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Effect of Chronic Jet Lag on Cognitive Flexibility Measured by the Attentional Set-Shifting Task in Mice

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Declaration of Independence

I assure that I have written this master thesis independently without external help and used only the given sources and aids. Literally or meaning points, which were obtained from other works are labeled with their source.

Signature: Place, Date:

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ABSTRACT

Background: Cognitive function deficits, such as impaired working memory and high error rates, have been observed in individuals with chronic jet lag (CJL), including airline crew. Furthermore, cognitive flexibility impairments have been reported in night shift workers and individuals with sleep deprivation. However, the effect of CJL on cognitive flexibility remains unknown. Therefore, the present study was performed to assess the effect of simulated CJL on cognitive flexibility using the attentional set-shifting task (ASST) in mice. **Methods**: C57BL/6J mice (aged: 12-14 weeks; 6 male and 5 female mice per group) underwent chronic shifts in the light-dark (LD) cycle. In detail, the light period was shortened by 6 h once weekly to simulate the effects of eastward travel (phase advance), yielding a total of 4 shifts (1-month CJL, 1MCJL) or 8 shifts (2-month CJL, 2MCJL) that were each maintained for a 1-week duration. A standard LD cycle was used for the control group. After 4 or 8 weeks, the ASST was performed and cognitive flexibility was assessed using the number of trials required to complete an ASST phase (trials to criterion) and the number of errors made until the trials to criterion was reached. Furthermore, the errors were categorized into perseverative errors and regressive errors. After an additional shift, the light-dark box (LDB) test was performed to assess anxiety. **Results**: The number of trials to reach criterion was generally higher in the 2MCJL than in the 1MCJL and control groups; however, this effect was not specific to a particular ASST phase. Additionally, the total number of errors was generally higher in the 2MCJL than in the 1MCJL and control groups. Female mice in the 2MCJL group made significantly more perseverative errors. Furthermore, animals in the 2MCJL group made significantly more regressive errors. Finally, the LDB test did not reveal any significant differences among the groups with regard to time spent in the light compartment; however, the frequency of crossings was significantly higher in the 2MCJL group than in the 1MCJL or control groups. **Conclusion**: 2MCJL had effects on cognitive flexibility as indicated by an increased number of trials to reach criterion in the ASST, an increase in the number of errors overall and regressive errors, including perseverative errors in females. Future studies should include increased sample sizes to confirm these effects and reduce variation in the data. Furthermore, nasal orexin as a therapeutic option should be explored in mice.

1 INTRODUCTION

Cognitive flexibility is an aspect of executive functioning that is essential for an individual to cope with changing circumstances. It enables an individual to identify that a change in rules is needed, disengage from these rules, and apply new rules in order to complete a task (Dajani and Uddin, 2015). In fact, cognitive flexibility is an important function as research has shown that it predicts the ability to generate novel ideas, identify multiple approaches to use an idea, and creative and academic performance (De Dreu, 2007; Hirt et al., 2008). Cognitive inflexibility, on the other hand, is characterized by perseverative behavior and an inability to learn from previous mistakes and meet the changing demands of a task and has been observed in several psychiatric disorders, such as autism spectrum disorder (ASD), ADHD, and schizophrenia (Uddin, 2021).

1.1 Components of cognitive flexibility

Cognitive flexibility is a top-down mental process that is categorized as a core executive function (EF) in addition to inhibitory control and working memory. These three EFs are required for top down control of goal-directed behavior, and support functions such as problem solving, reasoning, planning, and completing daily tasks (Diamond, 2013). However, although cognitive flexibility has been categorized separately as one of the core EFs, several subdomains of the EFs act together to implement cognitive flexibility (Tomiyama et al., 2019). These subdomains are salience detection, attention, working memory, and inhibition, in addition to set shifting, which is required for changing rules and applying a new appropriate set of rules. In a changing environment, individuals need to recognize the aspects that are changing (salience) and focus on them (attention); after identifying that the strategy in use is not appropriate for the new environment, they need to inhibit this strategy and their responses (inhibition) and use a new strategy (set shifting). This also requires individuals to process information in their surroundings and manipulate this information in real time to adapt flexibly to their environment (working memory).

1.2 Tests for cognitive flexibility

Various paradigms have been developed to test for different aspects of cognitive flexibility, such as reversal learning, attentional set shifting, and task switching in humans, non-human primates, and rodents. These paradigms have helped identify the neural correlates of cognitive flexibility as well as identify cognitive flexibility impairments in various disorders. Many of these tests have clinical relevance to disorders such as schizophrenia and traumatic brain injury, and can address different

aspects of cognitive flexibility. The following sections therefore provide an account of the various tests and their variations and improvements as applicable.

1.2.1 Reversal learning

Reversal learning is an essential test of cognitive flexibility that has been in use for a number of years to characterize cognitive flexibility deficits in several neuropsychiatric disorders including obsessive compulsive disorder and schizophrenia (Izquierdo et al., 2017). Reversal learning paradigms are used across species, including humans, monkeys, and rodents and therefore have major advantages in translational neuroscience. Here, subjects have to choose between options of at least two stimuli or responses, of which only one of them is the correct target. Thereafter, once a criterion level of performance is reached, unknown to the subject, the contingencies reverse, such that the target that previously resulted in positive feedback now results in negative feedback and vice versa (Highgate and Schenk, 2021). A change in the contingencies might still result in the previous response being selected as the subject works on determining the new rule. However, under certain conditions, such as a psychiatric disorder, this persistent response is exaggerated (Audet and Lefebvre, 2017).

1.2.1.1 Studies of reversal learning in humans and non-human primates

According to the aim of the experiment and the hypothesis tested or disorder studied, several different approaches have been taken to test for reversal learning in humans. Reversal learning paradigms can either be instrumental reward-related or based on the reversal of Pavlovian rules (Schiller et al., 2008).

Further, instrumental reversal learning can be either deterministic or probabilistic. In a deterministic reversal task, correct responses lead to 100% positive feedback, whereas negative responses lead to 100% negative feedback (Kanen et al., 2021). In contrast, to increase the task difficulty, previous studies have also followed a probabilistic model where the correct stimulus produces positive feedback more often than negative feedback (e.g. 80% vs 20%) and selection of an incorrect stimulus produces negative feedback more often (Highgate and Schenk, 2021). Studies on reversal learning have been conducted in Huntington's disease (Lawrence et al., 1999) and the study revealed that these patients are impaired in the reversal phase due to perseverative responding. Further, a probabilistic reversal learning paradigm was also used in patients with Parkinson’s disease, which revealed non-perseverative reversal learning deficits (Swainson et al., 2000).

1.2.1.2 Studies of reversal learning in rodents

Reversal learning paradigms used for human studies have been adapted for rodents. Overall, approaches to test reversal learning include tasks that involve olfactory and/or tactile, visual, or spatial discrimination. The T-maze, an example of a reversal task, is the earliest task that has been used to test for reversal learning (Hill et al., 1962). The maze has three sections, the left arm, right arