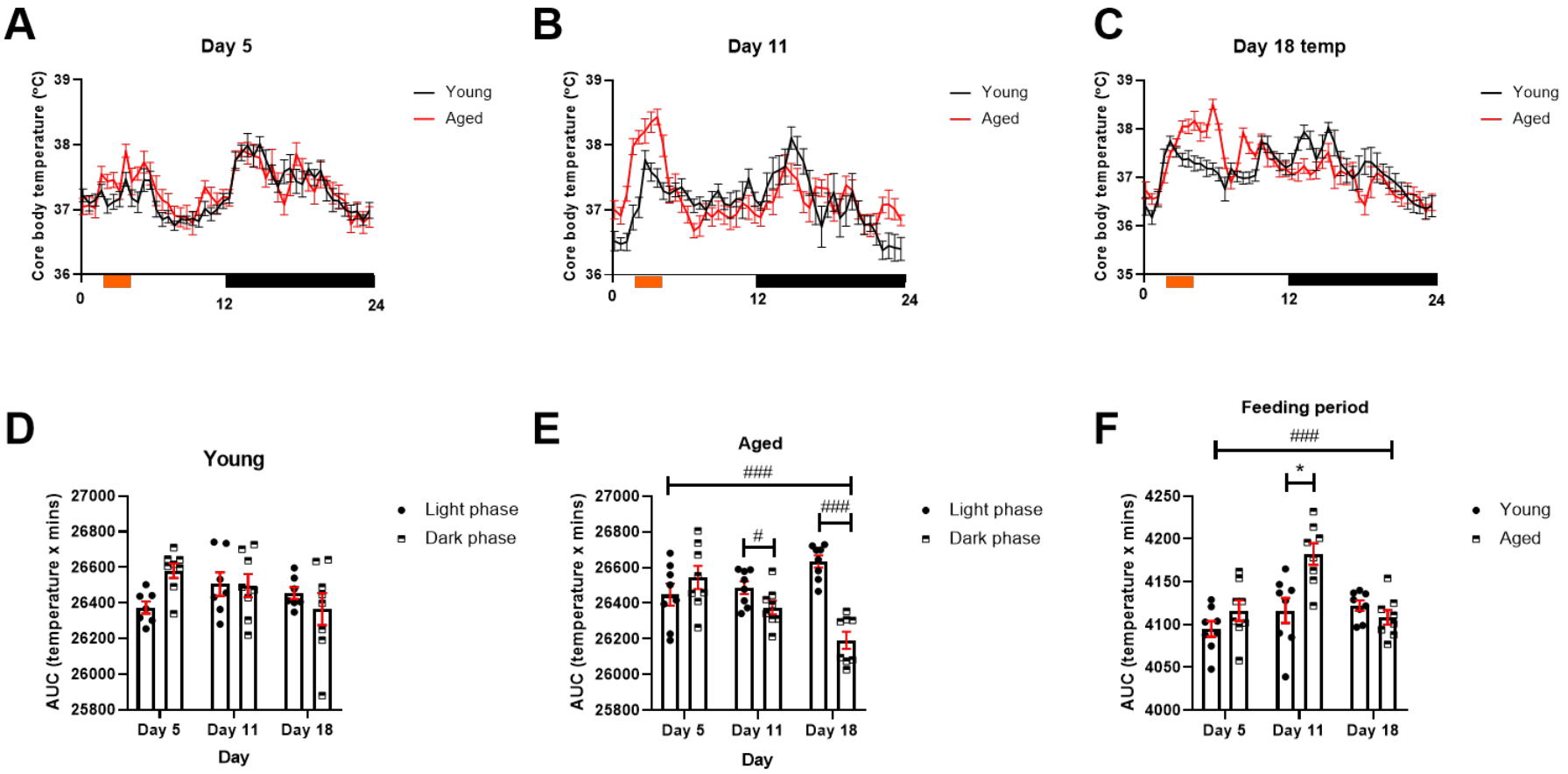


***Figure 7. Restricted feeding subverts core body temperature rhythm in aged rats and flattens in younger rats.*** *Young and aged rats were fed 16g food between ZT1-4 daily. Core body temperature was output in 10 min bins from implanted telemetry probes.* ***A*** *Average core body temperature trace on day 5. Orange bar indicates feeding period and black bar indicates the dark phase.* ***B*** *Day 11 and* ***C*** Day 18. **D** *There was no effect of day or age on core body*

*temperature in young rats (p> 0.05, RM two-way ANOVA).* ***E*** *At days 11 and 18 dark phase core body temperature was lower than light phase core body temperature in aged rats (p < 0.05 and p < 0.0001 respectively).* ***F*** *Aged rats had a higher core body temperature than young rats at day 11 (p < 0.05, RM two-way ANOVA with pairwise comparisons). Bars are mean ± SEM. N = 8 per group. # p < 0.05, ## p < 0.01, ###p< 0.001 (within-subject).*

### 4.3.7 CORT levels are greater at feeding time compared to the normal circadian peak.

Analysis of plasma CORT at ZT2-3 and ZT14-15 showed a main effect of time ($F_{(1,27)} = 55.62$, $p < 0.0001$). Post hoc comparison showed that CORT was lower at ZT14-15 compared to ZT2-3. There was no main effect of age or age*time interaction ($p > 0.05$) (**fig.8**).

### 4.3.8 There is no circadian rhythm of CRH neuronal expression in the PVN under a food restriction schedule.

CRH expression was analysed in the PVN at ZT2-3 and ZT14-15 and compared between age groups. There was no main effect, of time, age or time*age interaction ($p > 0.05$) (**fig.9.A**).

### 4.3.9 Aged rats show elevated levels of AVP neuronal expression at ZT14-15 but not ZT2-3.

AVP expression in the magnocellular and parvocellular areas of the PVN was analysed at the start of the light phase and the dark phase and compared between age groups. Within the magnocellular PVN, there was no main effect of time or of age group on AVP neuronal expression but there was a time*age group interaction ($F_{(1,12)} = 8.362$, $p = 0.0135$). Post hoc analysis revealed that AVP expression was lower at ZT14-15 compared to ZT2-3 in young but not aged rats ($p = 0.0179$ and $p > 0.05$ respectively). AVP expression was higher in aged rats compared to young rats at ZT14-15 ($p = 0.0207$) but not ZT2-3 ($p > 0.05$) (f**ig.10.B**). Within the parvocellular PVN, there was no effect of time and no time*age group interaction. However, there was a main effect of age ($F_{(1,12)} = 5.66$, $p = 0.038$). Post hoc comparison revealed that AVP expression was higher in aged rats compared to young rats at ZT14-15 ($p = 0.0248$) but not ZT2-3 ($p > 0.05$) (**fig.10.C**).

### 4.3.10 There is no circadian expression of GR in the PVN under food restriction.

Analysis of GR expression in the PVN at ZT2-3 and ZT14-15 in aged versus young rats revealed no main effect of time or time*age group interaction ($p > 0.05$). There was also no effect of age, though there was a trend ($F_{(1,12)} = 3.197$, $p = 0.099$) (**fig.11.B**).

### 4.3.11 POMC expression in the ARC is lower in aged rats than younger rats at ZT14-15 but not ZT2-3.

POMC expression in the Arc was analysed at two different timepoints and compared between age groups. There was no main effect of time or age ($p > 0.05$), but there was a time*age group interaction ($F_{(1,11)} = 4.902$, $p = 0.0489$). Post hoc comparison revealed that aged rats showed lower POMC expression at ZT14-15 compared to younger rats ($p = 0.0323$), but there was no difference between groups at ZT2-3 ($p > 0.05$). There was no difference in POMC expression between time points ($p > 0.05$), though in aged rats there was a trend towards aged rats showing lower expression at ZT14-15 compared to ZT2-3 ($p = 0.0763$) (**fig.12.B**).

**Table 2**

| Molecular target | ZT2-3 | ZT14-15 |
|---|---|---|
| CRH | No difference | No difference |
| AVP (magnocellular) | No difference | **Increase** |
| AVP (parvocellular) | No difference | **Increase** |
| GR | No difference | No difference |
| POMC | No difference | **Decrease** |

***Table 2. Summary of expression changes in targets of interest, aged relative to young.*** *CRH- corticotrophin releasing hormone, AVP-arginine vasopressin, GR- glucocorticoid receptor, POMC- Proopiomelanocortin.*

**Figure 8**

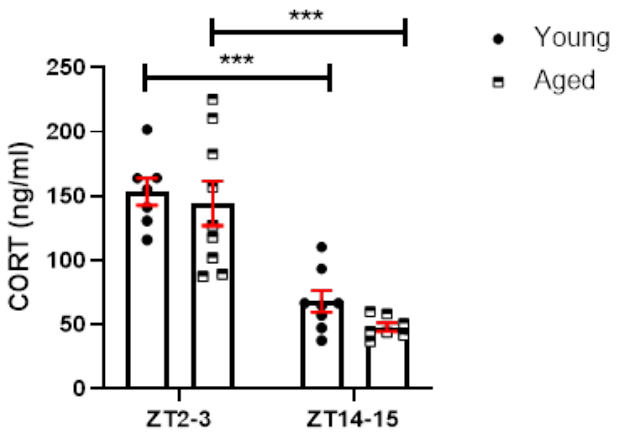


***Fig.8. ZT2-3 feeding raises nadir CORT above normal circadian peak levels.*** *Rats were killed at either ZT2 or ZT14 and CORT levels in the blood plasma were analysed. CORT levels were higher at ZT2-3 compared to ZT14. Bars are mean ± SEM with data points overlaid. N = 8 per group. ***p< 0.001 (between-subject).*

**Figure 9**

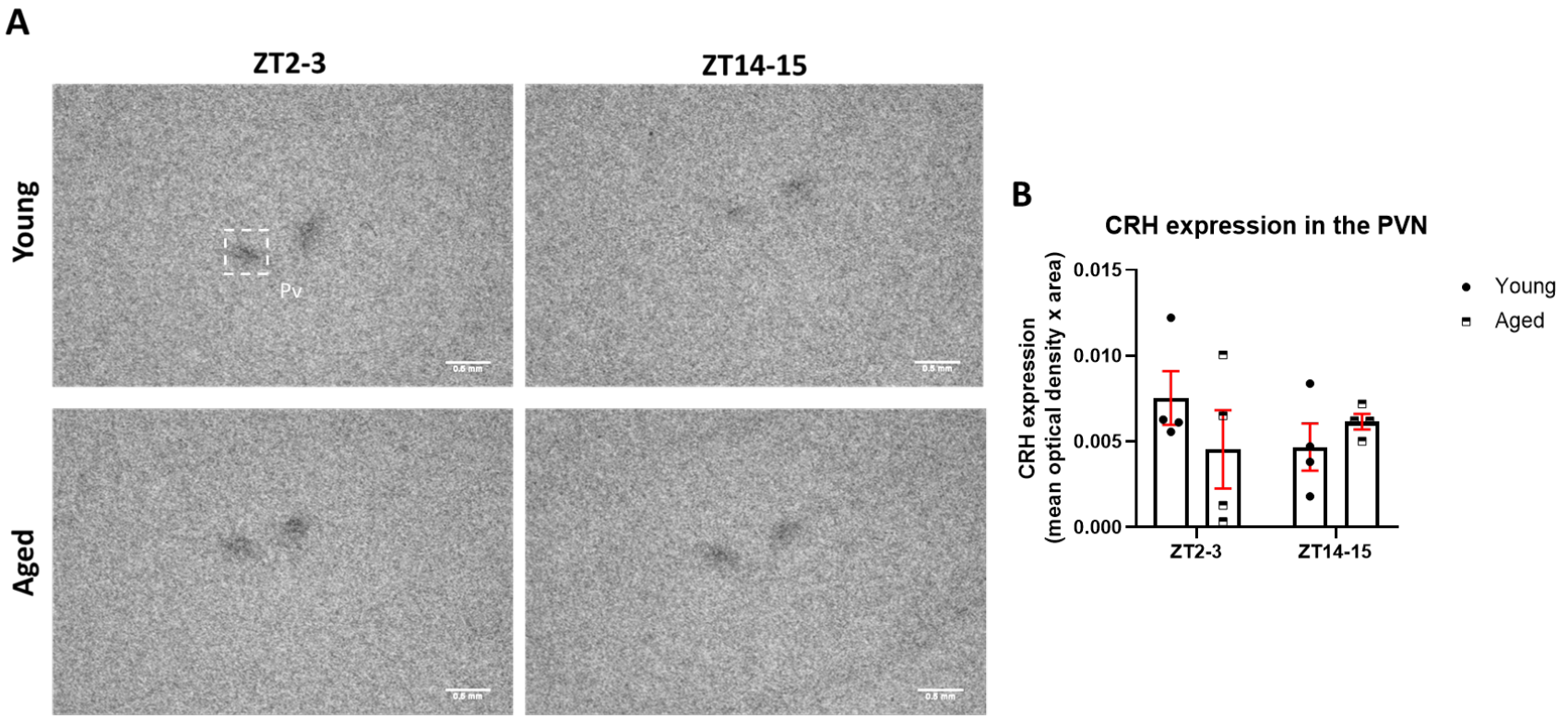


***Figure 9. There is no circadian expression of CRH under food restriction.*** *Young and aged rats were killed at either ZT2-3 or ZT14-15 and PVN sections underwent in situ hybridisation for CRH.* ***A*** *Example pictures of the PVN in each condition. Scale bars are 0.5mm.* ***B*** *There was no effect of time or age on CRH expression ($p > 0.05$, two-way ANOVA). Bars are mean ± SEM with data points overlaid. Pv-parvocellular.*

**Figure 10**

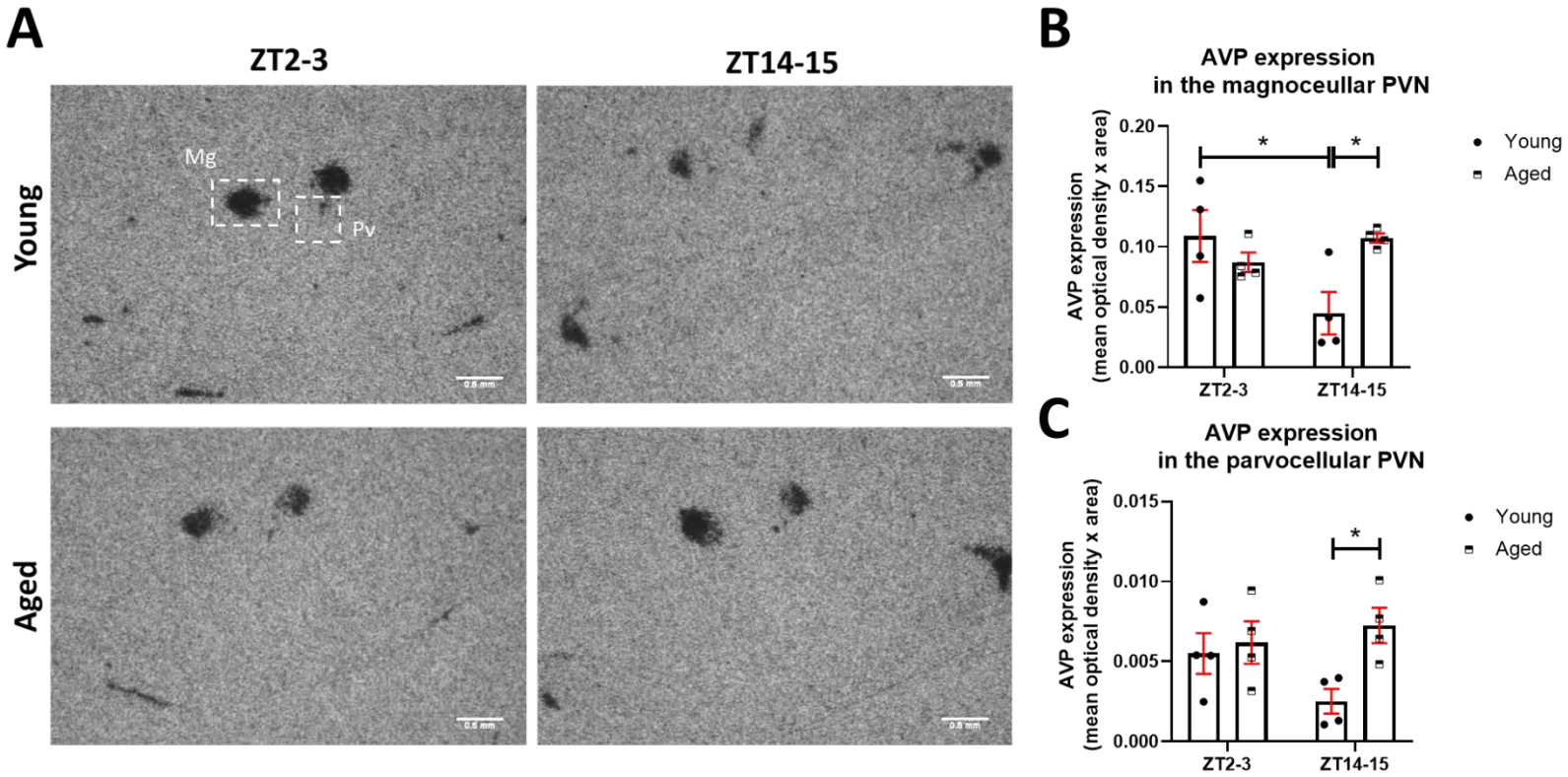


***Figure 10. Aged rats show elevated AVP neuronal expression in the PVN at ZT14-15 but not ZT2-3.*** *Young and aged rats were killed at either ZT2-3 or ZT14-15 and PVN sections underwent in situ hybridisation for AVP.* ***A*** *Example pictures of the PVN in each condition. Scale bars are 0.5mm.* ***B*** *AVP expression is lower at ZT14-15 compared to ZT2-3 in young but not aged rats ($p < 0.05$, two-way ANOVA with Sidak's post hoc comparison). AVP expression was higher in aged rats versus young rats at ZT14-15 ($p < 0.05$).* ***C*** *AVP expression was lower in aged rats compared to young rats at ZT14-15 but not ZT2-3 ($p < 0.05$, two-way ANOVA with Sidak's post hoc comparison). Bars are mean ± SEM with data points overlaid. n = 4 per time point. *p<0.05 (between-subject). Mg-magnocellular, Pv-parvocellular.*

**Figure 11**

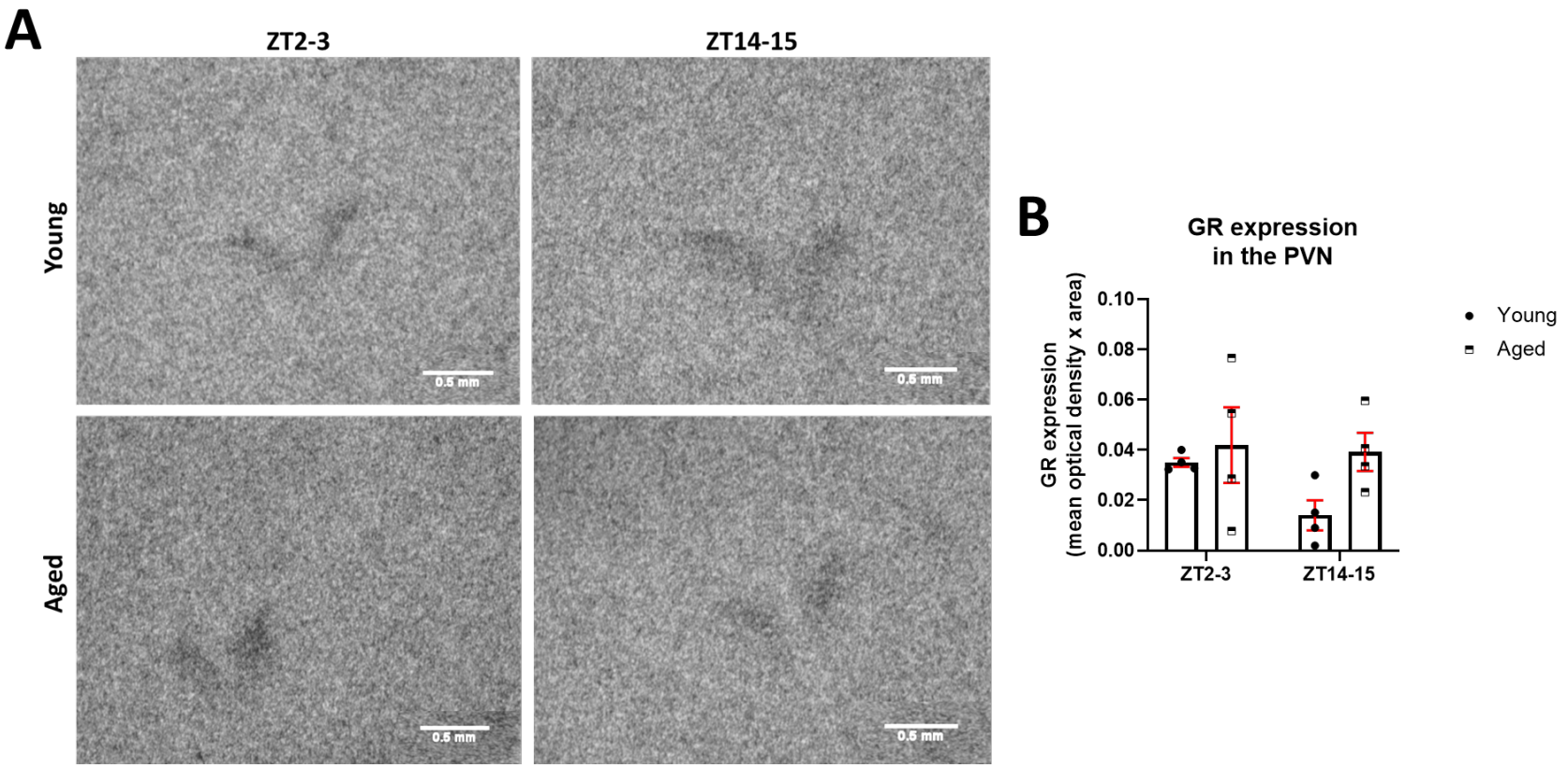


***Figure 11. There is no circadian variation in GR expression under a food restriction schedule.*** *Young and aged rats were killed at either ZT2-3 or ZT14-15 and PVN sections underwent in situ hybridisation for GR.* ***A*** *Example pictures of the PVN in each condition. Scale bars are 0.5mm.* ***B*** *There was no effect of time or age on GR expression in the PVN (p> 0.05, two-way ANOVA). Bars are mean ± SEM with data points overlaid. Aged and young N = 4 per time point. GR-glucocorticoid, PVN-paraventricular nucleus of the hypothalamus.*

**Figure 12**

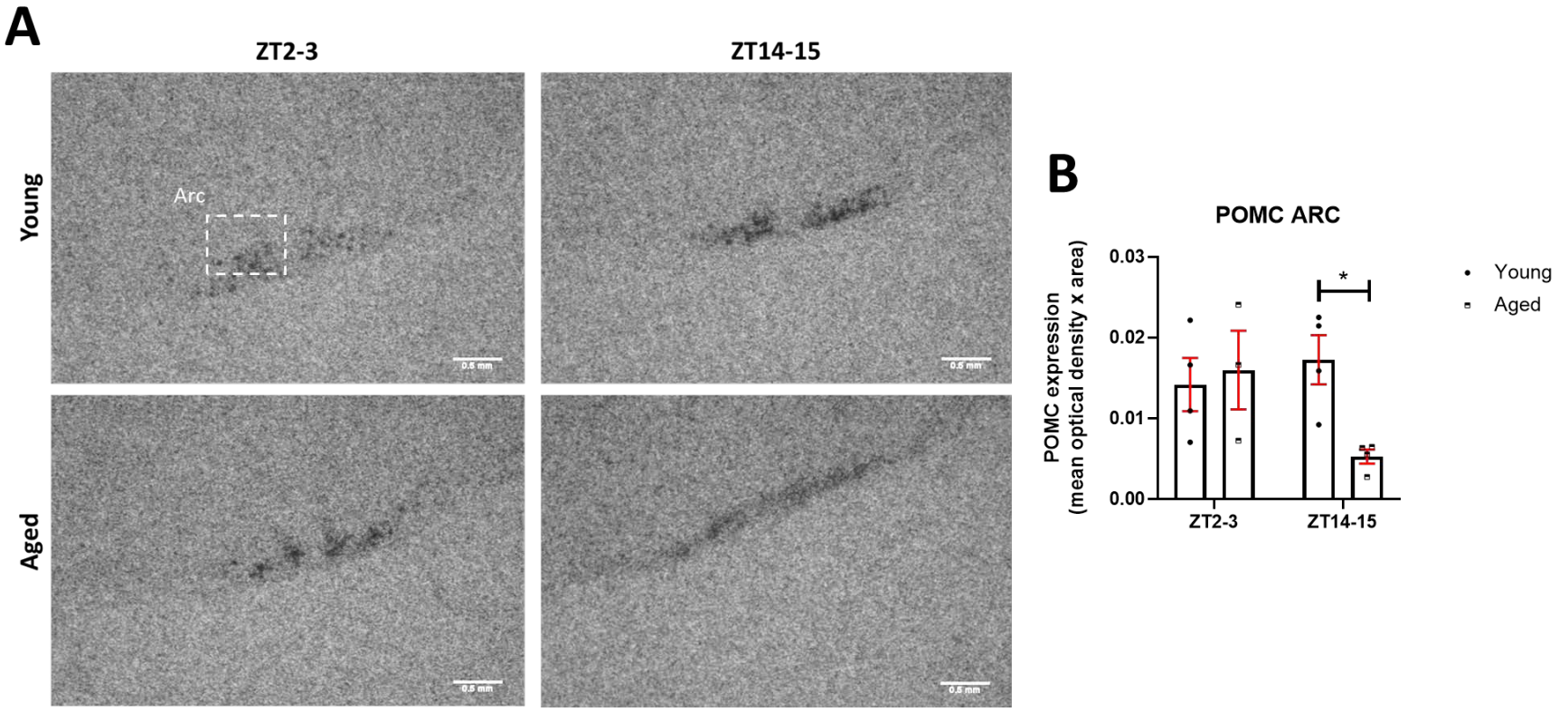


***Figure 12. Aged rats show lower expression of POMC at ZT14-15 but not ZT2-3.*** *Young and aged rats were killed at either ZT2-3 or ZT14-15 and ARC sections underwent in situ hybridisation for POMC.* ***A*** *Example pictures of the ARC in each condition. Scale bars are 0.5mm.* ***B*** *Aged rats showed a lower expression of POMC at ZT14-15 compared to younger rats ($p < 0.05$, two-way ANOVA with Sidak's post-hoc comparison). Bars are mean ± SEM with data points overlaid. Aged N = 3-4 per timepoint, young N = 4 per time point. *P< 0.05 (between-subject). Arc-arcuate nucleus.*

## 4.4 Discussion

Under *ad libitum* feeding conditions aged rats showed blunted dark phase activity driven by a reduction in activity immediately post-lights off. However, core body temperature was unaffected by age. Aged rats showed blunted CORT release with a loss of circadian variation and reduced stress response. When activity and core body temperature were recorded for a further 3 weeks while rats resumed a restricted feeding schedule in the light phase, aged rats showed an inversion of light-dark (LD) activity and core body temperature by day 11 but this did not occur in young rats. Analysis of CORT under food restriction revealed that CORT levels at time of feeding (ZT2-3) were higher than the normal circadian peak (ZT14-15). AVP expression was higher in aged rats in both the magnocellular and parvocellular PVN, while POMC expression in the Arc was lower. These age-related differences were specific to ZT14-15. CRH and GR in the PVN expression appeared unaffected by age or time. The following discussion considers how these results contribute to our current understanding of age-related changes to the circadian system and how the use of feeding in the light phase can potentially inform about the relative strength of different zeitgebers on circadian output.

### 4.4.1 Aged rats show blunted corticosterone release, dark phase activity and stress responsiveness under *ad libitum* feeding conditions.

Using automated blood sampling (ABS) to obtain a blood plasma CORT level every 10 mins for 24hr, the present study shows that aged rats show a blunted CORT circadian rhythm, with lower peak and nadir levels of CORT compared to younger rats. This blunting is consistent across different key time points, including anticipatory CORT (ZT11-12) and CORT immediately post lights off (ZT12-13) indicating an overall reduction of output. These timepoints were chosen because they most likely reflect changes in light zeitgeber sensitivity. In healthy rats under standard conditions, CORT rises in anticipation of lights off and is sustained for the first part of the dark phase before falling to a nadir. Any changes specific to these timepoints may suggest a reduction in circadian reactivity to light. While this blunted amplitude of change supports other studies indicating a blunting in amplitude of circadian CORT, it is in striking contrast to those studies which suggest that nadir CORT is elevated in ageing (Herman et al., 2016, Van Cauter et al., 1996). Higher levels of CORT are consistently reported in ageing and it has been suggested that ageing itself acts as a chronic stressor (Buechel et al., 2014).

A potential explanation for this contrast to the literature is the fact that the rats used in this study were food restricted for a proportion of their lives. It has previously been shown that aged rats food restricted for either 1 month or the duration of their lives show a blunted CORT level over the 24hr period compared to young rats and to aged rats that were fed *ad libitum.* Interestingly, food restricted young rats showed a greater CORT level over the 24hr period compared to *ad libitum* fed young rats, indicating that food restriction has opposing effects depending on age (Stewart et al.,

1988). It is important to note that in the present study during the blood sampling period the rats were put on *ad libitum* feeding to prevent room disruption which would lead to changes in CORT. The behavioural effects of restricted feeding are immediately masked under *ad libitum* (Pendergast and Yamazaki, 2018) but as changes to clock genes take several days to adapt to changing feeding schedules (Damiola et al., 2000) it may be that the central effects of restricted feeding persisted to some extent through this sampling phase. Similarly to CORT, a blunted amplitude of change in core body temperature has previously been reported in aged rats, driven by a rise in light phase temperature (Gordon, 2008). However, the present study shows a normal circadian rhythm in young and aged rats. Prior food restriction may also have driven this reduction in nadir core body temperature. Other studies have shown that calorie restriction results in lower core body temperature in rats (Aydin and Gordon, 2013). CORT and core body temperature have a relationship with metabolic activity (Jimeno et al., 2018), which in turn is altered by food restriction and it may be these physiological outputs in particular that are sensitive to changes to feeding schedule.

CORT has many downstream and central functions that affect a diverse number of systems, making a straightforward interpretation of HPA axis regulation extremely difficult. While an overall lower level of CORT seems beneficial in the context of age-related chronic stress, an overall reduction in 24hr CORT provides a weaker synchronising signal for peripheral and central clocks (Oster et al., 2017) and a loss of circadian variation indicates a reduction in output from the SCN to the PVN. Together this could lead to a loss in synchronisation of the peripheral and central clocks with the SCN which could have a wide range of health implications. Analysis of rhythmic clock gene expression in the peripheral tissues of young versus aged rats could provide more information about this. The lack of a significant CORT rise in response to a noise stressor in the aged rats further suggests that HPA axis regulation is weakened and could be driven by the loss of circadian rhythmicity as stress responsiveness is partially driven by time of day. Studies have also shown age-related temporal differences in CORT recovery following stress (Sapolsky et al., 1984), however this did not appear to be the case in the present study. There is the potential for the aged rats to show a reduced CORT response to a noise stressor because their hearing may be impaired. However, the aged rats showed an equivalent activity and temperature response to younger rats, suggesting they did hear the noise to similar extents. The lack of appropriate CORT response but normal activity and core body temperature response suggests a weakening of the aged HPA axis specifically.

Behavioural activity is unlikely to be affected by previous food restriction as activity changes induced by restricted feeding are immediately masked under *ad libitum* feeding conditions (Pendergast and Yamazaki, 2018). The aged rats show reduced levels of activity in the dark phase but not the light phase compared to younger rats. This reduction in dark phase activity appears to

be driven by activity immediately post lights-off, which has previously been characterised as the peak of the activity circadian cycle (Matikainen-Ankney et al., 2020). This suggests an age-related reduction in behavioural response to the light zeitgeber. It is not possible to conclude with the data obtained in the present study at what level this occurs, whether it is at the point of transmission through the eye, the entrainment of the SCN, or output from the SCN. There is evidence to suggest that elderly humans become less sensitive to light due to thickening and yellowing of the eye lens (Hood and Amir, 2017, Scheuermaier et al., 2010). However, a reduction in behavioural response to light has also been shown in rodents, where a reduction in light sensitivity is evidenced by delays in entraining to changes in the light cycle but unrelated to age-related changes to the eye (Lupi et al., 2012, Sellix et al., 2012, Zhang et al., 1996). Ageing has been shown to reduce SCN output, resulting in a reduction in behavioural output (Hood and Amir, 2017). The blunting of dark phase activity but not core body temperature provides an interesting disconnect and suggests that not all rhythms are dampened in ageing and may be system specific. In addition, a normal core body temperature rhythm suggests that light transmission through the eye is conserved in aged rats.

### 4.4.2 Differential effects of feeding in the light phase on activity and core body temperature circadian rhythms in young and aged rats.

Activity and core body temperature continued to be recorded for another 3 weeks after sampling and rats resumed being fed between ZT1-4 from day 5 of recording. The present study shows that providing food in the rest phase had a profound impact on both activity and core body temperature rhythms. Rats are nocturnal mammals and as such, 'normal' LD phase activity constitutes greater activity in the dark phase compared to the light phase. While young rats showed normal LD activity at day 11 but equivalent activity in both phases at day 18, aged rats showed inverted LD activity at day 11, where activity became greater in the rest phase. Feeding is a potent zeitgeber and feeding in the rest phase is a direct challenge to normal nocturnal activity (Edmonds and Adler, 1977). Core body temperature is similarly inverted in aged but not young rats where levels are equivalent between the light and dark phase. This is likely due to a competition between metabolic demand and normal LD circadian variation and the data suggests that in aged rats this metabolic demand overrides light-entrained circadian demand. By observing how LD patterns change under competing environmental stimuli, inferences can be made about the relative strength of these zeitgebers as entrainers of circadian rhythms. These results further highlight the differential impact restricted feeding has on the behaviour and physiology of the young versus the aged rat, as referenced above by (Stewart et al., 1988). The reason for this is yet unclear but given that young rat LD activity remains normal for longer and that aged rats appear to be less sensitive to the light zeitgeber, there is the potential that feeding time is a stronger zeitgeber than light in aged rats and is explored in more detail in the next chapter.

### 4.4.3 Age-related time-of-day changes in hypothalamic neuronal and receptor expression that may be driven by feeding in the light phase.

A new cohort of aged and young rats also food restricted and fed at the start of the rest phase were used to investigate the effects of ageing on molecular changes in the PVN. New rats were used to ensure there were no confounds associated with previous surgical intervention, single housing and long-term tethering that may have altered expression of targets of interest. The timepoints ZT2-3 and ZT14-15 were chosen as, under normal conditions, they represent the timepoints at the nadir and peak of the CORT, core body temperature and activity circadian rhythm under standard conditions. In addition, ZT2-3 represents the time at which the food restricted rats were fed, so provides an interesting contrast to changes at the circadian peak versus feeding time.

It should first be acknowledged that in the absence of a non-food restricted young and aged rat group it is not possible to conclude with any certainty that any effects or lack thereof discussed in this section are driven by feeding in the light phase. However, given the progressive loss of normal LD activity and core body temperature over time under food restriction discussed above and the wealth of literature showing the disruptive impact of light phase feeding on circadian rhythms it is the most likely explanation with which to speculate.

Despite previously obtaining a 24hr CORT profile, it was important to analyse CORT again at the two key time points while the rats were under food restriction, to put the CORT results in context with the molecular results. Analysis of CORT plasma levels at these two timepoints showed greater levels of CORT at ZT2-3 compared to ZT14-15 which is a complete inversion of what is expected under standard conditions and was observed in the 24hr CORT profile. While it is difficult to conclude with only two timepoints, it is unlikely the entire CORT circadian rhythm has completely inverted. Instead, it is likely that the ZT2-3 timepoint captured an anticipatory burst in CORT, which has previously been reported as occurring before feeding time and flattens the normal circadian peak (Xu et al., 1999) and is equivalent in aged versus young animals (Stewart et al., 1988). This anticipatory burst in CORT must be masked when rats are put back in *ad libitum* feeding, as observed in the 24hr CORT profile data.

Given that activation of CRH neurons is necessary for CORT release, there is the potential for CRH neuronal expression to reflect this change in CORT release, where CRH neuronal expression is lower at ZT2-3 due to higher levels of CORT. However, the present study shows no difference in CRH mRNA expression between ZT2-3 and ZT14-15 or between age groups. In standard conditions, while it has an intrinsically small amplitude of rhythm, CRH mRNA expression in the PVN is driven by circadian control from the SCN and shows higher levels around lights on and lowest levels around lights off, when CORT levels are at their highest (Watts et al., 2004, Kwak et al., 1993). Rhythmic expression of CRH mRNA is unrelated to levels of CORT and ACTH as it

persists in ADX rats (without circulating CORT), though sustained increases in CORT abolish this diurnal rhythm (Watts et al., 2004, Kwak et al., 1993). Feeding in the light phase may also produce a rise in CORT that is sufficient to disrupt normal rhythmic CRH mRNA expression in both young and aged rats, indicating that food restriction is disruptive to the HPA axis system at the central level. This is not particularly surprising as CRH neurons have diverse roles, including balancing homeostatic processes (Van Drunen and Eckel-Mahan, 2021). Previous work has found a loss of CRH mRNA rhythmicity in the PVN in aged rats, but no difference in the average level of CRH mRNA (Cai and Wise, 1996). It is likely that food restriction in the present study disrupted normal rhythmicity but is consistent with findings that levels of CRH mRNA expression remain unchanged with age.

Expression of AVP in the parvocellular and magnocellular portions of the PVN were considered separately due to some differences in function and underlying regulation of AVP between these two regions (Antoni, 2019). While AVP secreted from neurons of the parvocellular portion of the PVN acts mainly in synergy with CRH to release ACTH under conditions of stress, AVP released from the magnocellular portion of the PVN is part of the hypothalamic-neurohypophysial system, which drives the homeostatic regulation of body fluid and electrolyte balance (Wotjak et al., 2002). However, there is evidence to suggest that AVP from the magnocellular PVN (mPVN) also contributes to ACTH release (Saito and Soya, 2004). There is very little work on circadian expression of AVP in the PVN, a study showed that unlike CRH, there was no circadian variation in AVP in the pPVN but AVP is weakly rhythmic in the mPVN, where levels are low in the light phase and higher in the dark phase (Watts et al., 2004). This suggests that diurnal release of CORT is almost exclusively driven by CRH. These results are consistent with the present study findings, which show no time-of-day difference in AVP expression in young and aged rats in the pPVN. In contrast, there is a time-of-day difference in AVP expression in the mPVN, with young but not aged rats showing higher AVP expression at ZT2-3 compared to ZT14-15. This pattern is opposite to previous reports and may be due to feeding at ZT2-3 leading to an increase in water intake at this time point. In both the pPVN and the mPVN, aged rats show higher expression of AVP compared to younger rats at ZT14-15 but not ZT2-3. AVP gene transcription is extremely sensitive to CORT levels. In ADX rats, AVP shows a strong circadian rhythm in the PVN, showing that dynamic variation in CORT blunts rhythmic expression. At ZT14-15, when CORT levels are comparatively low in the food restricted rat, aged rat AVP expression may be higher than younger rats due to a different relationship between CORT and AVP transcription. However, further work is necessary to elucidate this.

While CRH and AVP neuronal activation results in the release of CORT, GR binding results in its inhibition. Therefore, changes in GR mRNA expression could provide further information about the impact of age or feeding on the negative arm of CORT regulation. The present study indicates

that GR mRNA expression in the PVN showed no significant time of day difference in either age group. Under standard conditions, rat skeletal muscle GR mRNA has previously been shown to peak in the early light phase of rodents and reach a nadir at the start of the dark phase as receptor transcription is inhibited by its own ligand (Yao et al., 2006). However, circadian variation in GR mRNA expression in the PVN has not previously been explored. It cannot be assumed that findings from peripheral tissues translate to the PVN due to temporal differences in SCN-mediated transcriptional regulation. It is unclear whether this observed lack of variation in GR mRNA expression is driven by feeding in the light phase as is the case for CRH mRNA expression and therefore first requires further study under standard conditions.

ARC was an additional target of interest due to its crucial role in hypothalamic metabolic and feeding regulation (Van Drunen and Eckel-Mahan, 2021). POMC neurons suppress appetite by releasing α-melanocyte stimulating hormone (α-MSH) (Huszar et al., 1997). Previous work has shown that under *ad libitum* feeding conditions, neurons of the ARC show circadian rhythmicity to modulate timing of feeding, with POMC mRNA expression peaking mid-light phase and reaching a nadir at the start of the dark phase. However if rats are food restricted and fed in the light phase, this rhythm disappears (Xu et al., 1999). This may explain why in the present study, young and aged rats do not show evidence of a time-of-day difference in POMC expression, though aged rats showed a trend towards a reduced expression of POMC at ZT14-15 compared to ZT2-3. It is interesting however that aged rats show reduced expression of POMC compared to young rats at ZT14-15 but not ZT2-3. This suggests that either aged rats retain an appetitive drive to consume food in the dark phase, or rhythmic expression of POMC is less effected by feeding in the rest phase compared to younger rats. However, analysis of POMC only considers one arm of appetite regulation. The ARC has a diverse population of neurons, and to get a more cohesive picture of the effects of restricted feeding and age on neuronal expression in the ARC, other populations such as neuropeptide Y (NPY), cocaine-amphetamine–regulated transcript (CART) and agouti-related protein (AgRP) neurons should also be considered (Zhang et al., 2019). However, this was not within the scope of the present chapter.

### 4.4.4 Conclusion

The original purpose of this study was to investigate the impact of otherwise healthy ageing on the CORT, core body temperature and activity circadian rhythm and the expression of key neuronal and receptor populations of the PVN. However, it became clear these were not ‘normal’ rats because they had been fed in the light phase and this was continued for most of the experiment. This confound could not be ignored and was considered in the interpretation of the results. Aged rats showed a blunted CORT rhythm, with a loss of significant rise of CORT over the 12:12 LD cycle and a lack of significant response to a noise stressor. This loss of rhythmicity is likely to result in a reduction in entrainment of the peripheral clocks and could have profound health implications.

Resuming feeding in the light phase caused a progressive loss of normal LD activity and core body temperature, with aged rats showing a complete inversion of normal LD patterns. This suggests that feeding may be a more potent zeitgeber than light in aged but not young rats. It also produced an anticipatory burst in CORT and appeared to flatten the circadian peak, showing feeding in the light phase profoundly impacts on CORT release. When considering the molecular data together, it is apparent that any age-related differences only occur at the ZT14-15 timepoint. This suggests that central changes induced immediately by feeding time are not differentially affected by age but the resulting impact of food restriction on neuronal/receptor expression post-lights off is differentially altered with age. This is consistent with the findings from (Stewart et al., 1988) and with the behavioural and physiological data in this chapter showing that food restriction affects young and aged rats differently. These findings may have important consequences for therapeutic intervention and is discussed further in chapter 6.

# Chapter 5: Investigating age-related changes in circadian flexibility

## 5.1 Introduction

A healthy circadian system must be able to flexibly adapt to a changing environment and anticipate important cues. The feeding entrainable oscillator (FEO) is a powerful driver of anticipatory activity for feeding time. When food availability is restricted to a certain time of day, mammals exhibit feeding anticipatory activity (FAA), a daily increase in locomotion and core body temperature 2-3hrs preceding presentation of food (Storch and Weitz, 2009). This persists in rodents even when food is presented in the light phase, when the rodent is typically inactive. This is an example of circadian flexibility, the ability to rapidly adjust behaviour to maximise survival (Riede et al., 2017).

FAA is a circadian mechanism as it persists in constant conditions, and only occurs when food is presented approximately every 24 hours (Bolles and De Lorge, 1962). However, feeding entrainment is completely independent of the SCN and known clock machinery. FAA persists even after bilateral lesioning to the SCN and in models with mutations in the core clock machinery. The location of the FEO has been researched for over 30 years and remains unknown (Pendergast and Yamazaki, 2018).

FAA is relatively well conserved in rodent ageing, with studies finding little to no change in the ability of aged rats to build FAA compared to younger rats under food restricted conditions (Mistlberger et al., 1990, Walcott and Tate, 1996). Two potential mechanisms for age-related blunting of circadian rhythms are 1) reduction in sensitivity to light and 2) the dissociation between the SCN and the peripheral clocks (Hood and Amir, 2017). Timed feeding is a powerful entrainer of the peripheral clocks and will eventually uncouple the peripheral clocks from the SCN such that they oscillate in distinct phases. However, the SCN remains unaffected by feeding time. Therefore, timed feeding could potentially re-entrain peripheral clocks to therapeutic benefit. However, the effects of manipulating zeitgebers are often studied in isolation and, in a complex environment, organisms are faced with multiple competing signals. Gaining a better understanding of how competing zeitgebers together impact on behavioural output (activity) can provide information on the relative strength of these stimuli as entrainers of circadian rhythms in the young and old system. It also has the potential to provide an indication of the flexibility of the aged versus young circadian system. Daily rhythms can be flexibly aligned to the light-dark cycle to improve fitness to nature (Riede et al., 2017). Thus, a circadian system that can balance the effects of light and other conflicting signals on physiological and behavioural output may be more flexible. Data from the previous chapter suggested that feeding in the light phase may affect normal nocturnal behaviour and seemed to affect young and aged rats differently, with aged rats showing a complete subversion of normal light-dark activity. This may suggest reduced circadian flexibility and that feeding is a more powerful zeitgeber than light in aged rats.

The aim of this chapter therefore was to explore the impact of age on circadian flexibility in more detail using zeitgebers that are translationally relevant and vital to normal human life: light, feeding, palatable reward and socialisation. Timed feeding was used to investigate (1) the gain and loss of feeding anticipatory activity in rats (2) its impact on light vs dark activity and (3) its impact on activity changes induced by the light zeitgeber. Timed social play was used to investigate whether anticipatory activity could be induced by a non-feeding zeitgeber. Mice were utilised to explore the impact of age on circadian flexibility by using timed delivery of reward and changes to the light-dark cycle. Aged rodents were hypothesised to show a reduction in circadian flexibility evidenced by disruption to the gain and loss of anticipatory activity for feeding and non-feeding signals, reduction in behavioural adaptation to competing zeitgebers and delays to light entrainment.

## 5.2 Methods

### Rats

### 5.2.1 Subjects

8 aged male Sprague Dawley rats, aged approximately 23 months old by end of experiment and 8 younger controls, aged around 10 months old by end of experiment were used. Animals were pair housed in enriched housing as previously described and housed in 12:12 standard lighting, lights on at 7:00 and lights off at 19:00. At Eli Lilly rats were previously kept on a restricted diet of 16g per animal, fed at ZT2-3. This was continued at the University of Bristol, until experimental manipulation. Rats were checked daily for health impairments that would affect general home cage activity. No animals were excluded based on ill-health.

### 5.2.2 Nanotag implantation

Rats were anaesthetised with gaseous isoflurane (5 % w/w/ liquid vapour, Merial, UK) and medical grade air and given 1 mg/kg sub-cutaneous injection of Rimadyl (Pfizer, UK) to manage post-operative pain. During surgical procedure and recovery, rats were placed on heat mats to maintain body temperature. Sc injection of 2.5 ml glucose saline (0.9 % NaCl, 5 % glucose) (Braun, Germany) was given post-surgery.

A single incision was made into the skin at the sub-cutaneous level several cm below the neck. A pocket was then opened using blunt dissection, and the nanotag was implanted. Rats were fed *ad libitum* for 48 h following surgery as per license requirements, and then switched back to a restricted feeding schedule. The nanotag was set to read activity every 5 mins. Data was collected from the animal every week by reading the nanotag through the skin. The use of nanotags allowed for freely moving rats to be group-housed while data was collected continuously, representing a refinement on the previous chapter which required long term tethering and single housing. The tag was read and data visualised using Nanotagviewer R1.1.2.1804.

### 5.2.3 Feeding manipulations

Rats were given 16g of standard lab chow at ZT2-3, ZT9-10 and then *ad libitum* in two-week periods (**fig 1**). Rats were fed in the light phase where activity is comparatively low, so that activity changes could be easily identified.

### 5.2.4 Social play

Rats were placed in a social play arena (1 x 1 m), filled with 400 plastic play balls (Chad Valley) and 20 reward pellets. Rats were placed in the arena between ZT5-6 in groups of 4 (of the same age). This allowed rats to interact with two unfamiliar rats while exploring and foraging in the arena

with lower risk of aggression (due to inability to balance effectively on the balls). All rats were brought into the room containing the ball pit at the same time. The order the groups were placed in the social play arena was counterbalanced. Rats were kept in the arena for 10 mins and then placed back in the home cage. Visible urine and faecal pellets were removed in between groups. This was repeated for 7 days. Rats were then left on *ad libitum* feeding for a further 7 days.

**Figure 1**

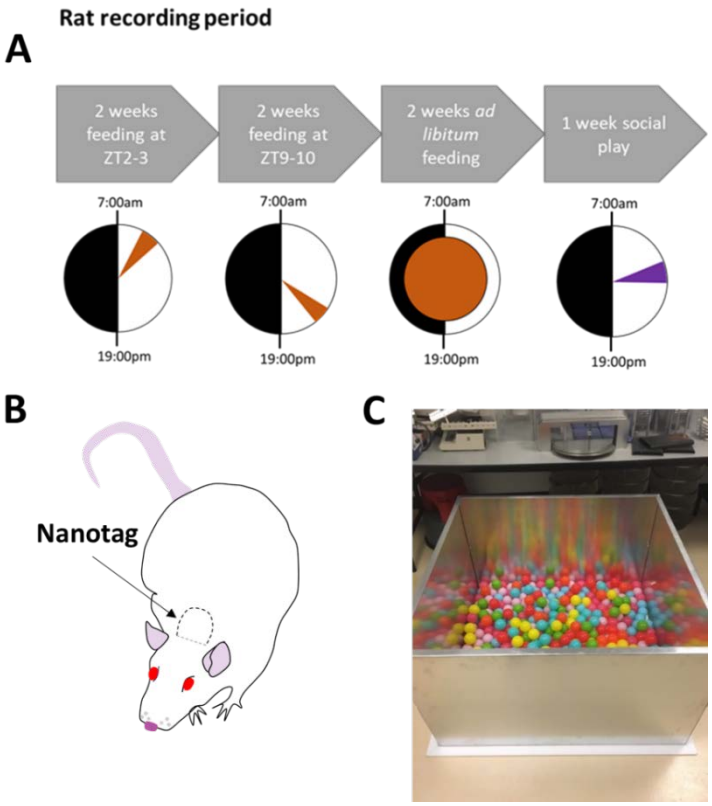


***Fig.1.Rat recording experiment design.*** ***A*** *Rat recording period* ***B*** *Placement of sub-cutaneous implanted nanotag and* ***C*** *Ball pit used for social play.*

**Mice**

**5.2.5 Subjects**

3 groups of c57bl/6j male mice were supplied by Eli Lilly aged 7 months, 16 months old and 22 months n = 12/group. Mice were singly housed using enrichment as described previously. Mice were provided with food and water *ad libitum.* Most experiments were conducted under standard 12:12 lighting, lights on at 8.15 am and lights off at 8.15 pm. However, lighting was shifted by 12h in the final experiment where lights off was 8.15 am and lights on was 8.15 pm. Mice were checked daily for health impairments that would affect general home cage activity. Where this occurred, mice were excluded from the analysis and reported in section 5.2.7.

### 5.2.6 Activity monitoring

A passive infrared activity monitoring system was built in-house by MJ to monitor home cage activity in individually-housed mice, based on a system previously developed by (Brown, 2016). 36 activity sensors with built-in amplifiers (AMN 2,3,4 series Motion Sensor, Panasonic) were read using an Arduino Mega 2560 with an Arduino Mega daughter board (SchmartBoard, Mouser). Data was read onto an SD card as CSV file using an SD card adaptor (TFT LCD w/microSD Breakout, Adafruit) and exact time was outputted alongside the data using a RealTime clock (Clock & Timer Development Tools PCF8523 RTC for RPi, Adafruit). Food was placed inside the cage to prevent blocking of mouse motion. Each sensor was placed above the middle of the cage, approximately 5 cm from the top of the cage. Screens were placed between cages to prevent sensors detecting movement in other cages. Proximity of age group to the door, shelf height and to each other was counterbalanced in case this affected activity. Movement was detected by the sensor every 100 msec. A percentage of movement within 10 secs was calculated and outputted in 10 second time bins. Data averaged into 1 min bins for analysis. Double-plotted actograms were built using the ActogramJ plugin used by ImageJ with a max value of 60. Chi-squared periodogram analysis was also conducted using ActogramJ. Actograms depicting readjustment to new lighting schedule were scored by two independent experimenters blind to age group. Actograms were scored by visually determining day at which the mouse re-entrained to the new light cycle. If there was disagreement between the two experimenters, then the median value was used. Mouse activity was recorded over several experimental manipulations (**fig 2**).

*Continuous monitoring of activity under standard conditions*

Mice were housed under standard conditions described above and activity monitored for a period of 3 weeks. N = 1 mouse was excluded from the 7 month and 16-month-old groups due to faulty sensors and n = 1 aged mouse was culled due to ill health induced by a skin condition.

*Continuous monitoring of activity during reward delivery*

For a period of 14 days, mice were given 5 reward pellets at exactly ZT8. In the following week, mice were given reward pellets every other day. On the days where reward was not delivered, the experimenter entered the room and stood for a couple of minutes before leaving to control for activity changes induced by entering the room. A further 2 16-month-old mice were excluded due to faulty sensors. A further 2 aged mice were excluded, one due to death from a random cardiac event and the other for missing teeth.

*Continuous monitoring of activity during change to light cycle*

Mice were moved from their standard 12:12 lighting room to a 12:12 reverse lighting room to monitor activity change in response to a lighting change. Recording began 10 mins after they were moved to the new room. A further 16-month-old mouse was excluded due to a faulty sensor.

**Figure 2**

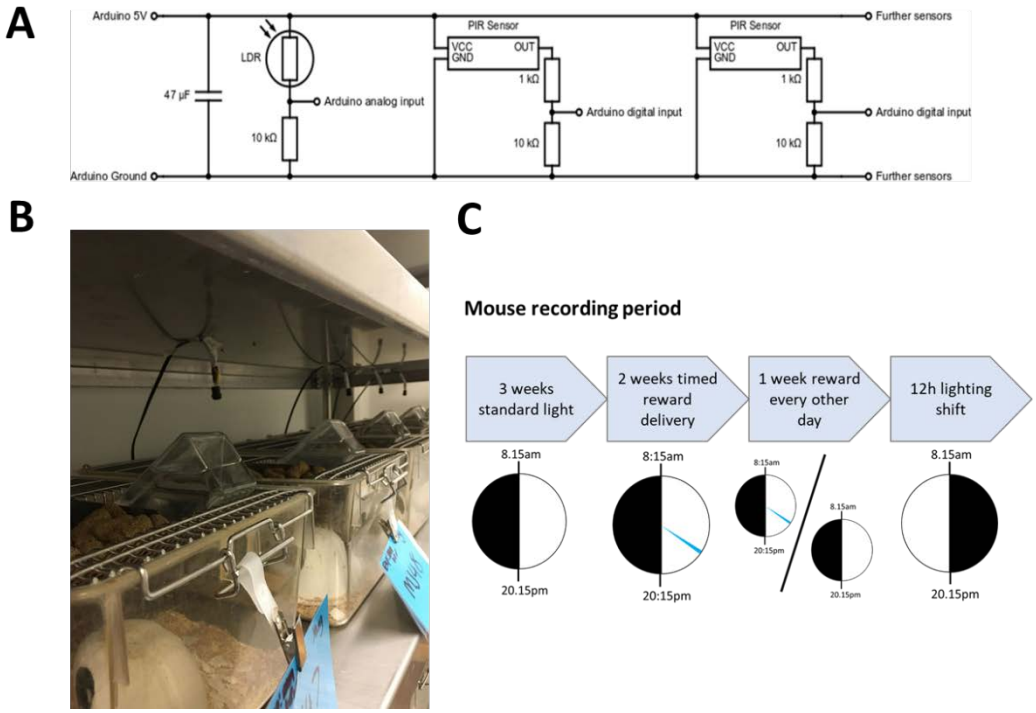


***Fig.2.A Circuit diagram of activity sensor system.*** *Diagram shows two activity sensors and a light sensor connected to an Arduino board* ***B*** *Example of sensor position over mouse cages (without screens).* ***C*** *Mouse recording period.*

### 5.2.7 Data analysis

As described in the previous chapter, time periods of interest were pre-selected prior to analysis and based off the literature. Periods of interest were whole light phase, whole dark phase, ZT11-12 (pre-lights off activity), ZT12-14 (post-lights off activity) and ZT22-24 (control period). Activity was analysed using area under the curve using GraphPad Prism v 7, calculated using the trapezoidal rule. For repeated measures data, if a rodent had ≥ 2 data points that was 2 SDs away from the mean then that rat was excluded from analysis. If the rat had ≤ 1 data points that was over 2 SDs away from the mean, then that value was replaced with the group mean.

Statistical analysis was conducted as described in chapter 2.

## 5.3 Results

### 5.3.1 Aged rats show differences in the loss and gain of anticipatory activity for feeding time but equivalent levels by the end of the feeding period.

Rats were fed for 2 weeks at ZT2-3 and were then switched to ZT9-10 for two weeks. To analyse loss of feeding anticipatory activity (FAA) over time, a comparison of activity 2 hours preceding the original feeding time of ZT2-3 FAA across 6 days was conducted, where day 1 was the first day feeding at ZT9-10. There was a main effect of day on activity ($F_{(5,65)} = 29.51$, $p < 0.0001$). Post-hoc pairwise analysis revealed that by day 6 in both young and aged groups, activity was significantly reduced compared to day 1 ($p = 0.002$ and $p = 0.028$ respectively). Strikingly, in the aged group, activity was significantly higher on day 3 compared to day 1 ($p < 0.0001$). This effect was not seen in the younger group ($p > 0.05$). There was also a day*group interaction ($F_{(5,65)} = 16.740$, $p < 0.0001$). Post-hoc pairwise analysis revealed activity in the aged group was higher on day 3 compared to the younger group ($p < 0.0001$) **(fig.3.A)**.

To analyse the gain of FAA for ZT9-10, a comparison of activity 2 hours preceding new feeding time (ZT9-10) across 6 days was conducted, where day 1 was the first day feeding at ZT9-10. There was a main effect of day on activity ($F_{(5,65)} = 22.621$, $p < 0.0001$). Post-hoc analysis revealed that FAA increased significantly by day 3 in the younger group ($p = 0.004$) and by day 4 in the aged group ($p = 0.019$) however this was not sustained in day 5 and 6 ($p > 0.05$). There was also a day*group interaction ($F_{(5,65)} = 3.223$, $p = 0.012$). Post-hoc analysis revealed that younger rats showed higher FAA on days 2 and 6. There was also a trend towards a between subject effect of age ($F_{(1,13)} = 4.342$, $p = 0.057$) **(fig.3.B)**. An average of the final 3 days of FAA of the new feeding schedule ZT9-10 was compared between groups. There was no difference between groups in FAA by the end of the new feeding period ($p>0.05$). One aged rat was excluded due to consistent outlying data and was also excluded from further analyses.

**Figure 3**

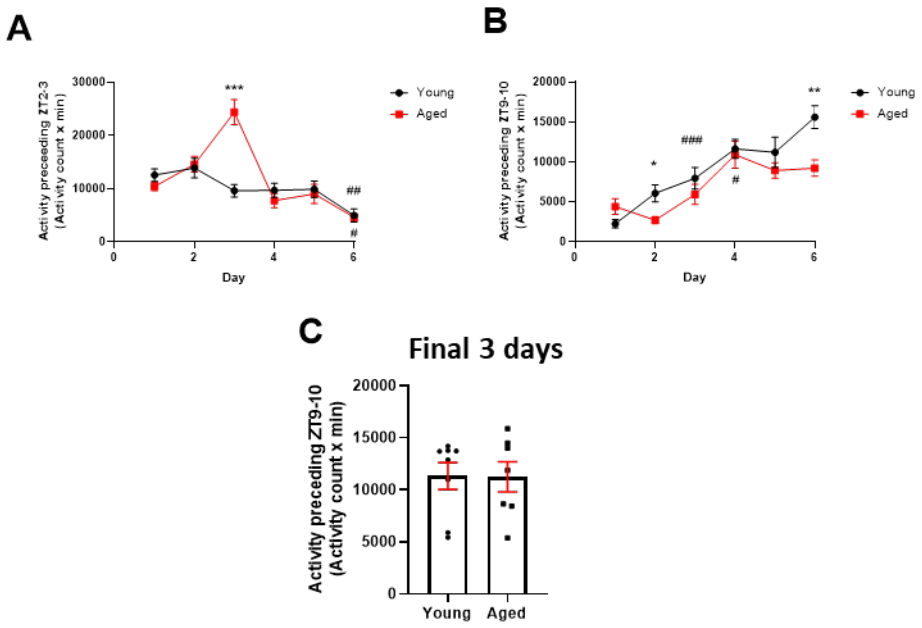


***Fig.3. Aged rats show differences in the gain and loss of feeding anticipatory activity.*** *Rats were fed at ZT2-3 for 2 weeks and then switched to being fed at Z9-10 for 2 weeks. Activity 2hrs preceding feeding time was defined as FAA.* ***A*** *FAA for original feeding time (ZT2-3) where day 1 is first day of ZT9-10 feeding. Both groups showed a reduction in ZT2-3 FAA by day 6 but aged rats showed greater FAA than younger rats on day 3 ($p<0.01$, RM two-way ANOVA with pairwise comparisons).* ***B*** *FAA for new feeding time (ZT9-10) where day 1 is first day of ZT9-10 feeding. Young rats showed an increase in FAA by day 3 and aged rats by day 4. Young rats showed greater FAA than aged rats at day 2 and 6 ($p< 0.05$, RM two-way ANOVA with pairwise comparisons).* ***C*** *An average of the final 3 days of ZT9-10 feeding. There was no difference between groups ($p > 0.05$). Bars are mean ± SEM with data points overlaid. Young n = 8, aged n = 7. *$p< 0.05$, ** $p< 0.01$, ***$p<0.001$, #$p<0.05$, ##$p<0.01$, ###$p<0.001$ (*between subject, #within-subject), FAA-feeding anticipatory activity.*

### 5.3.2 Aged rats show dysregulated light vs dark activity on a ZT9-10 schedule of feeding.

Analysis of total light vs dark activity using the final 3 days of the ZT2-3 feeding schedule showed both the young rats and the aged rats show greater activity in the dark phase compared to the light phase ($t_{(7)} = 4.061$, $p = 0.005$ and $t_{(6)} = 4.689$, $p = 0.003$) (**fig.4.A&B**). Analysis of the first 3 days on this schedule was not possible as this feeding regime began at Eli Lilly.

A comparison between light versus dark activity in the first 3 days of the new ZT9-10 feeding schedule and the final 3 days was performed. In young rats, there was a main effect of light phase on activity ($F_{(1,7)} = 10.456$, $p = 0.014$). Post-hoc analysis revealed there was no difference in activity

in the light phase vs the dark phase at day1-3 ($p > 0.05$) but there was by day 12-14 ($p = 0.002$). There was no main effect of day, but there was a light phase* day interaction ($F_{(1,7)} = 75.855$, $p < 0.0001$). Light phase activity reduced from day 1-3 to day 12-14 ($p = 0.004$) and dark phase activity increased ($p < 0.0001$) (**fig.4.C**). In aged rats, there was also a main effect of light phase ($F_{(1,6)} = 9$, $p = 0.024$). There was greater activity in the light phase compared to the dark phase at day 1-3 ($p = 0.006$) but was equal at day 12-14 ($p > 0.05$). There was no effect of day but there was a light phase* day interaction ($F(1,6) = 17.008$, $p = 0.006$). Light phase activity decreased from day 1-3 to day 12-14 ($p = 0.013$) but dark phase activity did not ($p > 0.05$) (**fig.4.D**).

The same comparison was also made under an *ad lib* schedule of feeding. In young rats, there was a main effect of light phase ($F_{(1,7)} = 57.899$, $p < 0.0001$). Post-hoc analysis revealed that activity was greater in the dark phase compared to the light phase over both sets of days ($p = 0.002$ and $p < 0.0001$ respectively). There was no main effect of day but there was a light phase*day interaction ($F_{(1,7)} = 66.832$, $p < 0.0001$). Light phase activity reduced from day 1-3 to day 12-14 and dark phase activity increased ($p < 0.0001$ and $p = 0.001$ respectively) (**fig.4.E**). In aged rats there was a main effect of light phase ($F_{(1,6)} = 74.104$, $p < 0.0001$). Dark phase activity was greater in the dark phase compared to the light phase at both day 1-3 and day 12-14 ($p = 0.001$ and $p < 0.0001$ respectively). There was no main effect of day but there was a light phase*day interaction ($F_{(1,6)} = 72.964$, $p = 0.00014$). Light phase activity reduced, and dark phase activity increased from day 1-3 to day 12-14 ($p = 0.001$ and $p = 0.003$ respectively (**fig.4.F**).

**Figure 4**

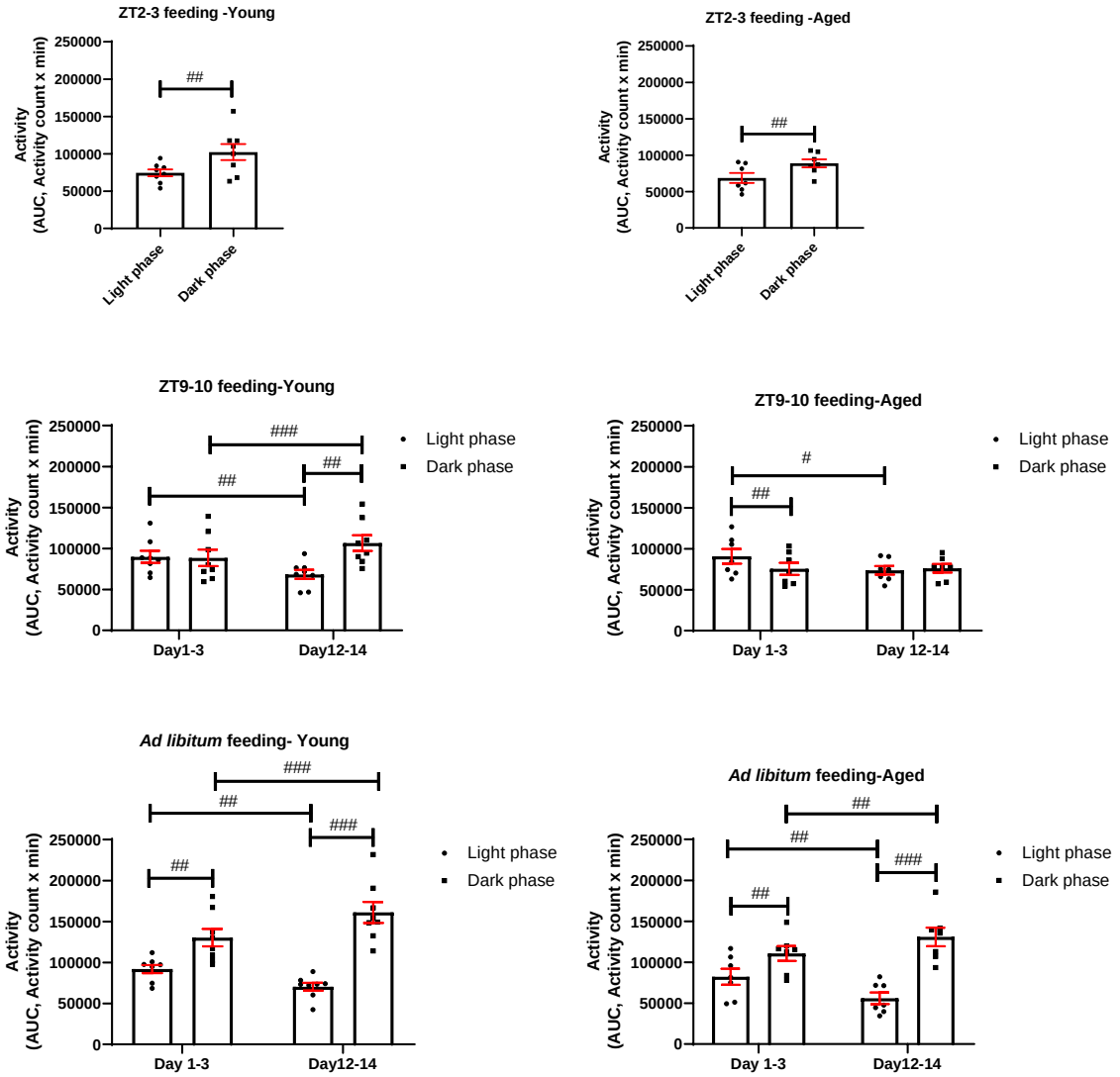


***Fig.4.Aged rats show dysregulated light-dark activity under a ZT9-10 schedule of feeding.*** *Rats were fed at ZT2-3, ZT9-10 and ad libitum in 2-week periods. Total activity in light/dark was compared.* ***A&B*** *Total light vs dark phase activity in young and aged rats respectively. Both groups showed greater activity in the light compared to the dark phase ($p < 0.01$, independent t-test).* ***C*** *Light vs dark activity and the beginning and end of the ZT9-10 feeding period in young rats. At day 1-3 there is no difference in light vs dark activity but by day 12-14 dark phase activity is greater ($p< 0.01$, RM two-way ANOVA with pairwise comparison).* ***D*** *In aged rats, light activity is greater than dark activity at day 1-3 and becomes equivalent to dark phase activity at day 12-14.* ***E*** *Light vs dark activity and the beginning and end of the ad lib feeding period in young rats. By day 12-14, dark phase activity increased, and light phase activity decreased ($p < 0.01$, RM two-way ANOVA with pairwise comparison)* ***F*** *and in aged rats. Bars are mean ± SEM with data points overlaid. N = 8 young rats, n = 7 aged rats. #p<0.05, ##p<0.01, ###p<0.001.*

### 5.3.3 ZT9-10 feeding weakens period amplitude in rats.

A chi-squared periodogram analysis was conducted using 14 days of activity over each feeding condition. Most rats show a period length of 1440 mins (24hrs) (**fig.5.B)**. Analysis of the effect of feeding time on period amplitude revealed a main effect of feeding time ($F_{(2,26)} = 11.71$, $p < 0.0001$, but no feeding time* age interaction or effect of age ($p > 0.05$). Post-hoc pairwise comparison showed that in both young and aged rats, ZT9-10 feeding weakened period amplitude compared to *ad libitum* feeding ($p = 0.019$ and $p = 0.018$ respectively) (**fig.5.C**).

**Figure 5**

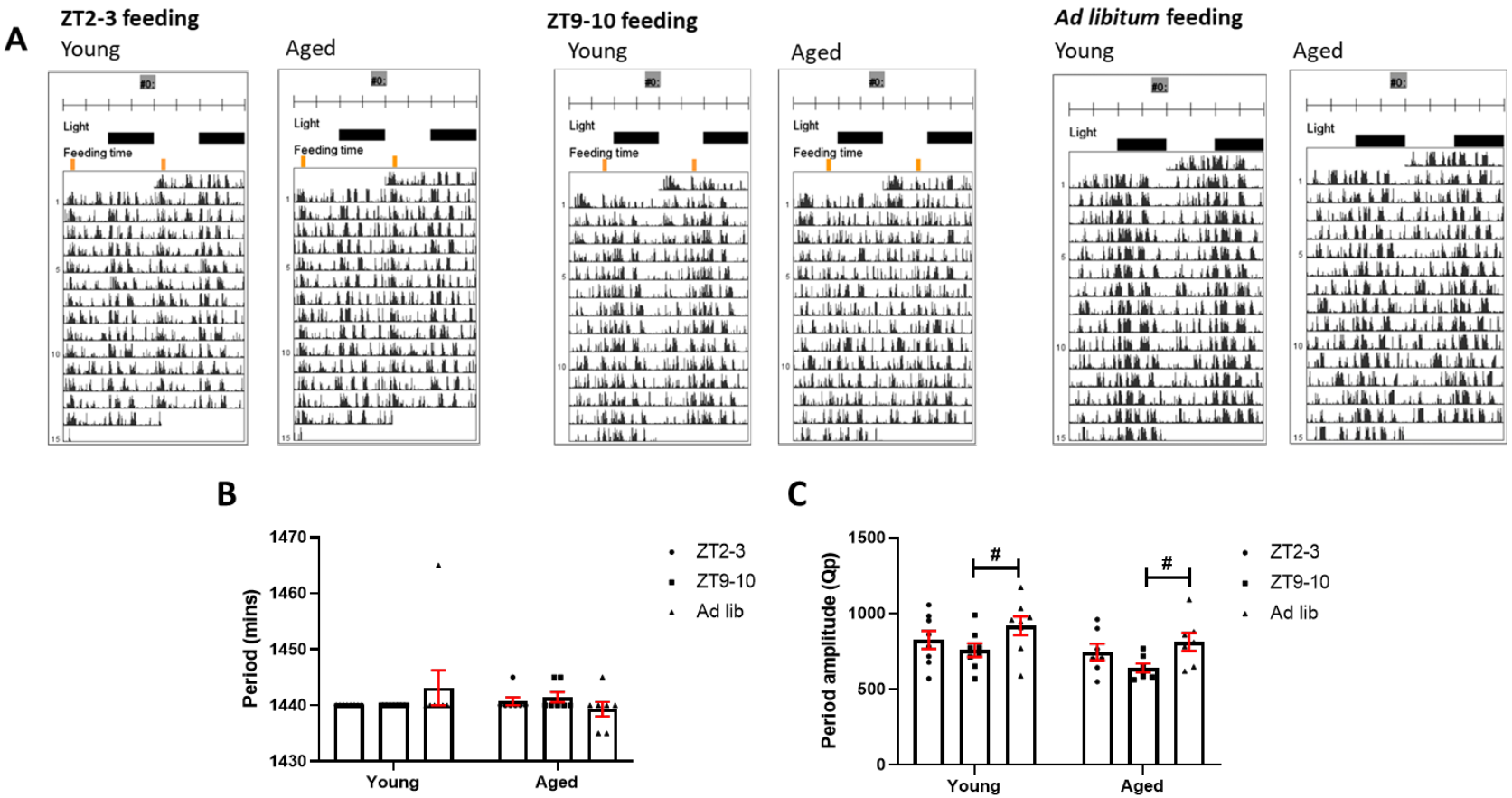


***Fig.5. ZT9-10 feeding weakens period amplitude compared to ad libitum feeding.*** *Rats were fed at ZT2-3, ZT9-10 and ad libitum over successive 2-week periods.* ***A*** *Double-plotted actograms show activity of one rat per group over feeding period. Black bars indicate dark period and orange bars indicate feeding time.* ***B*** *Visual representation of period length during feeding conditions.* ***C*** *Both young and aged rats showed a weaker period amplitude at ZT9-10 compared to ad libitum feeding ($p < 0.05$, RM two-way ANOVA with pairwise comparisons). Bars are mean ± SEM with data points overlaid. Young rats n = 8, aged rats n = 7. #$p<0.05$ (within-subject).*

### 5.3.4 Feeding time impacts pre- and post-lights off cage activity differently in young and aged rats.

Analysis of AUC activity at ZT11-12 under different feeding times and between young and aged rats revealed a main effect of feeding time ($F_{(2,26)} = 10.276$, $p = 0.001$) and feeding time*age group interaction ($F_{(2,26)} = 7.947$, $p = 0.002$) but no main effect of age group, though there was a trend ($F_{(1,13)} = 3.64$, $p = 0.079$). Post-hoc pairwise comparison revealed that younger rats showed greater activity at ZT11-12 under ZT9-10 feeding time compared to aged rats ($p < 0.0001$). There was a trend towards greater activity in the young rats under *ad libitum* feeding ($p = 0.067$) but no difference under ZT2-3 feeding time ($p > 0.05$). In young rats, activity was higher at ZT11-12 under ZT9-10 and *ad libitum* feeding conditions compared to ZT2-3 feeding conditions ($p < 0.0001$ and $p = 0.001$). However, feeding condition had no effect on activity at ZT11-12 in aged rats ($p > 0.05$) **(fig.6.D)**. Analysis of activity at ZT12-14 under different feeding conditions and between young and aged rats showed a main effect of feeding time ($F_{(2,26)} = 22.748$, $p < 0.0001$). There was no feeding time*group interaction or effect of group, though there was a trend ($F_{(1,13)} = 3.653$, $p = 0.078$). Post hoc pairwise comparison revealed that activity at ZT12-14 was higher under *ad libitum* feeding conditions compared to ZT2-3 ($p = 0.014$ and $p = 0.001$) and ZT9-10 ($p = 0.001$ and $p < 0.0001$) in both young and aged rats respectively (**fig.6.E**).

**Figure 6**

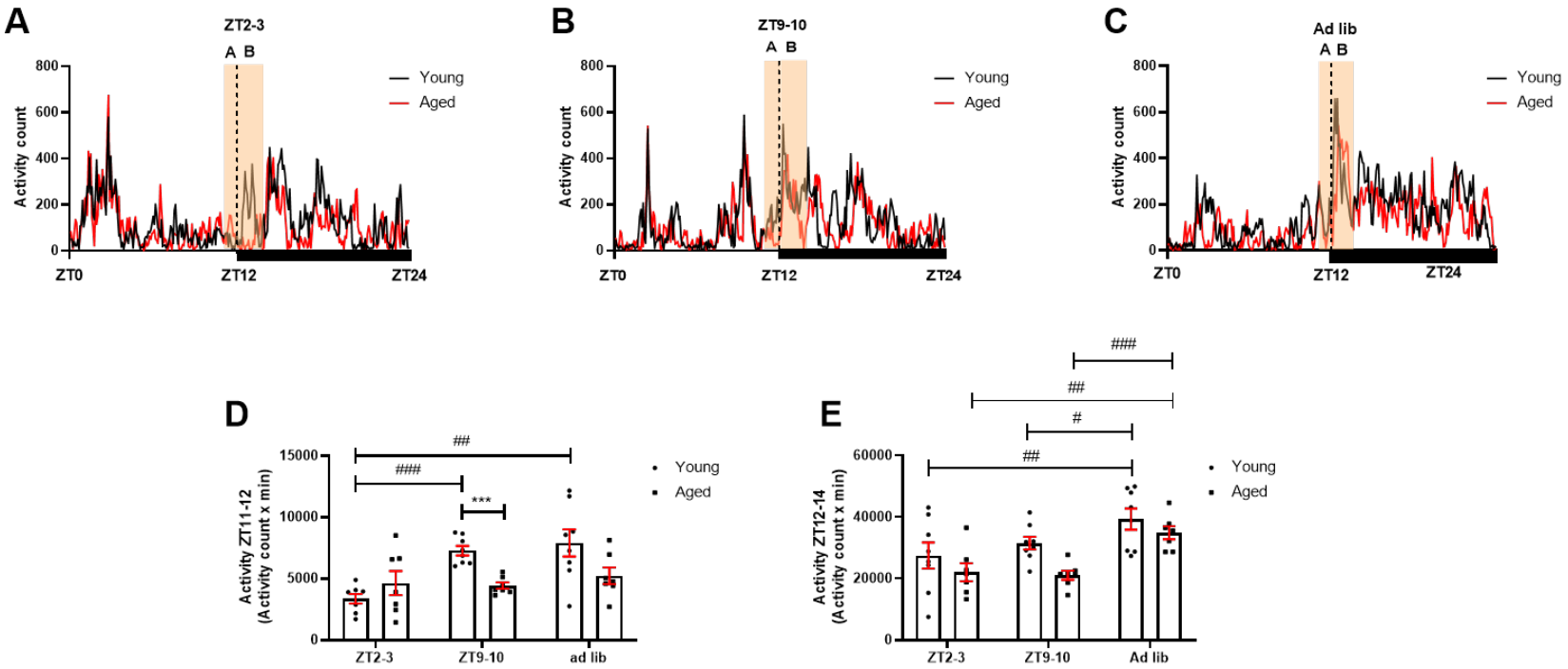


***Fig.6. Differing effects of feeding time on pre- and post-lights off activity in aged and young rats.*** *Rats were fed at ZT2-3, ZT9-10 and ad libitum for successive 2-week periods.* ***A*** *Example 24hr activity profile during ZT2-3 feeding with period of interest in shaded boxes with 'A' = ZT11-12 and 'B' = ZT12-14.* ***B*** *ZT9-10,* ***C*** *ad libitum.* ***D*** *Activity pre-lights off. Pre-lights off activity is higher under ZT9-10 and ad libitum feeding conditions compared to ZT2-3 in young but not aged rats. Pre-lights off activity is higher in young compared to aged rats (p< 0.05, RM two-way ANOVA with pairwise comparisons).* ***E*** *Post-lights off activity is higher under ad libitum feeding in both young and aged rats (p < 0.05, RM two-way ANOVA with pairwise comparisons). Bars are mean ± SEM with data points overlaid. Young n= 8, aged n = 7. ***p<0.001, #p< 0.05, ##p<0.01, ###p<0.001 (*between subject, #within subject).*

### 5.3.5 Rats do not show anticipatory activity for social play.

Change in AUC activity 1hr preceding timed social play was analysed across a 7-day period. Across this period one young rat and one aged rat had one data outlier. The identified outliers were replaced with the group mean. There was no main effect of day on AUC activity ($p > 0.05$). There was a day*age group interaction ($F_{(6,75)} = 2.418$, $p = 0.034$) but no main effect of age group ($p > 0.05$). Post-hoc pairwise comparison revealed younger rats showed greater AUC activity on day 2 compared to older rats ($p = 0.013$) and trended towards showing greater activity on days 6 and 7 ($p = 0.073$ and $p = 0.06$ respectively) (**fig.7.B**). Activity during the period of social play was additionally analysed across a 7-day period. One young rat was excluded due to having 2 data outliers within the 7-day period. There was a main effect of day ($F_{(6,72)} = 9.150$, $p < 0.0001$). There was no day*group interaction ($p > 0.05$) but there was a main effect of group ($F_{(1,12)} = 9.528$, $p = 0.009$). Post-hoc pairwise comparisons revealed younger rats showed greater activity during social play on days 1-6 ($p \leq 0.031$) but this difference was lost on day 7 ($p > 0.05$). A single pairwise comparison comparing activity within-subject revealed reduced activity at day 7 vs 1 in young rats ($p = 0.012$) but not aged rats ($p > 0.05$) (**fig.7.C**).

**Figure 7**

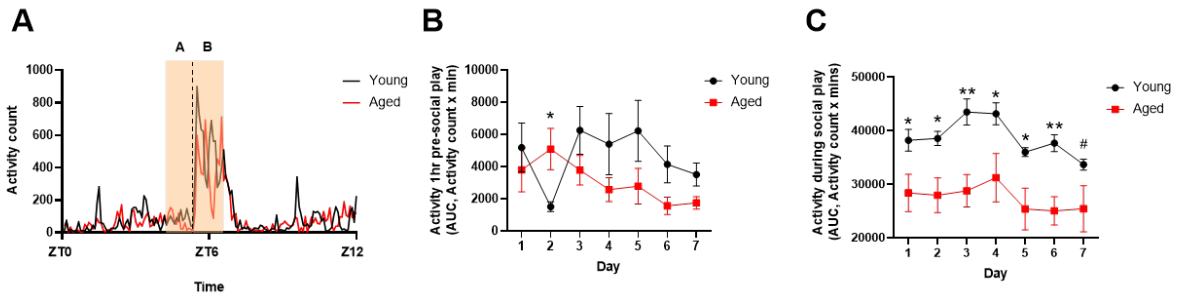


***Fig.7. Rats do not show anticipatory activity for timed social play over a 7-day period.*** *Rats were placed in a ball pit in groups of 4 every day at the same time for 7 days.* ***A*** *Example profile of activity over a 12h period, with activity 1hr pre-social play depicted as A and activity during social play as B.* ***B*** *Activity 1hr preceding social play. There was no effect of day on activity preceding social play ($p > 0.05$). Aged rats showed greater activity than young rats on day 2 ($p > 0.05$, RM two-way ANOVA with pairwise comparisons).**C*** *Activity during social play. Young rats showed greater activity during social play than aged rats ($p < 0.05$, RM two-way ANOVA with pairwise comparisons). Bars are mean ± SEM, young n = 7-8, aged n = 7, *p< 0.05, **p<0.01, ***p<0.001 (between-subject).*

**Table 1**

| | **ZT2-3 feeding** | | | | |
|---|---|---|---|---|---|
| | Normal LD activity? **Start** | Normal LD activity? **End** | Period amplitude (vs *ad libitum* feeding) | ZT11-12 (vs *ad libitum* feeding) | ZT12-14 (vs *ad libitum* feeding) |
| **Young** | N/A | Y | No difference | Reduced | Reduced |
| **Aged** | N/A | Y | No difference | No difference | Reduced |
| | **ZT9-10 feeding** | | | | |
| | Normal LD activity? **Start** | Normal LD activity? **End** | Period amplitude (vs *ad libitum* feeding) | ZT11-12 (vs *ad libitum* feeding) | ZT12-14 (vs *ad libitum* feeding) |
| **Young** | N | Y | Reduced | Reduced | Reduced |
| **Aged** | N | N | Reduced | No difference | Reduced |
| | ***Ad libitum* feeding** | | | | |
| | Normal LD activity? **Start** | Normal LD activity? **End** | Period amplitude (vs *ad libitum* feeding) | ZT11-12 (vs *ad libitum* feeding) | ZT12-14 (vs *ad libitum* feeding) |
| **Young** | Y | Y | N/A | N/A | N/A |
| **Aged** | Y | Y | N/A | N/A | N/A |
| | **Evidence for AA?** | | | | |
| | | Feeding | | Social play | |
| **Young** | | Y | | N | |
| **Aged** | | Y | | N | |

***Table 1. Rat data summary.*** *AA-anticipatory activity, N- no, Y-yes, ZT-zeitgeber time.*

### 5.3.6 Aged mice show reduced post-lights off activity and have a weaker 24 hr rhythm.

An average value over 21 days of recording for each animal was taken (**fig.8B&C**). Analysis of the total light phase activity and dark phase activity between age groups showed a main effect of light phase ($F_{(1,29)} = 118.268$, $p < 0.0001$). Post hoc analysis revealed that dark phase activity was higher than light phase activity in all three age groups ($p < 0.0001$). There was also a light phase*age group interaction ($F_{(2,29)} = 3.686$, $p = 0.037$) but post-hoc analysis revealed no difference between groups in either the light or dark phase ($p > 0.05$). There was also no main effect of age group ($p > 0.05$) (**fig.8D**). Analysis of activity between ZT11-12 showed no difference in activity between groups ($p > 0.05$) (**fig.8E**). Analysis of ZT12-13 showed a main effect of age group ($F_{(2,30)} = 8.04$, $p = 0.002$), where young mice showed greater activity than the other age groups (7mo vs 16 mo $p = 0.003$, 7 mo vs 22 mo $p = 0.01$, 16 mo vs 22 mo $p > 0.05$) (**fig.8F**). Analysis of activity between ZT23-24 showed no effect of age group ($p > 0.05$) (**fig.8G**). Chi-squared periodogram analysis of 21 days of activity (**fig.8H**) showed that younger mice have a stronger rhythm amplitude (Qp) compared to the older age groups ($F_{(2,31)} = 6.56$, $p = 0.004$. 7 mo vs 16 mo $p = 0.022$, 7 mo vs 22 mo $p = 0.006$, 16 mo vs 22 mo, $p > 0.05$) (**fig.8I**). Periodogram analysis also showed all age groups showed a rhythm of approximately 24hrs (7 mo 1440 mins ± 0.23, 16 mo 1441 ± 0.25 and 22 mo 1440 ± 0.27 (mean ± SEM)) (**fig.8J**).

**Figure 8**

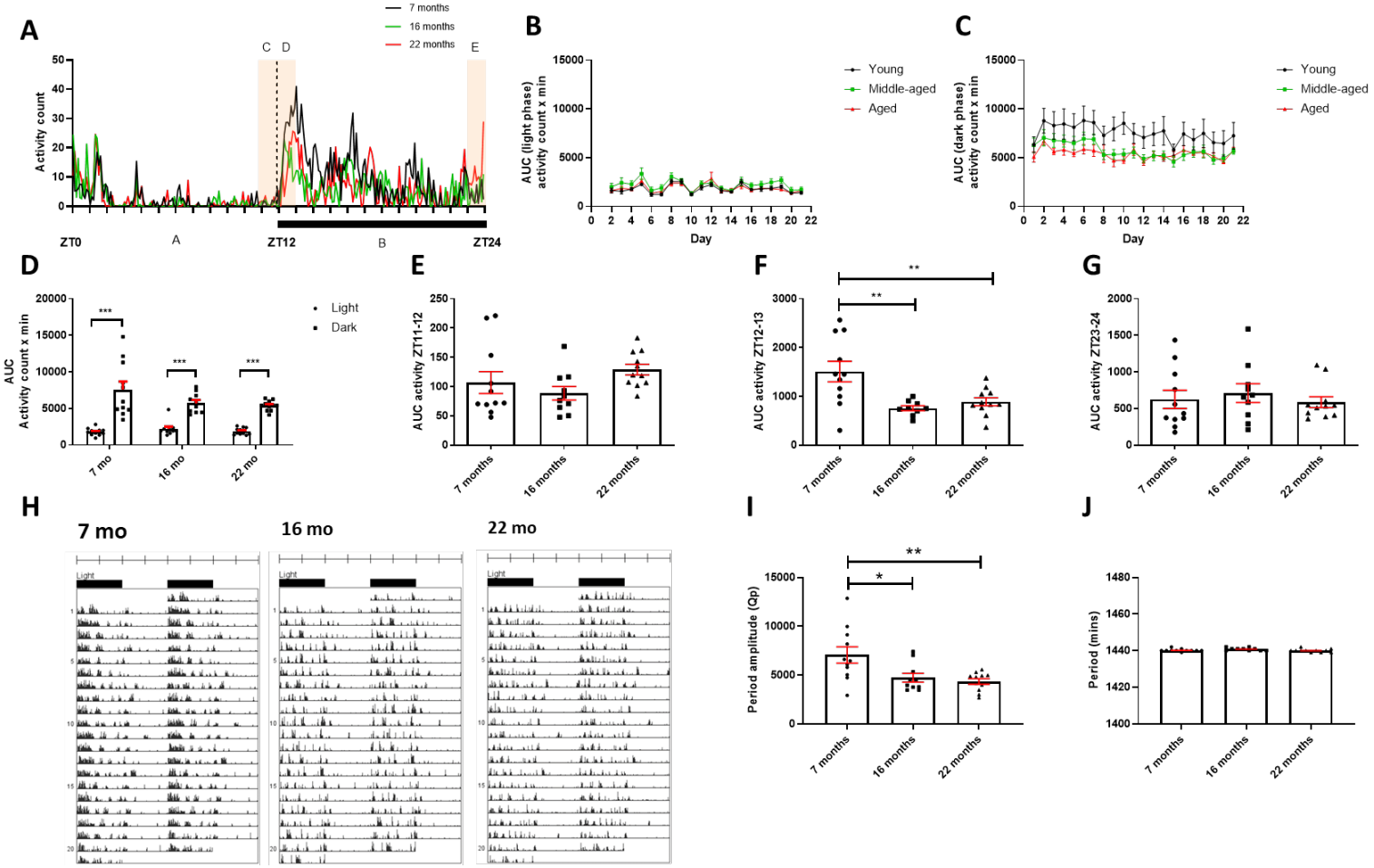


***Fig.8. Aged mice show reduced post-lights off activity and period amplitude in standard lighting conditions.*** *Activity in 3 age groups of mice was recorded over 21 days using a passive infrared sensor.* ***A*** *Example 24hr trace with shaded areas showing time periods of interest- total light phase (A), total dark phase (B), ZT11-12 (C) ZT12-13 (D) and ZT23-24 (E). The black bar indicates the dark phase.* ***B&C*** *Total AUC activity in the light phase (A) and dark phase (B) over 21 days of recording.* ***D*** *Total light phase vs dark phase activity. All age groups show greater dark phase activity than light phase activity*

*(p < 0.001, RM two-way ANOVA with pairwise comparisons).* ***E*** *Age comparison of pre-lights off activity. There was no difference between groups (p < 0.05, one-way ANOVA).* ***F*** *Age comparison of post-lights off activity. Young mice showed greater activity than older mice immediately after post-lights off (p < 0.01, one-way ANOVA with Tukey's post hoc analysis).* ***G*** *Age comparison of activity during ZT23-24. There was no difference between groups (p > 0.05).* ***H*** *Double-plotted actograms depicting 21 days of activity in each age group.* ***I*** *Younger mice showed a greater period amplitude than older mice (p < 0.05, one-way ANOVA with Tukey's post-hoc analysis).* ***J*** *All age groups showed a period length of 1440 mins (24hrs). Bars are mean ± SEM with data points overlaid. Young n = 11, middle-aged n = 10 and aged n = 11. *p< 0.05, **p< 0.01, ***p<0.001 (between subject).*

### 5.3.7 Mice do not show anticipatory activity for timed delivery of reward.

Mice did not show any change in activity in the hour preceding reward delivery over 14 days and there was no age*day interaction or main effect of age group ($p > 0.05$) (**fig.9.B**). Day 4 was excluded due to an unusually high level of activity unrelated to the experiment. Analysis of activity directly after reward was delivered showed a main effect of day on activity 30 mins post-reward delivery ($F_{(13,351)} = 13.999$, $p < 0.0001$). Post-hoc pairwise comparisons showed that younger mice had reduced activity on days 8, 11 and 12 versus 1 ($p \leq 0.014$) and middle-aged mice showed reduced activity on day 12 versus 1 ($p = 0.028$). There was no age*day interaction or main effect of age ($p > 0.05$) (**fig.9.C**). Analysis of activity directly after reward was delivered versus activity directly after experimenter enters the room without delivering reward showed a main effect of reward delivery ($F_{(1,26)} = 57.687$, $p < 0.0001$). Post hoc pairwise comparisons showed young, middle-aged and aged mice showed greater activity when reward was delivered ($p< 0.0001$, $p = 0.0003$ and $p = 0.0003$ respectively). There was no age*day interaction or main effect of age group (**fig.9.D**).

**Figure 9**

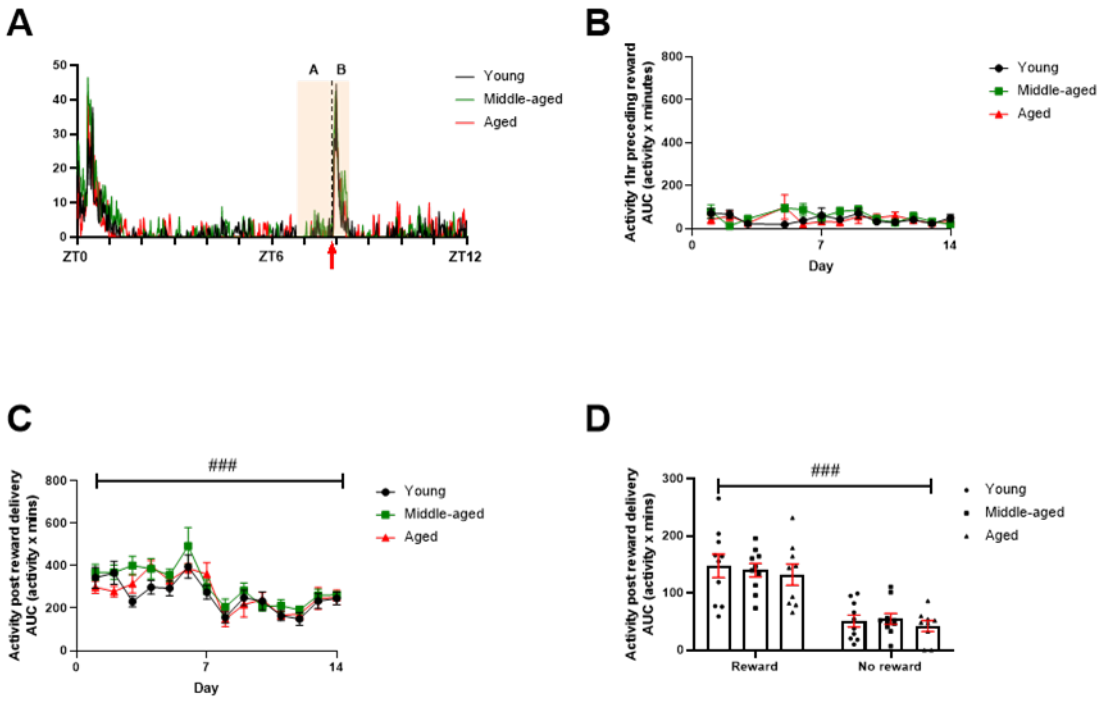


***Fig.9. Mice do not develop anticipatory activity for timed delivery of reward while under ad libitum feeding.*** *Mice were given 5 reward pellets at the same time every day for 14 days.* ***A*** *depicts a 24hr trace with shaded areas showing time periods of interest- A, 1hr pre reward delivery and B- 30 mins post reward delivery. Red arrow indicates when reward was delivered.* ***B*** *Activity 1hr before reward delivery. There was no effect of day or age on activity (p>0.05, RM two-way ANOVA).*

***C** Activity 30 mins post reward delivery. Activity changed over time but there was no effect of age (p<0.001, RM two-way ANOVA). **D** Comparison of activity 30 mins post reward delivery vs no reward. All age groups shower greater activity during reward delivery than without (p< 0.0001, RM two-way ANOVA). Bars are mean ± SEM with data points overlaid. Young n = 11, middle-aged n = 10 and aged n = 9. ###p<0.001 (within-subject).*

**5.3.8 Aged mice entrain to a new light schedule but take longer to restore normal light-dark activity.** Following a shift in light cycle by 12h, analysis of total activity in the new light phase over 12 days shows activity decreases over time in all age groups. There was a main effect of day ($F_{(4.188, 104.694)} = 88.486$, $p < 0.0001$) but there was no day*age group interaction or effect of age ($p > 0.05$) (**fig.10.A**). Analysis of total activity in the new dark phase (old light phase) shows activity increased over time in all age groups. First day of activity in the dark phase was excluded due to disruption associated with moving mice to a new room. There was a main effect of day ($F_{(4.870, 121.760)} = 55.234$, $p < 0.0001$). There was also a day*age group interaction ($F_{(9.741, 121.760)} = 2.158$, $p = 0.026$). There was also a trend level effect of age ($F_{(2,25)} = 2.924$, $p = 0.072$). Post-hoc pairwise comparison showed young mice had greater dark phase activity on days 5, 6 and 10 compared to the oldest group ($p = 0.034$, $p = 0.049$ and $p = 0.025$, respectively) (**fig.10.B**). Analysis of light vs dark activity over time was made to determine the point at which dark phase activity became greater than light phase activity in each age group. Dark phase activity became greater than light phase activity at day 6 in young and middle-aged mice, and day 8 in aged mice (**fig.10.C-E**). Analysis of the day at which mice entrained to the new light cycle, scored by two independent experimenters showed no difference in day of re-entrainment between groups, thought there was a trend level effect ($H = 5.641$, $p = 0.0596$) (**fig.10.G**).

**Figure 10**

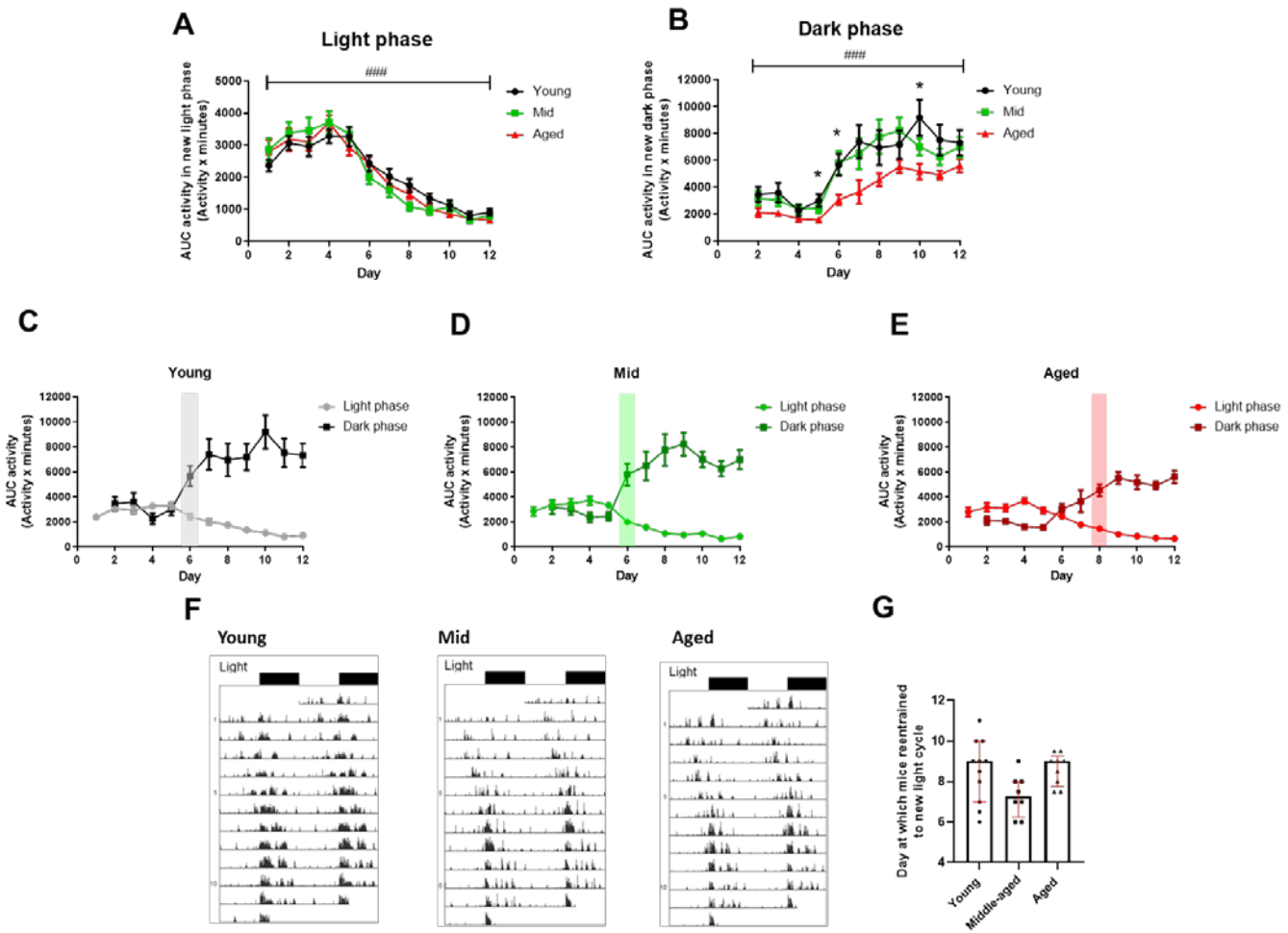


***Fig.10. Aged mice take longer to restore normal light-dark activity but re-entrain to a new light schedule at the same rate as younger mice.*** *Mouse activity was recorded for 12 days following a 12h light cycle shift.* ***A*** *Activity in the new light phase, where day 1 represents first day under new light schedule. Light phase activity decreased with time in all groups ($p < 0.001$, RM two-way ANOVA).* ***B*** *Activity in new dark phase. Activity increased with time in all groups, and young mice showed greater activity than aged mice day 5, 6 and 10 ($p < 0.05$, RM two-way ANOVA with pairwise comparisons).* ***C***

*A comparison of light vs dark activity in the youngest age group* ***D*** *middle-aged group and* ***E*** *oldest age group. Shaded block indicates day at which dark phase activity became greater than light phase activity ($p < 0.05$, RM two-way ANOVA with pairwise comparisons).* ***F*** *Actograms depict activity following a 12h light shift.* ***G*** *There was no difference in re-entrainment score between groups ($p > 0.05$, Kruskal-Wallis test). Bars are median ± interquartile range. Unless otherwise indicated, bars are mean ± SEM with data points overlaid. Young n = 11, middle-aged n = 8, aged n = 9. ###$p < 0.001$ (within subject).*

**Table 2**

| | Activity standard conditions | | | | | Reward delivery | Light shift | |
|---|---|---|---|---|---|---|---|---|
| **Age group** | Normal total LD activity? | ZT11-12 (*vs* 7mo group) | ZT12-14 (*vs* 7mo group) | ZT22-23 (*vs* 7mo group) | Period amplitude (*vs* 7mo group) | Evidence for AA? | Day normal LD was restored | Day of re-entrainment (*vs* 7mo group) |
| **7 months** | Y | N/A | N/A | N/A | N/A | N | 6 | N/A |
| **16 months** | Y | No difference | Reduced | No difference | Reduced | N | 6 | No difference |
| **22 months** | Y | No difference | Reduced | No difference | Reduced | N | 8 | No difference |

***Table 2. Mouse data summary.*** *LD-light-dark, N- no, Y-yes, ZT-zeitgeber time.*

## 5.4 Discussion

Aged rats showed differences in the gain and loss of FAA, but by the end of the feeding period showed normal FAA, consistent with previous reports. Feeding at ZT9-10 weakened period amplitude in both age groups but disrupted normal LD activity in aged rats only. Light anticipatory activity was sensitive to feeding time in young but not aged rats, and post-light activity was blunted under food restriction in both young and aged rats. While overall levels of light and dark activity were similar across three age groups of mice, activity immediately following lights off was blunted in the older group. Further, the older groups showed a blunted rhythm amplitude, and, while aged mice re-entrained to a new light cycle at the same rate as the younger group, it took longer to restore normal LD activity. Rodents did not show AA for delivery of palatable reward or social play. The following discussion considers how together these results may relate to age-related changes in circadian flexibility.

### 5.4.1 Young and aged rats show feeding anticipatory activity under a daily feeding schedule.

There is a wealth of evidence supporting the induction of FAA 2-3 hrs before food is made available when it is presented every 24 hrs (Storch and Weitz, 2009, Mistlberger, 1994, Mistlberger et al., 2012) and as such, an interval starting 2hrs preceding feeding time can be used to study changes in FAA (Martini et al., 2019). Both age groups show increasing levels of activity in the 2hrs preceding ZT9-10 feeding time over 6 days, with young rats showing increased levels of activity compared to the first day of feeding by day 3 and aged rats by day 4. This increase in activity over time suggests that the rats are exhibiting FAA. While this slight delay in the aged group suggests they are slower to build FAA than younger rats, by the end of the two-week period both age groups showed equivalent levels of FAA. This suggests aged rats show no impairment in anticipation of food delivery when given enough time to adjust. This finding is in line with previous work which demonstrates aged rodents show normal FAA (Mistlberger et al., 1990, Tahara et al., 2017, Walcott and Tate, 1996). Of note, the aged group showed a spike in FAA for ZT2-3 feeding, 3 days after feeding time was switched to ZT9-10. The reason for this is unclear but suggests that FAA persists more powerfully in aged rats when feeding time is switched. This persistence in activity for the old feeding time and delay in building activity for the new feeding time may be driven by a reduction in ability to rapidly adjust behaviour to account for changes in zeitgeber timing. This therefore suggests aged rats show a reduction in circadian flexibility. This spike in activity also suggests feeding time may be a more powerful entrainer of behaviour in aged compared to young rats as it suggests the feeding entrainable oscillator (FEO) is self-oscillating more powerfully in the absence of the zeitgeber.

Studies typically assess FAA under constant conditions, which in the context of feeding, means removing food completely for at least 24 hours (García-Gaytán et al., 2020). This is stressful to the

animal, may change normal home cage behaviour and does not allow for a meaningful time course to be analysed due to the restricted number of days a rodent can live without food. As an alternative, we shifted the feeding time so that FAA loss and gain could simultaneously be assessed without the need to remove food completely. This data demonstrates that the gain and loss of FAA can be observed using this method. Furthermore, studies investigating FAA often provide food *ad libitum* for a set time window of several hours and then remove it (Storch and Weitz, 2009, Takasu et al., 2012, Patton and Mistlberger, 2013). Restricting food availability to a narrow window can induce bingeing behaviour. This could potentially mean FAA is only induced by an extreme eating behaviour and is not reflective of normal eating behaviour. Importantly, this work shows that feeding a restricted amount of food at the same time of day can induce FAA in a way that is potentially healthier to the rodent.

### 5.4.2 Food restriction impacts the behavioural response to light change differently in young versus aged rodents.

As described above, daily rhythms can be flexibly aligned to the light-dark cycle, such that nocturnal activity can occur while also maximising chances of obtaining resources such as food. Assessing how feeding impacts normal LD activity may provide an indication of circadian flexibility as well as the relative strengths of competing zeitgebers.

As demonstrated in the previous chapter, feeding in the light phase can have a profound impact on normal nocturnal activity. In this study, both groups of rats showed normal LD activity while fed at ZT2-3, which is at odds with the previous chapter where, by day 11, aged rats fed between ZT1-4 showed inverted LD activity. This may be due to several different reasons; confining timing of feeding to a narrower window of time may have also restricted the activity changes surrounding it. Or it may be driven by the change in home cage conditions, where the rats in this chapter were group housed while the rats in the previous chapter were singly housed. In the single housing context, the rodent has more room to move around the home cage and with greater distance available to cover, an activity deficit in the aged animal may become more pronounced. The presence of cage mates may also alter general activity, where social interaction may drive some behaviour.

When feeding time was shifted by 7 hours to ZT9-10, LD phase activity became disrupted in the first few days of the new feeding period in both age groups, with young rats showing equivalent levels of activity in the light and dark phase, and aged rats showing a more profound disruption with greater levels of activity in the light phase versus the dark phase. An average of the first and last 3 days of light versus dark phase activity in each feeding period was used because clock gene phase and physiological changes associated with restricted feeding begin after several days of the regimen (Damiola et al., 2000). This analysis therefore provides an insight into activity changes

before and after clock gene and physiology has adjusted. Therefore, this initial disruption in LD activity is likely because behavioural adaptation to the shift in zeitgeber timing had not yet begun. At the end of the feeding period, young rats showed restored normal LD activity, while aged rats showed equal levels of light and dark phase activity. This suggests that over the two-week period young rats showed behavioural adaptation to manage both the feeding and light zeitgeber while aged rats did not. This finding adds to data in the previous chapter suggesting that feeding time is a stronger driver of activity than light change in aged rats and suggests aged rats show a reduction in circadian flexibility. As aged rats showed normal LD activity under the ZT2-3 feeding condition it suggests that timing is important in this disruption. ZT9-10 is in closer proximity to lights off, which may increase competition between the two zeitgebers. This is evidenced by ZT9-10 weakening period amplitude in both age groups.

Rodents typically show a rise in activity in anticipation around an hour before lights off (Bains et al., 2016, Loos et al., 2014). The younger group show a blunting in pre-light activity under ZT2-3 feeding conditions compared to ZT9-10 and *ad libitum* feeding, indicating a restructuring of behaviour around the two zeitgebers. However, feeding time had no impact on pre-light activity in aged rats. This suggests that aged rats show limited circadian flexibility in adjusting anticipatory activity (AA) to two zeitgebers. Alternatively, aged rats may not show sufficient light-induced AA that is sensitive to changes in feeding time, particularly as there is a trend towards lower pre-light activity under *ad libitum* conditions compared to younger rats. In addition to a rise in pre-light activity, rodents also show a burst in activity directly after lights off which can persist for several hours (Matikainen-Ankney et al., 2021). Feeding at ZT2-3 and ZT9-10 reduces this activity relative to *ad libitum* feeding conditions in both young and aged rats, which suggests that feeding in the light phase blunts the behavioural response to lights off and suggests that normal post-lights off activity may be in some part be driven by foraging for food.

When put on *ad libitum* feeding, both groups showed normal LD activity, even within the first 3 days. As discussed in the previous chapter, it has been reported FAA immediately disappears during *ad libitum* feeding (Pendergast and Yamazaki, 2018). Equivalent levels of activity in the dark phase between age groups is at odds with reports in the literature (Hood and Amir, 2017) and the previous chapter which report a blunting in activity amplitude under *ad libitum* feeding. This difference may be driven by housing conditions as described above, or alternatively methods of activity assessment in the home cage. For example, many studies use wheel running as a measure of activity. Wheel running may reflect behaviours beyond general home cage activity as it is thought to have a rewarding component (Greenwood et al., 2011) and potentially be more effortful. As such, these studies may have picked up a behavioural rather than a circadian deficit. Even when putting this potential rewarding confound aside, the use of running wheels has been found to intensify activity levels, with rodents supplied with running wheels showing far greater levels of activity than those

without (Novak et al., 2012). It may be that age-related activity deficits only emerge when rodents can reach higher levels of physical activity.

All age groups of mice showed an activity-rest period of 24hrs under 12:12 LD conditions, consistent with previous reports (Eckel-Mahan and Sassone-Corsi, 2015, Ebihara and Tsuji, 1980), showing that the PIR system can be used to capture activity changes over the LD cycle effectively and non-invasively. While aged mice did not show an overall blunted LD amplitude as previously reported by (Valentinuzzi et al., 1997), aged mice showed blunted activity 2hr post lights off, but equivalent activity preceding lights off (ZT11-12) and preceding lights on (ZT23-24), used as a control. In line with this, aged mice show a weaker activity period amplitude, suggesting that light is a weaker inducer of rhythmicity in aged mice. These findings are consistent with findings from chapter 4 showing that light is a weaker inducer of activity in aged rats. This shows that these findings are consistent across mice and singly housed rats.

In addition to using competing zeitgebers to test potency of the light zeitgeber, shifts in the light cycle can be used, with a faster rate of re-entrainment indicating greater sensitivity to light change. In the present study, there were no differences in number of days to re-entrainment following a 12h light shift between age group. This is at odds with previous work which has shown that older humans show delays in recovering from jet lag (Moline et al., 1992) and aged mice show delays in entraining to a new light cycle (Valentinuzzi et al., 1997, Sellix et al., 2012). It is not clear why the data does not align with these studies. However, work of this nature typically uses short shifts of 4-6h. Potentially, 12h is such a dramatic shift that group differences cannot emerge. Despite equivalent levels of entrainment between groups, it takes longer for the oldest group of mice to restore normal LD activity. While activity in the light phase decreases at a similar level as the younger mice, dark phase activity takes longer to rise. This suggests aged mice show reduced flexibility in adjusting their overall activity in accordance with the light cycle.

### 5.4.3 Rodents do not show anticipatory activity for timed stimuli that isn't food delivery.

To gain a more comprehensive view of the effect of age on the circadian system, activity preceding social play was also examined. As well as feeding, socialisation is an important aspect in both human and rat daily functioning. There is evidence to suggest that timed social interaction, while not as potent as light, can act as a zeitgeber in both humans and animals (Ohta et al., 2002, Mistlberger and Skene, 2004b). However, there is limited evidence for the induction of AA from social interaction. Therefore, it was important to first establish whether social play could induce AA in both young and aged rodents. The present study showed that social play did not induce AA in either age group. This finding contrasts with a previous study which showed that daily timed sexual interaction with female mice induced moderate levels of AA in singly housed male mice (Hsu et al., 2010a). However, this was not seen under timed interaction between two female mice,

suggesting this AA was driven by a sexual rather than a social component. Social interaction while already group-housed in enriched cageing may represent a secondary reward that is not sufficiently motivating to induce AA. In support of this, it has been shown that standard-housed rats showed greater AA for reward than rats housed in enriched cageing, suggesting behavioural needs impact on AA (Spangenberg and Wichman, 2018). The aged rats showed consistently reduced activity during the period of social play across the 7-day period. Ageing is associated with a reduction in social interaction and exploration (Salchner et al., 2004), and therefore could explain this reduction in activity. Of additional importance, no aggression or fighting was observed between rats of different cages in either age group while in the ball pit. As such, we suggest the use of a ball pit as a potential form of social and environmental enrichment for rats housed over long periods of time, particularly those that are singly housed.

Delivery of reward under *ad libitum* feeding conditions was used to test whether an appetitive but secondary reward could induce AA. The ability to induce AA without the need to food restrict could represent a refinement when considering measures of circadian flexibility. However, supporting evidence above that secondary reward does not induce AA, reward pellets delivered every 24 hrs to mice for two weeks did not induce AA. This was not due to mice sleeping and missing the reward pellet delivery as mice showed a clear activity response to the delivery of reward that was not induced when the researcher entered the room but did not deliver a reward. As above, this may be due to the salience of the reward. Mice were fed *ad libitum* and therefore were likely not hungry when the reward was delivered. The literature surrounding AA for palatable reward under *ad libitum* feeding conditions in heterogenous. A study showed rats show AA for reward despite being fed *ad libitum* (Mistlberger and Rusak, 1987). Conversely, a different study showed mice do not reliably show AA for a palatable reward (Hsu et al., 2010b). It has been suggested that AA is sensitive to nutritional value and fat content of the palatable reward, explaining the heterogeneity in studies (Mistlberger and Rusak, 1987). Therefore, the delivery of 5 reward pellets may not be of sufficient nutritional value to induce AA. As activity post reward delivery was lower on some days in the latter half of the experiment, mice may have additionally become less interested in reward delivery over time.

Together, this data suggests that AA may not occur for secondary reward and therefore may not be useful to test circadian flexibility. This suggests that the salience of the signal is important for inducing AA. This also shows that AA for feeding time is not a learned association to a cue but a result of entrainment to a specific zeitgeber.

### 5.4.4 Conclusion

Timed feeding and light change were used to assess circadian flexibility in rodents. Social play and reward delivery were used to investigate whether presentation of secondary reward could induce

AA in the same way as timed feeding. Changes in the gain and loss of FAA, dysregulated LD activity under the ZT9-10 feeding condition and a lack of effect of food restriction on pre-light activity together indicates a reduction in circadian flexibility in aged rats. This data also supports findings from the previous chapter suggesting feeding time is a stronger zeitgeber than light. However, normal LD activity under the ZT2-3 feeding condition and a lack of blunted dark phase activity in the aged rats under the *ad* libitum feeding condition is at odds with findings from the previous chapter. This highlights the potential effects home cage environment and activity recording method can have on experimental outcome. A weaker period amplitude, reduced activity post-lights and longer time to restore normal LD activity following a light shift suggest aged mice also show reduced circadian flexibility and shows loss of sensitivity to the light zeitgeber is consistent across species. This finding could be potentially used to explore the relationship between apathy-related behaviour and biological rhythms and is discussed in more detail in chapter 6. Daily social play in rats and daily reward delivery in mice did not induce AA in the same way restricted feeding did, showing timed presentation of a stimulus does not necessarily lead to AA. This raises important questions about an interaction between the salience of stimuli and the induction of AA and shows behavioural effects of timed feeding are specific to the zeitgeber and not presentation of a cue.

# Chapter 6: General discussion

## 6. General discussion

The two overarching aims of this thesis were to investigate whether aged rodents show apathy-related behaviour across multiple domains, and whether they show disrupted biological rhythms at the physiological, behavioural, and molecular level, with the view that this data could be used to test a relationship between apathy and disrupted biological rhythms in the future. This thesis found that aged mice show a reduction in motivated behaviour and emotional blunting, making them a potential model of apathy-related behaviour. In contrast, aged rats do not show this profile of deficits, indicating a striking species difference in ageing which is important to consider when choosing a model for apathy research. This thesis also shows that circadian CORT is blunted in aged rats, which has important implications for the entrainment of peripheral clocks. Both aged mice and rats show a blunted CORT response to stress, though this appears to be dependent on the stressor. They also show blunted activity in the hours immediately post-lights off, indicating a reduction in sensitivity to the light zeitgeber. Daily feeding in the light phase of rats has a profound impact on their normal LD activity, but this is particularly prominent in aged rats. This suggests feeding time may be a more potent zeitgeber than light in ageing, which could have important therapeutic implications. The following discussion considers how these findings could be used to better understand the underlying neurobiology of human apathy and the relationship between apathy and disrupted biological rhythms in ageing.

### 6.1 The suitability of aged mice as a preclinical model for apathy-related behaviour.

It should first be acknowledged that using natural ageing as a model for apathy is unusual, because it implies that ageing itself is a pathogenic mechanism, or a disease state. This is a subject of longstanding debate (see (Franceschi et al., 2018)). If in this context we accept that it is, and consider ageing at its broadest level, a progressive senescence over time that can, in a way currently unknown, lead to the development of apathy, then its validity as a model can be assessed.

Using pre-validated measures of motivated behaviour and emotional reactivity, data from chapter 2 suggest that aged mice show deficits in behaviours relevant to apathy-related behaviour across multiple domains. This multidimensional approach meant that the phenotype could be more meaningfully integrated across domains and dissociated from the behavioural profile typically seen in depression models, increasing the likelihood that the behaviours observed are specific to apathy-related behaviour. In addition, a multidimensional approach is more reflective of the human condition, where patients must show behaviours in two domains of apathy to be clinically diagnosed (Robert et al., 2018). By showing similarity of behaviours relating to the human pathological state across domains, aged mice show robust ethological validity (for more details see (Belzung and Lemoine, 2011)). As previously discussed in chapter 1, the current approach to assessing apathy behaviour is to quantify motivated behaviour using EBDM tasks, explorative tasks or home cage

observations and fails to consider other dimensions of apathy such as the emotional-affective domain. Compared to other apathy models that test behavioural changes in a single domain, the approach used in this thesis therefore represents an improvement.

It has been argued that a translatable animal model of a psychiatric symptom should fulfil a multidimensional set of criteria of validity (Belzung and Lemoine, 2011). As such, other criteria should also be considered. Mechanistic and predictive validity are important because the testing and development of effective therapeutic agents depends on these. Mechanistic validity refers to the similarity of the mechanism driving disease in the animal model to that driving the disease in a human and should be sensitive to the same therapeutic agents. The assessment of mechanistic validity can be achieved by back-translation to the human condition. However, the underlying neurobiological mechanism of apathy in aged humans remains unknown. Predictive validity refers to the similarity of the effect of a therapeutic agent in an animal model as to that in the clinic. However, there is a lack of consensus on the pharmacological approach to human apathy (Tanaka and Hamaguchi, 2019). Due to limitations in the human literature we therefore heavily depend on an apathy model with good ethological validity that allows us to work backwards to investigate the underlying neurobiology of apathy and address the question of mechanistic and predictive validity in the future. Given its potentially more robust ethological validity, the use of aged mice may be a valuable model with which to address these important questions.

It should be noted that the ethological validity of one apathy model may not extend to another. As previously described, apathy occurs with healthy ageing, neurodegenerative and neuropsychiatric disease but it is not clear whether apathy is driven by a common mechanism. As previously outlined in chapter 1, the mechanistic view of apathy domains posits that each apathy domain results from disruption to distinct neurocircuits (Levy and Dubois, 2006). Given the varying aetiologies of these disease states the presentation of apathy symptom domains may differ between conditions. Like other models of apathy, such as HD and PD mouse models, aged mice show deficits in motivated behaviour, evidenced by a reduction in PR responding and explorative behaviour (Heath et al., 2019, Tanaka and Hamaguchi, 2019, Menalled et al., 2009). However, it is unclear whether these similarities extend to other apathy domains such as emotional blunting, as they have not been tested in these models. Therefore, in addition to improving ethological validity, a multidimensional approach to the study of apathy-related behaviour would benefit comparison between models.

There are some limitations associated with interpreting the ethological validity of aged mice as a model for apathy. Non-specific physiological changes in aged mice can make behavioural interpretation difficult. Where possible, these have been tested, such as appetite differences, overt motoric differences, and pain. In addition, aged rodents were only used if overtly healthy and the study was ended if it was clear that they had reached a decline in health. However, it is difficult to

fully rule out metabolic or motoric differences which may change the drive to work for reward in appetitive tasks. These differences may change effort and reward contingencies, where a change in motoric ability may increase the activity-related cost of obtaining reward, and changes in appetite may devalue the reward. These in turn could lead to a reduction in motivated behaviour and makes it difficult to conclude whether aged mice 'can't' or 'won't'. Data from chapter 2 showed that one aspect of complex motor performance on the raised beam test was sensitive to the effects of anti-inflammatories and suggests aged mice do experience some pain that may influence performance. It is therefore important to select tasks that do not require complex or high-level motor performance, or to demonstrate comparable responses under devalued conditions to show any change in behaviour under higher value reward conditions is not driven by a physical deficit. For example, when reward was devalued by *ad libitum* feeding in the progressive ratio task, aged and young mice showed equivalent levels of responding. To account for potential changes in metabolic demand, tests of motivation to work for non-appetitive reward such as bedding could be used. Nest quality has previously been used to assess apathy behaviour in mice (Cathomas et al., 2015) though this gives limited quantitative output and interpretation can be subjective. It is also important to note that many of the motivational deficits observed in the battery of tests, including exploratory behaviour and operant responding, may also be driven by fatigue, a distinct psychological construct that results in a similar motivational deficit to that induced by apathy. Fatigue can be potentially ruled out by assessing changes in task performance within a session. A more rapid decline in task response over time may indicate fatigue rather than an overall blunting of motivation to respond. In addition, fatigue does not have an emotional component, and so further highlights the importance of taking a multidimensional approach to the preclinical study of apathy. Fatigue may also affect home cage measures of activity, resulting in a reduction in overall activity levels. This could potentially be explored further by assessing bouts of waking inactivity, or periods of inactivity immediately following higher levels of exertion.

An important limitation of the study more generally is that only male mice were used to study apathy-related behaviour. Human studies of age-related apathy in the elderly population consider both sexes and therefore, the inclusion of females would benefit the overall translatability of findings. Sex-differences in motivated behaviour and anxiety have been reported (Song et al., 2018, Kokras and Dalla, 2014) but there is little work on the differential effects of sex on age-related changes in this behaviour, with the exception of measures of exploration where declines in both sexes have been observed (Adelöf et al., 2019).

### 6.2 Species differences in apathy-related behaviour: potential causes and implications for preclinical apathy research.

The striking differences in age-related behavioural changes between species highlights the importance of considering these differences before choosing a rodent model for the study of apathy-related behaviour. The literature surrounding model species differences is relatively small, presumably driven by the assumption that mice and rats are very similar. The reality is rats and mice are very different species and the evidence goes beyond what is reported in this thesis. A study found discrepancies between a HD mouse and rat model, which share the same genetic construct (bacterial artificial construct (BAC)-HD). While BAC-HD mice showed an increase in fear conditioning behaviour (Abada et al., 2013b), BAC-HD rats showed a reduction in the same behaviour (Abada et al., 2013a). This further emphasises the importance of comparing models when attempting to ask a specific question in neuroscience research (Ellenbroek and Youn, 2016). Homological validity in an animal model requires that an adequate species and strain be selected (Belzung and Lemoine, 2011). By demonstrating that aged rats are unsuitable for testing the described domains of apathy (at least at the age tested), it informs which species is a better choice for preclinical apathy research and therefore contributes to homological validity.

There are several potential explanations for this species difference in the behavioural effects of ageing. 1) The role of the aged mouse/rat within the social group, 2) differing basal levels of anxiety between rats and mice or 3) different relative ages between mice and rats. On the first explanation, rats and mice differ considerably in their social dynamics both in the wild and in the laboratory (Lore and Flannelly, 1977) (Schmid-Holmes et al., 2001) (Kummer et al., 2014). As such, the role of the aged rodent within the hierarchy may differ between species. This in turn, has the potential to drive profound age-related behavioural differences between the two species. There is currently no work on the role of the aged rodent within the hierarchy but could provide insight into these behavioural differences. Furthermore, it should be noted that the tests in this thesis only focused on behaviours relevant to the behavioural-cognition and emotional domains of apathy and did not consider the social domain of apathy. It may be that considering the collaborative and complex nature of human social behaviour, and the greater levels of social interaction and hierarchy complexity in rats, that the rat is more appropriate than mice to study the social domain of apathy. Therefore, it may be more pertinent to conclude that it is important to choose the correct rodent model for the apathy domains of interest, not apathy-related behaviour in general.

On the second explanation, it has previously been reported that mice experience more stress and anxiety than rats in the laboratory setting. This is reflected in mouse protocols, which in comparison to the rat, typically require longer habituation to behavioural arenas, and require more sessions to complete training (Ellenbroek and Youn, 2016). It has previously been suggested that mice show greater levels of anxiety that are potentially truer to the wild mouse. This may be due to experimenter selection bias, rather than a true evolutionary difference. The smaller size of the mouse and its reduced potential to inflict serious wounds has resulted in less pressure to remove

defensive behaviours whereas the rat has become more domesticated over time (Blanchard et al., 1997). Differences in task experience induced by fundamental differences in anxiety-related behaviour may drive conflicting age-related behavioural changes between species, across multiple domains of apathy. In this thesis, emotional blunting was assessed by using measures of anxiety behaviour and stress-reactivity, which could be particularly sensitive to these differences. While aged mice showed blunted anxiety behaviour, aged rats showed some evidence of increased anxiety behaviour. As discussed in chapters 2 and 3, these differences in anxiety behaviour also have the potential to affect behaviours relating to other domains, such as exploration of novel objects. Interestingly, aged mice showed blunted stress responsiveness after restraint stress, and aged rats showed blunted stress responsiveness to noise but not restraint stress, suggesting some commonality on the effects of age on stress responsiveness across species, but potentially stressor-dependant.

Finally, there is the potential for differing lifespans between rats and mice to affect study results. As outlined in chapter 1, while survival in laboratory mice drops off markedly at 24 months, laboratory rats are thought to have potentially longer lifespans of 2 - 3.5 years. There is the potential therefore that the aged rats were not 'aged' enough to show evidence of apathy-related behaviour. This could be addressed by testing rats at a more advanced age, however as discussed in chapter 1, it is important to test aged rats when they are in a relatively healthy state to be able to meaningfully interpret behavioural output. If aged rats must be tested at an advanced age where they are more susceptible to non-specific health impairments, then the aged mouse may be the more suitable model going forwards. It should be noted, as pointed out in chapter 3, mice and rats were not tested at precisely the same ages, both were tested in most motivation-based tasks at similar time periods after they are considered 'aged' (4-5 months and 3-4 months in rats and mice respectively). However, 'aged' is a vague term, and the length of aged phase may differ between species.

### 6.3 How could a relationship between apathy and disruption to normal biological rhythms be examined?

As previously outlined in chapter 1 section 3, there is evidence to suggest that motivated and affective behaviour show circadian rhythmicity (Clark et al., 2009, Keith et al., 2013) and disrupted circadian rhythms have been linked to psychiatric and neurodegenerative disease, which apathy commonly occurs with (Germain and Kupfer, 2008, Kuhlmei et al., 2013). Given that previous work suggests healthy aged humans have disrupted circadian rhythms (Hood and Amir, 2017) and higher levels of apathy (Brodaty et al., 2009), a relationship between the two may exist. It has previously been shown that synchronous neuronal firing in the SCN becomes disrupted in old age, which leads to the blunting or fragmentation of circadian rhythms (Nakamura et al., 2016). This may include disruption to the rhythms of dopaminergic activity and affective state, which may, over time, disrupt normal reward processing. Previous reports of a disrupted activity-rest cycle may

reflect these changes in motivated behaviour. This thesis set out to test apathy behaviour in both aged mice and rats and to characterise age-related changes to the circadian system in standard and complex conditions with the view that this data could be used to test a relationship between disrupted biological rhythms and apathy in the future.

To investigate a relationship between the two, the aged rodent would need to show a clear circadian deficit and a readout of apathy-related behaviour. The observed blunted CORT circadian rhythm in aged rats could be a suitable choice for a clear circadian deficit with the potential to be sensitive to therapeutic intervention. For example, rodent models of disrupted CORT regulation such as Cushing's disease have been shown to be sensitive to pharmacological intervention to therapeutic benefit (Yasuda et al., 2019). However, a greater understanding of the mechanism driving this blunted CORT rhythm is required. This was addressed to some degree by assessing circadian expression of key neuronal and receptor populations in the PVN, but results were confounded by the restricted feeding schedule, and, while it provided valuable information about the relative strengths of the light and feeding zeitgebers, it made it difficult to interpret the results in the context of standard conditions. Crucially, a clear behavioural readout of apathy related behaviour in rats was not obtained and therefore it would not be possible to conclude whether treating this deficit has any effect on apathy-related behaviour. This blunting of circadian rhythms but no evidence of apathy-related behaviour has important implications for their relationship. It suggests that either there is no relationship between apathy and disrupted biological rhythms, the behavioural tasks used to obtain an apathy readout were not sensitive enough to reveal a deficit in aged rats or, as suggested above, it may be that aged rats were not aged enough to show apathy behaviour. While circadian deficits were present at the age tested, this may precede onset of apathy-related behaviour, if a relationship between the two does indeed exist. It is unlikely that the behavioural tests used lacked sensitivity, as deficits were observed using the same tests in aged mice. While a clear behavioural readout of apathy was obtained in aged mice, less work was completed on the presence of a circadian deficit beyond the level of activity. Conducting a more comprehensive analysis of circadian changes in aged mice, including that of CORT and core body temperature could provide further clarification on whether a relationship between apathy and disrupted biological rhythms exists, and whether this relationship is species-specific.

In aged mice, a reduction in sensitivity to the light zeitgeber was evidenced by a specific reduction in post-lights off activity, weakened period amplitude and increased time taken to restore normal LD activity following a light shift. The reduction in post-lights off activity is particularly interesting as it has previously been shown that goal-directed behaviour in the home cage, measured by operant responding for reward, is increased in the first few hours after lights off (Matikainen-Ankney et al., 2021). This coincides with the circadian peak in extracellular dopamine levels in the striatum (Ferris et al., 2014). Therefore, there is the potential for this reduction in activity to reflect a reduction in

motivated behaviour and more specifically, accumbal dopamine function. This suggests that a reduction in sensitivity to the light zeitgeber can impact on motivated behaviour and provides an interesting mechanistic link by which the observed deficit in motivated behaviour and circadian rhythms may be linked in aged mice. However, at this point it is purely speculative. There is the potential to integrate these findings to test the relationship between the observed post-lights off activity deficit, the therapeutic potential of timed feeding and apathy-related behaviour. For example, the apathy behavioural battery could be used to test changes in apathy related behaviour following attempts to strengthen or restore circadian rhythm deficit. This could potentially be achieved by restoring a robust activity peak in aged mice by using daily feeding in the dark phase.

There are alternatives or methods that may be complementary to the apathy behavioural battery used in this thesis. While the use of discrete behavioural tasks can provide information on different domains of human apathy, taking the rodent from the home cage where circadian rhythms are tested and placing them in various contexts for relatively short time periods may make it difficult to link changes in circadian rhythmicity with changes in apathy-related behaviour. Home cage activity is driven by the same behavioural processes underlying the tasks used in this thesis, such as foraging, feeding, interacting with conspecifics and exploring, and it is these behaviours that drive the difference between light and dark activity. Adding in a degree of effort to obtain these primary rewards in the home cage and quantifying output may give a more meaningful readout of the effects of circadian disruption on motivated behaviour, particularly as behaviour can be monitored over the 24hr period. This approach may be particularly valuable given the finding of a specific reduction in post lights off activity. The continual development of open-access automated software to measure primary reward intake may drive new measures of motivated behaviour in the home cage. For example, the Feeding Experimentation Device version 3 (FED3) is a recently developed device used to measure food intake and operant responses in the home cage, allowing for motivation for food reward to be measured over the 24hr light cycle (Matikainen-Ankney et al., 2021). This approach could also be adapted to non-appetitive reward, such as willingness to work for bedding. This would be particularly valuable given the potential age-related metabolic confounds described above.

If a relationship between apathy and biological rhythms in the context of old age was established, then circadian variation in reward behaviour could be tested in aged versus young animals by assessing their performance in reward-related tasks across the light cycle. We would hypothesise that young animals would show normal circadian variation, while aged animals would show a blunting or complete loss of variation. Going further, models of disrupted biological rhythms could be generated to probe the underlying mechanism and to test its feasibility as a target for therapeutic intervention. For example, by chronic disruption of sleep, or use of adrenalectomy to disrupt CORT rhythms. This would allow any resulting behavioural effects to be localised to circadian disruption,

independent of other age-related changes. We would hypothesise that a rodent with an induced disrupted sleep-wake cycle would show a disruption in normal circadian variation of reward, and consequently progressively worsening apathy-related behaviour. This apathy-related behaviour would improve when the rat was returned to a normal sleep-wake cycle.

### 6.4 Unmasking age-related circadian deficits: constant or complex conditions?

To investigate deficits in SCN-entrained rhythms, studies often examine activity circadian rhythms under constant darkness. In this way, the self-driving oscillation of the SCN can be studied in the absence of entrainment to zeitgebers. In the study of rodent age-related changes to circadian activity, it has been suggested that rodent SCN-related deficits that affect behavioural output are 'unmasked' by constant conditions (Nakamura et al., 2015) but appear normal in standard LD conditions. If aged rodents, at least at the level of behavioural activity, are relatively normal under an approximation of normal environmental lighting conditions, how translatable are these findings to humans? Most organisms, including elderly humans, spend their time in an environment that cycles through light and dark, with perhaps the exception of hospital stays. Entrainment in the natural environment results from the combined influence of multiple zeitgebers (Edmonds and Adler, 1977), and the findings in this thesis show that by challenging the aged rodent circadian system with multiple zeitgebers, deficits in light-entrained behavioural activity emerge. Therefore, we provide some evidence for a translatable alternative to 'unmasking' age-related deficits in the light-entrained circadian system. Going forward, studies of age-related activity changes to light-entrained circadian rhythms may benefit from analysis in a complex rather than a constant environment.

### 6.5 The potential therapeutic benefit of timed food restriction.

As outlined in previous chapters, changes in light sensitivity or SCN synchronicity are thought to uncouple the SCN from the peripheral clocks and result in age-related phase shifts or blunting in circadian amplitude. Given the feeding zeitgeber entrains rhythms independently of the SCN and is a powerful entrainer of peripheral clocks, time restricted feeding could be used to replace light as the main entrainer of circadian rhythms to therapeutic benefit. Both previous reports (Mistlberger et al., 1990) and data from this thesis shows aged rats can build FAA, indicating that the behavioural output of the feeding entrainable oscillator (FEO) is well conserved. At the level of activity there is evidence to suggest that restricted feeding can restore normal activity rhythms that have become fragmented in aged rodents (Walcott and Tate, 1996) as well as more extreme models of activity fragmentation such as a HD mouse model (Maywood et al., 2010). The therapeutic effects of timed restricted feeding on other biological rhythms are less well studied. However, data from chapter 4 demonstrates the potential profound effect of feeding time on normal core body temperature rhythm suggests that it can powerfully affect other rhythms too. It is important to note that while feeding in chapter 4 & 5 was conducted in the light phase, this was only to reflect the conditions at Eli Lilly

and to visualise the effects of feeding more clearly. Feeding out of phase with other biological rhythms in normal, healthy rodents could have a negative impact on health by inducing metabolic desynchrony and it could be far more beneficial to feed during the active phase (Bray et al., 2013).

More work on the effects of the feeding zeitgeber on the human circadian system is necessary, particularly in the context of ageing. In addition, investigating whether eating habits change in ageing is crucial. In healthy, working adults, while food is technically available "*ad libitum*", daily life imposes temporal windows where food is typically consumed. Breakfast is consumed on waking for work, lunch is consumed in the middle of day, and dinner is consumed at the end of the working day. In retirement (and potentially during lockdown?), it is more likely for this consistent daily eating routine to be lost. Studies investigating the beneficial impact of time restricted eating on sleep in individuals with disrupted eating patterns are beginning to emerge. For example, it has been shown that time restricted eating (10hr window during the day versus >14hr) improved sleep quality in healthy, overweight participants (Gill and Panda, 2015). Daily socialisation may also be beneficial to the aged circadian system, particularly as one third of individuals > 60 years old report feeling lonely (Landeiro et al., 2017). While results from chapter 5 suggest social play does not induce anticipatory activity in rats, it is important to remember that different species may have different requirements for optimal survival. There is the potential for socialisation to be a stronger signal to the human circadian system than the rat circadian system.

To manage circadian disruption non-pharmacologically would be particularly valuable to the aged community where polypharmacy is prevalent. The mean number of medications taken by individuals > 65 years old is four and ever-growing (Charlesworth et al., 2015). Polypharmacy is a challenge in the geriatric community as it can result in medicine non-adherence and increases risk of drug-drug interactions and adverse drug events (Charlesworth et al., 2015). In addition, these medications often lead to non-specific effects such as fatigue and confusion and end up compounding other symptoms.

## 6.6 Future directions

### *6.6.1 Inclusion of additional domains of apathy*

The behavioural tasks utilised in this thesis mapped onto the behaviour-cognition and emotional domains of human apathy but omits the third domain which pertains to changes in social behaviour. Social withdrawal is a key symptom of apathy. To gain a greater understanding of apathy related behaviour in preclinical rodent models, the effects of ageing on the social domain of apathy should also be considered. There are several tests that are used to assess social motivation in rodents. For example, the three-chamber paradigm test, which tests the exploration preference of rodent for a novel rodent, a familiar rodent, or an empty chamber. A behaviourally normal rodent would prefer to explore the chamber containing the novel mouse (Kaidanovich-Beilin et al., 2011). Ultrasonic

vocalisations could also be utilised to assess age-related changes in communication which is an additional important aspect of social behaviour and also provides an additional readout of motivational and emotional state (Simola and Granon, 2019, Hinchcliffe et al., 2020) . The inclusion of an additional domain of apathy to the behavioural battery could reveal further species differences, allowing a more informed choice of which species to use for the apathy domain of interest.

The selection of the correct behavioural task for the species chosen may be an important consideration. Aged rats showed a profound deficit in bowl digging probabilistic reversal learning which appears to be driven by perseverative behaviour, but no evidence of a deficit in reward learning, memory, sensitivity, or general cognition. Therefore, this perseverative behaviour may be driven by a lack of willingness to exert cognitive effort. Given that rats are capable of more cognitively complex tasks, it may be that motivation impairment in this species is more sensitive to cognitively effortful rather than physically effortful tasks. The principle of the EfR task could be used to examine preference for high cognitive effort but high value reward versus low cognitive effort but low value reward. A variation of the human Effort Expenditure for Reward Task (EEfRT) has been developed to test cognitive effort for reward and found differences in willingness to exert cognitive versus physical effort under different reward probabilities and values (Lopez-Gamundi and Wardle, 2018).

*6.6.2 Application of the apathy behavioural battery to alternative models*

At present, it is not clear if this battery of behavioural tests will reveal the same behavioural profile as aged mice in other currently used models of apathy, such as transgenic mouse models of neurodegenerative disease. While previous work has demonstrated reductions in motivated behaviour, and concluded apathy behaviour from this, it is not clear whether these models will show apathy-related behaviours in the emotional-affective domain. The inclusion of this domain will benefit the overall interpretability of findings. Furthermore, there is high variability between studies in how motivated behaviour is quantified, including changes in operant responding, explorative behaviour, and home cage behaviour. Using a more consistent approach, findings can be more accurately compared between models. This is particularly important as it is not clear whether apathy in healthy ageing shares a common mechanism with apathy that occurs with neurodegenerative or neuropsychiatric disease. This could have important implications for therapeutic intervention.

*6.6.3 Taking a more ethological approach to operant-based tasks*

Use of the PRLT revealed a discrepancy between bowl digging and operant-based methods. While aged rats were unable to reverse in the bowl digging version of the PRLT, this deficit was not observed in the operant version of the task. This raises important questions about the sensitivity of operant-based tasks to tests of cognitive flexibility. Responding in the operant box version of the task is largely dependent on the visual system, which, while dominant in humans, is not the

dominant sense of the rodent (Lankford et al., 2020). In contrast, the bowl digging version required digging in different substrates to find a reward which may be more reflective of the natural foraging behaviour of the rodent. The use of more ethologically relevant stimuli may increase within-session sensitivity while the presentation of unchanging visual stimuli over large numbers of trials and repeated sessions may result in learning becoming procedural and resulting in a lack of true cognitive engagement with the task. This may result in a lack of sensitivity to age-related deficits in cognitive flexibility. This loss in sensitivity may also extend to the effort for reward task. Over time, the gap between young and aged mice trial performance closed, with the Rimadyl effort for reward study finding no difference in high effort trial performance between ages in the vehicle group, in contrast to the start of the study where greater trial performance in young mice was observed. The use of more ethologically relevant versions of the tasks may produce more meaningful data without the need for repeated sessions and should be explored in more detail.

*6.6.4 Testing the therapeutic potential of timed feeding in rodents in the context of apathy*

Feeding every 24hr in the light phase revealed an interesting differential impact of feeding time on aged versus young rats. This finding should certainly be explored further, due to the therapeutic potential timed feeding could have on the aged circadian system as described above. Expanding the number of time points for molecular analysis of the PVN could provide more detailed information about how feeding affects the rhythmic dynamics of the molecular findings reported. The inclusion of a non-food restricted young and aged group would allow for the effects of timed feeding to be compared to *ad libitum* feeding and provide insight into age-related changes in molecular targets of interest under standard conditions. While the thesis results show that feeding in the light phase affects nocturnal activity differently in aged versus young rats it does not tell us whether it has therapeutic benefit. It is important to assess whether timed feeding in the dark phase has a beneficial impact on commonly reported circadian changes in ageing such as circadian activity amplitude and sleep quality, as well as other physiological measures such as core body temperature and CORT release. This could provide further therapeutic insight.

If this is the case, there is the potential to use timed feeding in the dark phase to strengthen circadian amplitude and test whether this improves apathy symptoms. However, given much of the current apathy behavioural battery requires food restriction to complete appetitive tasks this may not be possible. It is therefore important that non-appetitive measures of behaviours relating to apathy domains be utilised or developed.

**Appendix**

**Article A1**



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# Evidence for deficits in behavioural and physiological responses in aged mice relevant to the psychiatric symptom of apathy

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**Abstract**

Apathy is widely reported in patients with neurological disorders or post viral infection but is also seen in otherwise-healthy aged individuals. This study investigated whether aged male mice express behavioural and physiological changes relevant to an apathy phenotype. Using measures of motivation to work for reward, we found deficits in the progressive ratio task related to rate of responding. In an effort-related decision-making task, aged mice were less willing to exert effort for high value reward. Aged mice exhibited reduced reward sensitivity but also lower measures of anxiety in the novelty supressed feeding test and an attenuated response to restraint stress with lower corticosterone and reduced paraventricular nucleus c-fos activation. This profile of affective changes did not align with those observed in models of depression but suggested emotional blunting. In a test of cognition (novel object recognition), aged mice showed no impairments, but activity was lower in a measure of exploration in a novel environment. Together, these data suggest aged mice show changes across the domains of motivated behaviour, reward sensitivity and emotional reactivity and may be a suitable model for the pre-clinical study of the psychiatric symptom of apathy.

**Keywords**

Ageing, apathy, emotional blunting, motivation, stress reactivity

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## Introduction

Ageing is associated with widespread physiological changes, including disruptions to normal behaviour. A prevalent behavioural change is the onset of the psychiatric symptom, apathy (Brodaty et al., 2009), which is defined as a quantitative reduction in self-generated or voluntary behaviours (Levy and Czernecki, 2006). While apathy is a common feature of neurodegenerative diseases, and in some people following viral infections (Kamat et al., 2012), it is also seen in otherwise-healthy ageing. Apathy can have a profound effect on the daily functioning of the individual and the caregiver. It is associated with cognitive decline, nutritional deficit and an overall poorer quality of life as well as significant caregiver stress (Gerritsen et al., 2005; Ishii et al., 2009). It therefore represents an important potential target for pharmacological interventions.

While generally considered a motivational disorder, the work of Marin, Levy and Dubois has conceptualised apathy into three domains: an emotional/affective component, an auto-activation/behavioural component and a cognitive component (Levy and Dubois, 2006). Efforts have been made to map these components onto distinct brain circuits, particularly those of the frontal cortex-basal ganglia (Levy and Dubois, 2006), though there have been few studies to understand apathy in the context of normal ageing or using animal models. The development of an approach to study these domains of apathy in non-human species could facilitate insight into its underlying neurobiology and potentially elucidate therapeutic targets. Clinical assessment of apathy is traditionally conducted using self-report or questionnaire-based methods (e.g. Apathy Evaluation Scale (Marin et al., 1991) and Lille Apathy Rating Scale (Sockeel et al., 2006)). These forms of assessment rely on the individual to recognise changes in their own behaviour and have limited sensitivity to track changes in behaviour over time. These subjective methods cannot be directly

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translated to rodent tasks. When considering animal models in psychiatry, particularly where a diagnosis is related to deficits in several symptom domains, it is useful to consider the framework set out in RDoC (Research Domains of Criteria, (Cuthbert, 2015)). Rather than looking at the effects of ageing on one specific area in isolation, using a battery of measures can provide a more detailed insight into the changes which are present across multiple domains. Where behavioural tasks involve more than one underlying construct, using a test battery will also benefit the overall interpretation of the findings.

In this study, we tested a cohort of normal, healthy aged mice using behavioural assays specifically designed to quantify domains of emotional behaviour, motivation, reward and cognition. In contrast to previous studies, we tested the same animals across different tasks so we could achieve an overall profile of deficits and then relate this back to what has been observed in human ageing and apathy. We selected tasks based on RDoC and where, as much as possible, translational analogues were available.

To investigate changes in motivation, we chose two different behavioural tasks which have been successfully back translated to human studies: the progressive ratio (PR) task (Chelonis et al., 2011; Richardson and Roberts, 1996) and effort for reward task (EfR) (Heath et al., 2015; Salamone et al., 1991; Treadway et al., 2009). PR requires the rodent to expend increasing amounts of effort for each subsequent reward with the point when the animal stops responding, the breakpoint, used as a measure of motivation. In the EfR, the animal is given a choice between a high effort, high value reward option or a low effort, low value reward option providing a measure of effort-related choice behaviour which is sensitive to changes in motivational state and manipulations of dopaminergic transmission (Salamone et al., 2018).

Emotional blunting, defined as a diminished response to emotionally salient stimuli, is a core feature of apathy but is often overlooked in favour of effort-based paradigms to study motivation, particularly in rodent models (Magnard et al., 2016). Some groups have reported changes in anxiety/depression behaviour in models of neurodegenerative disorders (Taylor et al., 2010) and more recently, healthy ageing (Shoji and Miyakawa, 2019), and changes in fear processing have been used as a model of emotional blunting in a rodent model of schizophrenia (Pietersen et al., 2007). One of the challenges is the limitations associated with animal models of anxiety and depression (Commons et al., 2017), with conventional measures such as behavioural despair used to predict antidepressant efficacy but with poor validity in terms of quantifying phenotypic changes (Reardon, 2019) and hence may have limited value in relation to emotional blunting. We chose to use the novelty supressed feeding test (NSFT) and then quantify both baseline and stress-induced corticosterone and post-mortem c-Fos expression to investigate the effects of ageing on stress reactivity at a behavioural, physiological and cellular level. The NSFT has been used to investigate stress-related behaviours in depression and anxiety research in phenotypic models, and studies in models such as early life adversity (ELA) has shown both behavioural changes in the NSFT and stress reactivity at a physiological and molecular level (Stuart et al., 2019; Uchida et al., 2010). We also tested animals using the sucrose preference test to quantify reward sensitivity and performed a novel object recognition test to look at their associative recognition memory. By assessing the aged mouse phenotype across these different domains and comparing the findings to those seen in other models, for example, depression, we predicted being able to show a distinct behavioural profile and establish a test battery which could be used to study the relevant underlying neurobiology of apathy.

## Methods

### Subjects

A cohort of 12 aged male C57bl/6J mice (15 mo at experiment onset and 24 mo by end of experimentation, 31.6–38.9 g at onset and 33.6–40.0 g by end) supplied by Eli Lilly and a cohort of C57bl/6J strain and sex-matched controls (3 mo at experiment onset and 12 mo by end of experimentation, 21.8–27.6 g at experiment onset and 30.5–32.4 g by end) supplied by Charles River were used. Sample size was based on previous behavioural studies using both spontaneous behavioural assays such as the open-field arena and operant methods (Gourley et al., 2016; Rex et al., 1998). However, this type of ageing work is novel, and effect sizes may be smaller than those more typically seen with manipulations in these assays. Prior to arrival in Bristol, aged mice were group-housed in enriched caging and fed a restricted diet of 3 g to promote healthy ageing. On arrival in Bristol, we noticed significant in-fighting in the aged group and as a consequence, all mice were individually housed in open-top cages. Cages were enriched with a plastic house, cardboard tube and wooden chew block. They were kept in temperature-controlled conditions (21°C) and a 12:12-h light–dark cycle (lights OFF at 08:15, lights ON at 20:15). Standard laboratory chow (Purina, UK) was provided *ad libitum*, apart from during operant training where mice were fed a restricted diet of 2 g chow per mouse. Weights were monitored at least once a week and maintained to at least 85% of their free feeding weight relative to their normal growth curve. Water was provided *ad libitum*. The same cohorts of animals were used for all studies and a timeline indicating the order of testing is given in Figure 1. As the tests were performed sequentially, there is the potential that increasing age may have affected some measures, and hence the design randomised the tasks between measures of motivation-related and emotional behaviour. It should be noted that this design does not fully mitigate an effect of order of testing or increasing age of the animals. Within each behavioural assay, age was counter-balanced to account for time of day differences. Where possible, the experimenter was blind to age group, though this was not always possible due to obvious physical differences between age groups. Aged mice were checked daily for changes in health that would impair performance in behavioural tasks, including overt changes in motoric function. If this occurred, mice were removed from the study. However, no animals were excluded based on health. All experiments took place in the animals' active phase and were performed in accordance with the Animals (Scientific Procedures) Act (United Kingdom) 1986 and were approved by the University of Bristol Animal Welfare and Ethical Review Body (AWERB).

### Exploration of a novel arena

Mice were placed in a novel, circular open-field arena, 85 cm in diameter under red lighting. Movement was captured for 15 min using a Logitech HD Pro Webcam c920 suspended 1 m above the arena. Videos were analysed using Ethovision Xt10 software (Noldus Information Technology, Wageningen) and total distance travelled (cm) and velocity (cm/s) were output.

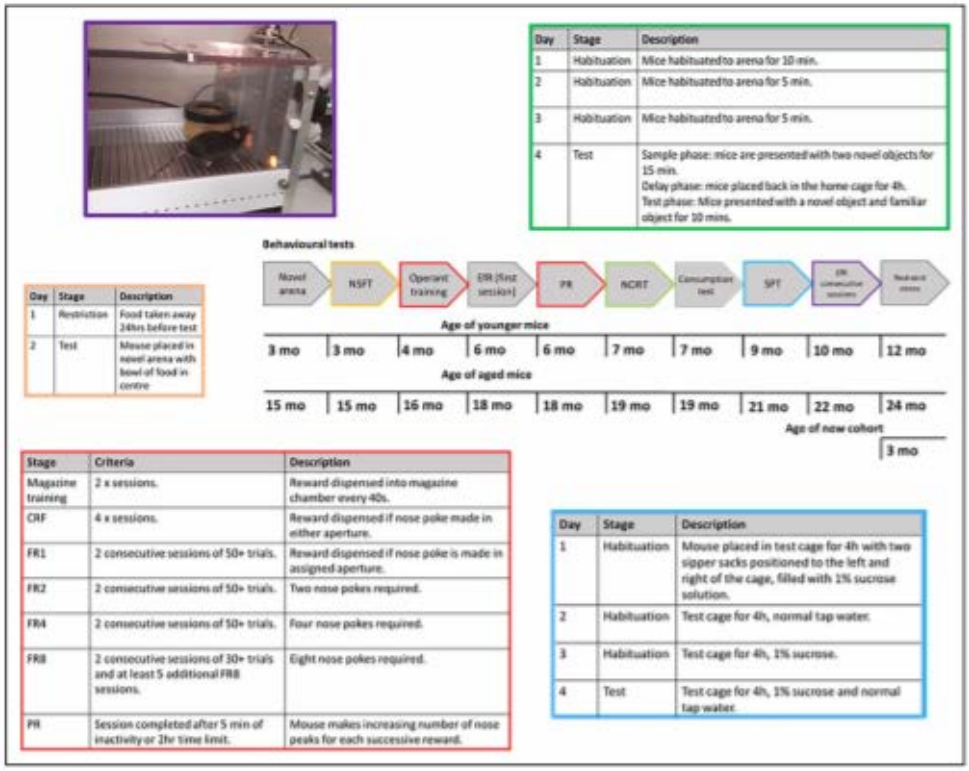


**Figure 1.** Experimental timeline and training protocols. Experimental timeline illustrating the different behavioural procedures used and when these occurred. Where more complex behavioural tasks have been used, additional information about training is also included and colour coded to link to the specific task within the timeline.
CRF: continuous reinforcement, EfR: effort for reward, FR: fixed ratio, NORT: novel object recognition test, NSFT: novelty supressed feeding test, PR: progressive ratio, SPT: sucrose preference test.

### *Novelty suppressed feeding test*

The protocol used was similar to that reported by Shephard and Broadhurst (1982). Mice were food deprived for 24 h before being placed in the left-hand corner of a novel arena (clear Perspex, 40 cm × 40 cm and lined with sawdust). A ceramic bowl of food was placed in the centre of the arena. Time taken for the mice to approach the bowl and to eat was manually recorded. Faecal pellets were removed after each mouse, and the sawdust was shaken to redistribute odour cues but reduce the aversive nature of the context, the food bowl was cleaned, and fresh food added between mice.

### *Operant training*

Training was similar to that reported by Stuart et al. (2019). Mice were trained in sound-proof operant boxes (Med Associates Inc) which were run on Klimbic software (Conclusive Solutions Ltd., UK). Each operant box consisted of two nose poke apertures positioned either side of a centrally located food magazine. The magazine was connected to a reward pellet dispenser (20 mg rodent tablet, TestDiet, Sandown). Mice were run once per day during their active phase (9:00–17:00). Mice first learned to associate the magazine with the delivery of a reward pellet (one pellet every 40 s) over two 30-min sessions. Mice then progressed to continuous reinforcement training (CRF), where a response made in either the left or right assigned nose poke aperture resulted in a single reward pellet. The magazine was illuminated until the mouse collected the pellet. In the third stage of training, the mice were required to advance through ascending fixed ratios (FRs) of reinforcement, making responses in either the left or right aperture only (counter-balanced across cohorts). The mice progressed through FR1, 2, 4 and 8, where the number refers to the number of nose pokes into the active aperture required for the delivery of one reward pellet. Mice completed each FR level when they obtained 50+ pellets over two consecutive sessions until FR8 where criteria was 30+ pellets over two consecutive sessions. Once all mice were trained, mice then completed a minimum of

five additional FR8 sessions to manage differences in time to train (Figure 1).

### EfR task

Directly after FR training was completed, the mice underwent a single FR8 session with access to low value, *ad libitum* powdered standard laboratory chow presented in a pot placed in front of the inactive nose poke aperture similar to that previously described by (Salamone et al., 1991). Animals were required to make a sequence of eight nose pokes to obtain a single reward pellet (20 mg rodent tablet, TestDiet, Sandown) which represents the high effort, high reward option. The freely available chow represents the low effort, low value reward option as it is their standard diet and lacks the higher sucrose content of the reward pellets. The pot was accessed via a 0.5 inch hole in the lid. Chow consumed was measured using change in weight of chow pre and post session, in gramme. Later in the behavioural battery (Figure 1), mice were re-tested in the EfR task, but this time, over five consecutive sessions.

### PR task

Mice were tested in a PR task, in which each successive reward ($n$) required an increasing number of nose pokes using the algorithm $F(n) = 5 \times \text{EXP}(0.2n)-5$ (Roberts and Richardson, 1992). A PR session consisted of a maximum of 100 trials or 120 min and included a 1-s intertrial interval. 5 mins of inactivity ended the session. This test was conducted under both *ad libitum* and food-restricted conditions. Breakpoint was defined as the last ratio competed before 5 min period of inactivity. Mice underwent one session of the PR task under each feeding condition. A PR session was preceded by an FR8 session to check for stability in performance. Preceding the described PR conditions, mice were tested with a PR session that lasted a maximum of 60 mins or 10 mins of inactivity. However, it was clear a breakpoint would not be reached under these conditions (data not shown).

### Consumption test

Mice were food restricted overnight, and the following day were presented with free access to either powdered chow or reward pellets for 10 min over two different days in the home cage. Total amount consumed in gramme was calculated.

### Novel object recognition test

This protocol was similar to that previously developed by Ennaceur and Delacour (1988). Mice were habituated to a Perspex arena (40 cm ×40 cm) lined with paper liner for 10 min (Day 1) or 5 min (Days 2 and 3). This arena was differentiated from the NSFT arena by changing the room in which experiment was run and changing the sawdust floor to a paper floor to change context. On Day 4, animals were tested using a sample phase where each mouse was presented with two novel objects for 15 mins. Mice were returned to their home cage during the 4-h delay phase before being returned to the same arena for the test phase. Each animal was presented with both a novel and familiar object for 10 mins (Figure 1). During both the sample and test phase, exploration (defined here as the animal pointing its nose towards the object at a distance of approximately <2 cm) was captured using a Logitech c920 webcam and then scored manually using DOSBox 0.74 software. Criteria for inclusion in the analysis was 20+ s of total exploration in the sample phase as described by Lueptow (2017). A discrimination ratio was calculated using (time spent exploring novel object − time spent exploring familiar object)/(time spent exploring novel object + time spent exploring familiar object).

### Sucrose preference test

The protocol used was similar to that by Willner et al. (1987). Mice were water restricted overnight for −16 h before both the habituation and test sessions. On Days 1 and 3, the mice were placed into test cages which contained sawdust and a cardboard tube and were presented with a 1% sucrose solution in two sipper sacks (Edstrom-Avidity Science) with drip-free drinking valves, placed to the left and the right of the cage. On Day 2, the mice were presented with two sipper sacks containing normal tap water. On the test day, they were presented with one sipper sack containing 1% sucrose solution and the other containing tap water (Figure 1). Position was counter-balanced across the cohort and swapped at 1 h and 2 h. The habituation and test sessions lasted 4 h, and liquid consumed was weighed at the 1-, 2- and 4-h time points. Sucrose preference was calculated using (total amount of sucrose consumed/(total sucrose + total water consumed)) × 100.

### Acute restraint stress

At the end of behavioural experiments, groups were approximately 12 months and 24 months old. As such, an additional cohort was brought in as a young group. $N = 12$ male C57bl/6J mice from Charles River (3 mo at experiment onset) were singly housed in open-top cages upon arrival at the unit. Mice were kept in the same lighting conditions as described above and were given the same cage enrichment. Food and water were provided *ad libitum*. Mice spent 1 month in the unit before this experiment and were handled for 3 weeks to prevent potential confounds of stress from handling. The new mice weighed 23.2–26.9 g at the experiment end. One mouse from the oldest group died before this final experiment.

Mice were individually restrained in a restraint tube (Ad Instruments Ltd), and their tail warmed for 3 minutes on a heat pad to promote blood flow in the tail vein. The tail vein was then opened with a 25G needle (Sigma-Aldrich, Germany) and blood was collected using a Mitra Microsampling sponge (Neoteryx, USA), with a calculated average blood wicking volume of 10 µL. The mouse was left in the restraint tube for a further 24 mins, before having the tail vein warmed again for 3 mins and another blood sample was taken. Throughout the period of restraint and blood sampling, the animals were not in olfactory or auditory contact with other mice and the restraint tube was cleaned fully between animals. Sampling was also counter-balanced across the different groups. As an additional measure faecal pellets during testing were counted as it has previously been shown psychological stress significantly increases faecal output in rodents (Mönnikes et al., 1993). Sampling took place between zeitgeber

time (ZT) 17–20. Blood samples were stored at room temperature with a bag of desiccant until analysis.

### Mass spectrometry analysis of corticosterone

Following sample extraction corticosterone levels were analysed using high-performance liquid chromatography/electrospray ionisation tandem mass spectrometry (HPLC-ES/MS-MS) (for full experimental details see supplementary methods).

### Tissue collection

Mice were returned to their home cage after the 30 min restraint stress and then killed by cervical dislocation after a further 60 min (90 min after onset of restraint stress). Brains were immediately removed and placed in 4% paraformaldehyde (PFA) solution prepared in phosphate buffer saline (PBS) overnight and then transferred to a 25% sucrose solution (Sigma-Aldrich, UK) prepared in PBS. They were then frozen in OCT (Cryomatrix, Thermofisher) and stored at −20°C.

### c-Fos immunohistochemistry

Brains were sliced in 40 µm coronal sections using a freezing microtome (Reichert, Austria). Sections were blocked for 30 mins in TBS-T (Trizma buffer saline with 0.1% Triton X) with 3% normal goat serum (Vector Laboratories). Sections were incubated with primary antibody rabbit anti c-Fos (1:4000, ABE457, Merck Millipore) overnight at 4°C. Sections were then incubated with secondary antibody goat anti-rabbit (1:500, A32731, Alexa Fluor 488, Invitrogen) for 3 h and then with 4′,6-diamidino-2-phenylindole (DAPI) for a further 3 min (for full experimental details see supplementary methods).

Images were taken on a Leica widefield microscope with DFC365 FX camera with LasX software. The paraventricular nucleus of the hypothalamus (PVN) was captured with a 10× objective and the central (CeA) and basolateral amygdala (BLA) with a 5× objective. A picture of the PVN was taken across three different sections, bregma level −0.58 to −0.94 mm. A picture of the amygdala (left or right) was taken across three different sections, bregma level (−1.22 to −1.40 mm). Some brains/sections were lost due to tissue damage. $N = 10$ per age group amygdala was obtained. $N = 10$ PVN for aged and young group were obtained, $N = 9$ for middle-aged group. c-Fos positive cells that co-localised with DAPI were counted manually using ImageJ cell counter. Contrast was adjusted uniformly across all images. c-Fos count was normalised by dividing c-Fos count by area of region.

### Data analysis

Where data were normally distributed (tested using the Shapiro–Wilk test) or required two-factor repeated measures (RM) analysis, RM two-way analysis of variance (ANOVA), one-way ANOVA, or independent samples *t*-test were performed where appropriate. Where normality was violated, the Kruskal-Wallis test or Mann–Whitney *U*-test were performed. Where significant main effects or interactions were observed ($p < 0.05$), these are reported in the results section with appropriate post hoc pairwise comparisons to explore age differences. Where main effect or interaction statistics found a trend-level effect ($p < 0.1$), these are mentioned in the text but were not further analysed. When conducting ANOVA tests, where data did not meet the assumption of sphericity, the Huynh–Feldt correction was used to adjust degrees of freedom. When conducting single independent *t*-tests, Levene's Test for Equality of Variances was used to determine whether equal variances were assumed with correction being applied if this was not valid. Statistical outliers were defined as values 2 standard deviations away from the group mean. Analysis was performed on IBM SPSS statistics v. 24 for Windows and all graphs were made using GraphPad Prism 8.3.0 for Windows.

## Results

### Aged mice show reduced exploration of a novel arena but show no deficit in the novel object recognition task

In a novel arena, aged mice covered less distance and had a slower mean velocity than younger mice ($t_{(22)} = 2.536$, $p = 0.019$ and $t_{(22)} = 2.613$, $p = 0.016$, respectively, independent *t*-test) (Figure 2(a) and (b)). When data were split into 5-min time bins, there was a main effect of time ($F_{(2.44)} = 35.330$, $p = 7.06 \times 10^{-10}$, RM two-way ANOVA) where distance travelled reduced with time. There was also a main effect of age group ($F_{(1,22)} = 6.434$, $p = 0.019$), but there was no age group*time interaction ($p > 0.05$). Pairwise comparisons revealed no difference in distance travelled between age groups in the first 5 mins, but a difference emerged in the second- and third-time bin $p = 0.013$ and $p = 0.049$, respectively (Figure 2(c)).

In the NOR task, there was no difference in discrimination ratio between age groups ($p > 0.05$) (Figure 2(d)), and there was no difference in exploration of the objects between age groups during the sample phase (Figure 2(e)) or test phase (Figure 2(f)).

### Aged mice show changes in motivated behaviour

Under food restriction, aged mice ended the PR session at a lower final ratio completed ($t_{(12.464)} = 4.563$, $p = 0.001$, independent *t*-test); however, mice failed to reach a true breakpoint, that is, they kept working until session time limit. Analysis of time taken to complete each ratio within the PR session showed a main effect of ratio ($F_{(1.405, 30.843)} = 22.812$, $p < 0.0001$, RM two-way ANOVA), where time taken to complete a ratio increased with ratio. There was also a ratio*age group interaction ($F_{(1.405, 30.843)} = 13.581$, $p < 0.0001$) and a main effect of age ($F_{(1,22)} = 19.960$, $p < 0.0001$). Ratio 62 was the final ratio all mice completed so was used as a cut off for analysis. Pairwise comparisons showed younger mice completed ratio 6–62 faster than aged mice ($p \leq 0.016$) (Figure 3(a)). Under *ad libitum* feeding conditions eight young mice and nine aged mice reached breakpoint, that is, ended session with 5 mins of inactivity. There was no difference in breakpoint between age groups ($p > 0.05$). However, when all mice were added to the analysis, including those that did not reach breakpoint, a difference emerged ($t_{(14.108)} = 2.619$, $p = 0.019$). There was no difference in time taken to complete each ratio between age groups (up to ratio 40). RM ANOVA was not

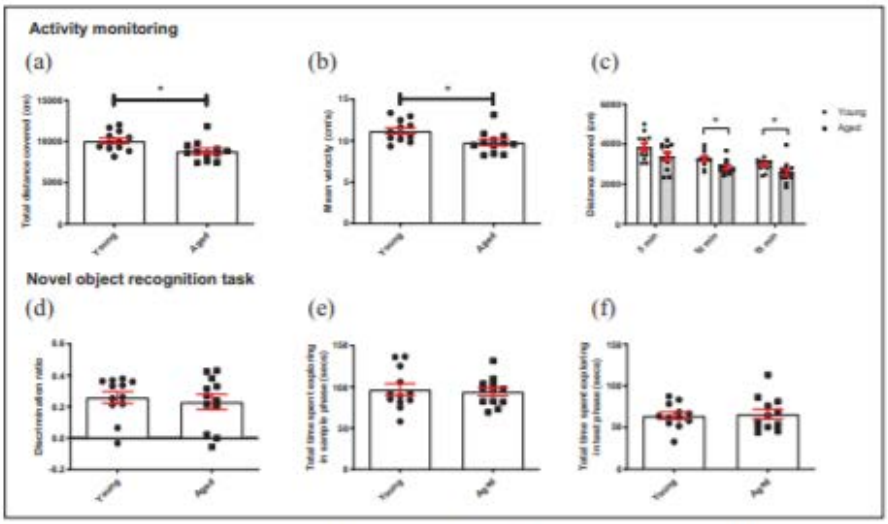


**Figure 2.** Aged mice show reduced exploration in a novel arena but normal cognition in the novel object recognition test: (a) aged mice covered less distance overall in a novel environment compared to younger mice (independent t-test), (b) aged mice were slower than younger mice (independent t-test), (c) distance covered was not different between age groups in the first 5 mins of the task but was by 10 and 15 mins. Mice also covered less distance over time (RM two-way ANOVA with pairwise comparisons). (d) There was no difference in discrimination ratio during the test phase of the NOR task. Bars are mean ± SEM, with data points overlaid. (e) There was no difference in time spent exploring objects in the sample phase of the NORT between age groups, (f) or in the test phase. *p < 0.05, ###p < 0.001—refers to main effect of time. N = 12 per age group.

possible for this analysis as there were missing data points due to animals completing different final ratios before the break point or end of the session. As the main interest was the effect of age group, a series of independent *t*-tests were conducted comparing the speed to ratio completion for each ratio independently and for old versus young mice. There was no difference in speed to ratio completion between age groups ($t_{(15)} \leq 1.565$, $p \geq 0.113$, independent *t*-tests) (Figure 3(b)).

On first exposure to the EfR task, aged mice obtained less of the high value reward pellets than younger mice ($t_{(15.982)} = 5.088$, $p = 0.0001$, independent *t*-test) but consumed more of the low value chow ($t_{(15.819)} = 2.453$, $p = 0.023$). One younger mouse was excluded as an outlier (Figure 3(c) and (d)). In a subsequent 5-day test, analysis of number of trials completed over sessions showed a main effect of session ($F_{(3.22,\ 70.833)} = 41.553$, $p < 0.0001$, RM two-way ANOVA), and there was a session*age group interaction ($F_{(3.22,\ 70.833)} = 2.73$, $p = 0.047$). There was a trend-level effect of age ($F_{(1,22)} = 3.038$, $p = 0.095$). Post hoc pairwise comparison showed younger mice completed more trials only in the final two sessions ($p = 0.046$ and $p = 0.032$, respectively) (Figure 3(e)). However, there was no effect of session or group on consumption of chow ($p > 0.05$) although there was a trend towards a chow*age group interaction ($F_{(3.50,\ 77.4)} = 2.237$, $p = 0.08$) (Figure 3(f)). $n = 1$ young mouse was excluded for digging in chow over consecutive sessions. In a single instance of digging, value was replaced with group mean to permit RM ANOVA analysis ($n = 1$ young mouse). Analysis of average speed (speed at which FR8 was completed, averaged over a session) across the sessions showed a main effect of session on speed, where speed increased over sessions in both age groups ($F_{(2.302,\ 50.636)} = 34.476$, $p < 0.0001$). However, there was no age*session interaction or effect of age ($p > 0.05$) (Figure 3(g)). Consumption tests showed aged mice consumed more reward pellets in 10 mins than younger mice ($t_{(22)} = 2.587$, $p = 0.0168$, independent *t*-test) while consumption of chow did not differ ($p > 0.05$) (Figure 3(h) and (i)).

Analysis of CRF training performance revealed a main effect of session on number of trials completed ($F_{(3,66)} = 44.471$, $p < 0.0001$, RM two-way ANOVA), as well as a session*age group interaction ($F_{(3,66)} = 5.815$, $p = 0.0014$) and a main effect of age ($F_{(1,22)} = 8.365$, $p = 0.008$). There was no difference in performance between groups in the first two sessions, but younger mice completed more trials in the final two sessions ($p \leq 0.003$) (Figure 3(j)). There was no difference in session at which FR training was completed between age groups (Mann–Whitney *U*-test, $U = 54.50$, $p = 0.3058$) (Supplementary data S1).

## *Aged mice show changes in hedonic behaviour, anxiety-like behaviour and stress reactivity*

Analysis of % sucrose preference showed a main effect of time ($F_{(1.781,\ 39.191)} = 12.146$, $p < 0.0001$, RM two-way ANOVA) where sucrose preference increased with time. There was also a time*age group interaction ($F_{(1.781,\ 39.191)} = 7.076$, $p = 0.003$) and an effect of age group ($F_{(1,22)} = 16.692$, $p < 0.0001$) (Figure 4(a)). There were three leaks in water sacks during testing (2 aged, 1 young)

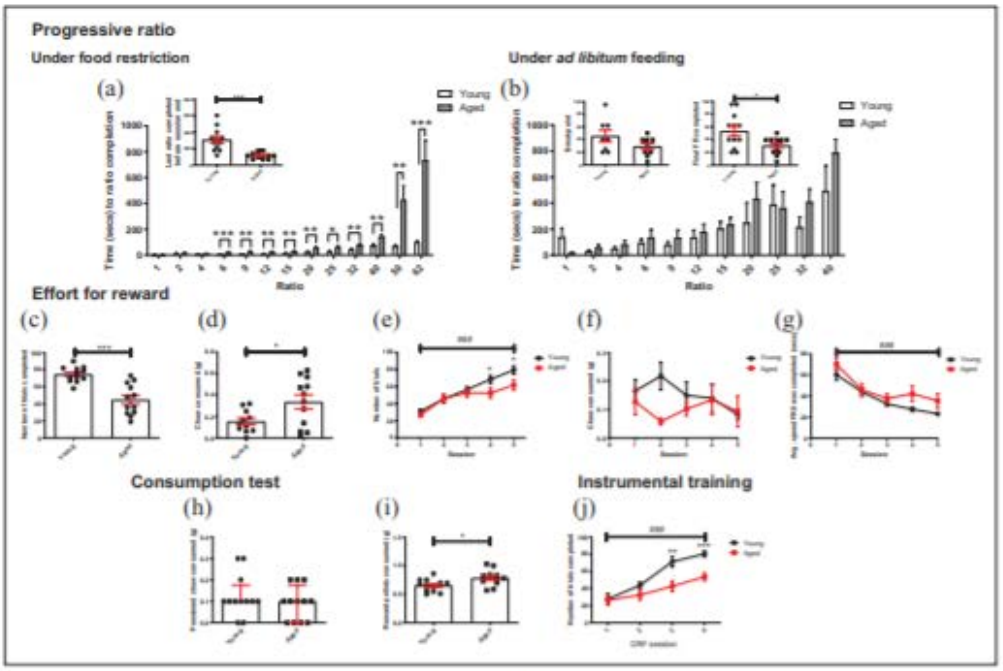


**Figure 3.** Aged mice show changes in motivation under different conditions: (a) under food restriction, aged mice completed the task on a lower final ratio in the progressive ratio schedule than younger mice (independent t-test). Younger mice completed ratios 6–62 faster than aged mice (RM two-way ANOVA with pairwise comparisons). (b) Under ad libitum feeding conditions and for animals which achieved a true breakpoint, there was no difference between age groups (independent t-test). However, when all mice were included in the analysis, aged mice finished on a lower ratio than younger mice (independent t-test). There was no difference in speed across age groups under free feeding conditions (independent t-tests). (c) On first exposure to the EfR task, aged mice completed less trials than younger mice (independent t-test). (d) In the same session, aged mice consumed more chow (independent t test). (e) When the task was repeated over five consecutive days and at the end of the PR training and testing, aged mice completed less trials than younger mice only in sessions 4 and 5. Number of trials completed increased across the week in both age groups ($p < 0.05$, $p < 0.001$, RM two-way ANOVA with pairwise comparison). (f) There was no effect of session or age group on chow consumption. (g) Mice became faster at completing trials over sessions ($p < 0.001$, RM two-way ANOVA). (h) In a 10 min consumption test, there was no difference in chow consumed between age groups (Mann–Whitney U test, bars are median ± interquartile range). (i) Aged mice consumed more reward pellets than younger mice (independent t-test). (j) Aged mice completed less trials in CRF training session 4 and 5 (RM two-way ANOVA with pairwise comparison). Bars are mean ± SEM with data points overlaid (unless otherwise indicated). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ###$p < 0.001$—refers to main effect of session. N = 10–12 per group.

and where this occurred the value for this sack was replaced with the group mean to permit RM ANOVA analysis. Post hoc pairwise comparison showed younger mice had a higher sucrose preference than aged mice at each time point ($p \leq 0.043$). There was a main effect of time on total liquid consumed in the SPT (two-way RM ANOVA, $F_{(2,44)} = 70.192$, $p < 0.0001$, a time*age group interaction ($F_{(2,44)} = 14.672$, $p < 0.0001$ as well as a main effect of age group ($F_{(1,22)} = 26.977$, $p < 0.0001$). Pairwise comparison revealed that young and aged mice drank an equivalent volume of liquid in the first hour of the test ($p > 0.05$) but aged mice drank less liquid at hour 2 and 4 of the test ($p < 0.0001$) (Supplementary data S2A). These differences in total consumption were accounted for in the % sucrose calculation. During habituation to the SPT, there was a main effect of habituation day on total liquid consumed (two-way RM ANOVA, $F_{(2,44)} = 29.950$, $p < 0.0001$). There was also a habituation day*age group interaction ($F_{(2,44)} = 4.44$, $p = 0.018$) and a main effect of age group ($F_{(1,22)} = 6.032$, $p = 0.022$). Pairwise comparison revealed that aged and young mice drank equivalent volumes on Days 1 and 2 of habituation ($p > 0.05$) but young mice drank more liquid on the final day of habituation ($p < 0.0001$) (Supplementary data S2B). Aged mice took less time to eat from the bowl than younger mice in the NSFT ($t_{(14.208)} = 3.411$, $p = 0.004$, independent $t$-test) (Figure 4(b)) but latency to approach the bowl was not different ($p > 0.05$) (Figure 4(c)). Mice were weighed before and directly after the 24-h food restriction. Younger mice lost a greater percentage of their body weight compared to aged mice (unpaired $t$-test, $t(22) = 5.654$, $p < 0.0001$; Supplementary data S3).

Under acute restraint stress, baseline corticosterone was similar for all age groups but aged animals had a lower corticosterone

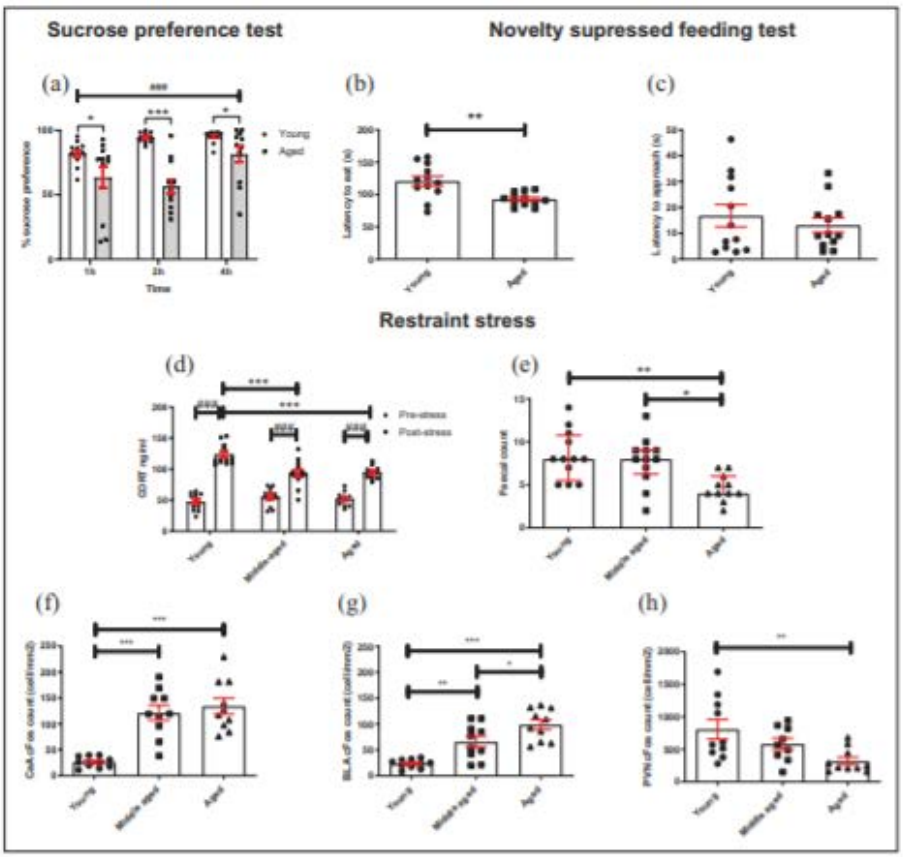


**Figure 4.** Aged mice show changes in reward sensitivity and stress reactivity: (a) aged mice showed a reduced sucrose preference compared to younger mice across all time points, and preference changed across time (RM two-way ANOVA with pairwise comparisons). (b) In the NSFT, aged mice took less time to eat from the bowl than younger mice (independent t-test). (c) There was no difference in latency to approach bowl between age groups (independent t-test). (d) Following 30-min restraint stress, CORT was increased in all groups, but aged mice showed a reduced CORT response to stress compared to younger mice but not middle aged (RM two-way ANOVA with pairwise comparisons). (e) Aged mice had a lower faecal count following stress compared to young and middle-aged groups (Kruskal–Wallis test with Dunn's post hoc (bars are median with interquartile range)). (f) Following stress, middle-aged and aged mice had a greater c-Fos count in the CeA compared to younger mice ($p < 0.001$, one-way ANOVA with Tukey's post hoc analysis). (g) Aged mice had a greater BLA c-Fos count compared to middle-aged and young mice. Younger mice had a lower c-Fos count in the BLA compared to middle-aged mice (one-way ANOVA with Tukey's post hoc analysis). (h) Younger mice had a greater c-Fos count in the PVN compared to aged mice (one-way ANOVA with Tukey's post hoc analysis). Unless otherwise indicated bars are mean ± SEM with data points overlaid. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ###$p < 0.001$—refers to main effect of time or pairwise effect of stress. N = 9–12 per group.

response to restraint (main effect of stress $F_{(1,32)} = 179.608$, $p < 0.0001$, age $F_{(1,32)} = 5.772$, $p = 0.007$, and stress*age group interaction $F_{(2,32)} = 9.862$, $p = 0.0005$, RM two-way ANOVA). Pairwise comparison revealed CORT was increased following stress in all age groups, but this was higher in the young mice (young vs aged or middle aged: $p = 0.001$ and $p = 0.0003$, respectively) (Figure 4(d)). There was also an effect of age on faecal count ($H_{(2)} = 12.424$, $p = 0.002$, Kruskal–Wallis test). Dunn's post hoc test showed aged mice had a lower faecal count than middle-aged mice ($p = 0.011$) and young mice ($p = 0.004$) (Figure 4(e)). Post-mortem analysis of neuronal activation following restraint stress showed CeA c-Fos count was higher in both the aged and middle-aged animal (main effect of age $F_{(2,29)} = 22.102$, $p < 0.0001$, one-way ANOVA) with Tukey's post hoc analysis ($p < 0.0001$ vs younger mice) (Figure 4(f)). There was also an effect of age on BLA c-Fos count ($F_{(2,29)} = 19.915$,

$p < 0.0001$), where middle-aged and young mice had a lower BLA c-Fos count than aged mice ($p = 0.029$ and $p < 0.0001$, respectively). Middle-aged mice also had a higher BLA c-Fos count than younger mice ($p = 0.004$) (Figure 4(g)). PVN c-Fos count was higher in younger mice compared to aged mice (main effect of age $F_{(2,28)} = 5.326$, $p = 0.012$, Tukey's post hoc, $p = 0.008$) (Figure 4(h)).

## Discussion

Aged male mice displayed behavioural deficits in both motivation and affect which suggests they may develop a phenotype with deficits in behavioural domains relevant to apathy. Using measures related to motivation, we observed that aged mice showed a more rapid decline in activity in a novel environment, responded with reduced vigour in the PR task and were more likely to choose a low-value low-effort reward under certain conditions. These effects did not appear to be related to changes in appetite, cognition or gross changes in locomotor function. When we reduced motivation across all age groups using free feeding, we found that the deficit in the aged animal's performance in the PR task related mainly to rate of responding. In the NSFT, aged mice exhibited lower levels of anxiety-related behaviour and were faster to consume the food; however, they also showed reduced reward sensitivity contrasting with effects typically observed in rodent models of depression (Planchez et al., 2019). When we consider these changes in emotional behaviour alongside the blunted corticosterone and PVN c-Fos activation in response to restraint stress, we propose that aged mice show emotional blunting. The following discussion considers these two domains associated with the psychiatric symptom of apathy and the potential to use aged mice to better understand the underlying neurobiology.

### *Aged mice show a reduction in motivated behaviours*

Aged mice were slower and covered less distance in the open-field arena. When the analysis was broken down by time bin, the effect was more apparent after 10 and 15 min, suggesting that initial exploration of the environment and locomotor function did not differ. However, it should be noted the effects were marginal. These effects may be due to declining motivational drive to explore, though it is important to acknowledge that age-related deterioration in motoric capability and fatigue may also play a role. Ageing has been associated with psychomotor slowing (Tombaugh, 2004), where slowing is due to a reduction in motivation and emotional arousal, which can be independent of age-related effects (Seidler et al., 2010).

During operant conditioning aged animals initially responded at a similar level but then failed to increase their rate of responding to the same level as the younger animals over time. However, both age groups showed learning across sessions, suggesting reduced vigour rather than an overt learning impairment. In the initial studies carried out under food restriction, aged animals appeared less motivated and completed less ratios for reward under a PR schedule of reinforcement. They were slower to complete each ratio but a true breakpoint, in which the mouse gives up before the session ended was not reached. Some PR studies are limited by time rather than lack of responding (Heath et al., 2015) or a combination of both (Gourley et al., 2016). This may mean that conclusions are confounded by changes in rate of response and an assumption that the last ratio completed before the task times out is also break point. When the motivational state was reduced by providing food *ad libitum*, we found no difference in breakpoint or the speed of ratio completion. This suggests rate of responding is driving the motivational deficit in aged mice and the effects are more apparent when control animals are in a high motivational state. These findings are in line with studies using a Huntington's disease (HD) model, in which apathy is a core symptom, where they found a reduction in rate of response in a PR schedule of reinforcement, relative to controls (Heath et al., 2019; Oakeshott et al., 2012). This was consistent with a study using HD patients with apathy, and apathy questionnaire scores negatively correlated with breakpoint scores (Heath et al., 2019).

An alternative way to look at motivation is the EfR task, an effort-based decision-making (EBDM) task. By offering a choice to the mouse, this task provides a more complex measure of motivation involving economic choices such as cost/benefit analyses (Salamone et al., 2018). In this task, a shift from the high-effort to low-effort reward option is thought to indicate a change in motivation whereas an overall suppression of consumption would indicate a change in appetite. The integration of the findings across both PR and EfR has been used to try to address limitations associated with any one task in isolation. In an initial single test session following FR training, aged mice completed less trials but consumed more chow, indicating preference for the lower effort option compared to younger mice. However, after animals had undergone multiple sessions under FR8 and PR schedules and, when the task was presented over consecutive days, an age difference emerged only in the final two sessions. There is a potential confound with this format which links to our PR data. Animals were run under food restriction, but mice are given food *ad libitum* over the weekend and food restricted across the week. In this second study, changes in chow consumption were not observed; however, variability was higher, possibly resulting from excessive digging/waste from the chow bowl. Changes in EBDM have been reported in Schizophrenia patients and Parkinson's disease patients with apathy (Chong et al., 2015; Le Heron et al., 2018). However, much of pre-clinical work on this type of EBDM focusses on investigating the role of the dopaminergic system, rather than probing for apathy behaviour in disease/ageing models (Le Heron et al., 2019).

Together, these data suggest aged male mice have a deficit in the activational phase of motivated behaviour, which is characterised by speed, vigour and persistence (Salamone et al., 2016). These changes appear not to be explained by changes in appetite or a cognitive impairment as no differences were observed in consumption tests or in the NOR task. There is conflicting evidence in the human literature whether motoric vigour is reduced in apathy, where some studies suggest vigour in pursuit of reward may be conserved in apathy patients, while other work points to a reduction (Le Heron et al., 2019).

### *Aged mice show emotional blunting*

Rodents are known to supress feeding in response to novelty and the time taken to eat in this novel context provides a behavioural

readout of this stress response. The NSFT is based on this principle and has been used for decades in the pre-clinical testing of anxiolytics and antidepressants (Nestler and Hyman, 2010; Shephard and Broadhurst, 1982) and in phenotypic studies of depression models including ELA and chronic social defeat stress (Stuart et al., 2019; Venzala et al., 2012). In the NSF test, aged mice were quicker to consume food in a novel environment, suggesting lower novelty-induced hyponeophagia. These findings are consistent with reduced anxiety and contrast effects seen in rodent models of depression where increased feeding latencies are consistently observed (Planchez et al., 2019). While young and aged mice ate equivalent levels of chow in a separate consumption test, younger mice lost a greater proportion of their body weight following food restriction. Therefore, there is a potential for metabolic differences that may influence appetite and therefore impact measures of motivation driven by hunger. The quantification of ghrelin/leptin levels could provide further information on potential differences in appetite. Conversely, aged animals had reduced reward sensitivity in the SPT, similar to the effects observed in rodent models of depression (Willner, 2016). Human studies have shown reward sensitivity deficits in ageing (Muhammed et al., 2016). A probabilistic reinforcement learning task showed monetary loss had a larger impact on subsequent behaviour than gain in elderly versus younger participants (Hämmerer et al., 2010). This disruption may be due to age-related changes to the efficiency of dopaminergic and serotonergic neuromodulation (Eppinger et al., 2011; Schott et al., 2007). These behavioural findings provide an interesting contrast to depression-like phenotypes and importantly suggest emotional blunting rather than negative affective state.

Studies in the ELA model of depression have shown that stress reactivity is linked to deficits in the NSFT (Stuart et al., 2019; Uchida et al., 2010). To explore the apparent reduction in stress reactivity seen in the NSF test at a physiological level, we used an acute restraint stress which reliably increases the stress hormone corticosterone (CORT) (Harizi et al., 2007; Nohara et al., 2016). There were no differences in CORT under baseline conditions and all age groups showed an increase following 30-min restraint. However, in line with behavioural findings, we show aged mice had a blunted stress-induced CORT response and the oldest group also had a reduced faecal count consistent with reduced stress reactivity. We also found age-related changes in the response to stress centrally, where c-Fos activation in the paraventricular nucleus of the hypothalamus (PVN) was reduced in the 24 mo group compared to the youngest group, yet central (CeA) and basolateral amygdala (BLA) activation was increased in the aged groups. The PVN is a core part of the stress response, its activation results in the release of CORT (Benarroch, 2005). This reduced activation could explain the reduced CORT response in the aged mice. The CeA plays a key role in the stress response, integrating behaviour and autonomic response to aversive stimuli (Day et al., 2005). Previous research has shown that there is relatively little activation of this region in response to processive stressors and is more responsive to homeostatic disruption or systemic stress (Day et al., 2005). However, aged mice show greater activation of the BLA/CeA during stress compared to the young mice. The reason for this and its functional significance is unclear. However changes to the functional gating of limbic information by local PVN projections may explain age-related changes to the HPA axis (Herman et al., 2002). Stress reactivity and active coping in response to aversive experiences has previously been shown to reduce with age in mice (Oh et al., 2018) as well as rat and human studies (Brugnera et al., 2017; Buechel et al., 2014). There is some variability in findings possibly due to a lack of standardisation across studies including the nature and intensity of the stressor (Novais et al., 2017; Segar et al., 2009). An interesting next step would be to test whether this blunted physiological response to an acutely aversive event translates to changes in aversive learning in a contextual fear conditioning paradigm.

It is important to acknowledge that only male mice were used in this study and translatability of findings would benefit from the use of female mice to account for any potential sex differences in the behavioural domains tested. While sex differences in motivated behaviour have been reported (Song et al., 2018), it is unclear if there is a sex difference in how ageing affects these behaviours, with the exception of exploratory behaviour where parallel declines have been observed (Adelöf et al., 2019).

## Conclusion

It is not possible to fully translate the psychiatric symptom of apathy to non-human animals, and there is no one behavioural test that will tell us whether an animal is apathetic. However, by using a battery of behavioural tests that probe aspects of motivated and emotional behaviour we show that aged male mice have deficits in behaviours relevant to multiple domains of apathy. Specifically, deficits measured by the exploration of a novel arena, the PR task and the EfR task indicate a reduction in motivated behaviour. A reduction in stress reactivity and reduced reward sensitivity provide evidence for emotional blunting in aged mice. Together, these data suggest naturally aged mice have the potential to provide a model to investigate the underlying neurobiology of behaviours relevant to apathy. The findings relating to stress reactivity add to previously published work (Oh et al., 2018) and raises an important issue when considering data obtained from stress-driven cognitive tasks such as the commonly used Morris Water Maze (Morris, 1981). Changes in stress reactivity could influence learning independent of a specific cognitive impairment.

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### Author contributions

M.G.J. performed the research, analysed data, wrote and edited the paper. S.L.L. supervision, designed the research and edited the paper. G.G. supported the data analysis and interpretation and edited the paper. H.M. provided resources, designed the research and edited the paper. E.S.J.R. supervision, designed the research and wrote and edited the paper.

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## Data statement

All data used in this manuscript are available and can be accessed on the Robinson Laboratory Open Science Framework upon acceptance https://osf.io/gjv4m/.

## Supplemental material

Supplemental material for this article is available online.

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**Table A2**

| Falls | | Foot slips | | Traversal time (s) | |
|---|---|---|---|---|---|
| Fall number | Score | Foot slip number | Score | Group mean + s | Score |
| 0 | 0 | 0 | 0 | ≤ Mean + 0.5s | 0 |
| 1 | 5 | 1-2 | 1 | Mean + 0.5-1s | 1 |
| 2 | 10 | 3-4 | 2 | Mean + 1-1.5s | 2 |
| 3 | 15 | 5-6 | 3 | Mean + 1.5-2s | 3 |
| 4 | 20 | >6 | 4 | Mean + 2-2.5s | 4 |
| 5 | 25 | | | Mean + > 2.5s | 5 |

***Table A2*** *Composite score used in the raised beam test. This composite score was developed by LJB and previously published in her thesis. s- seconds.*

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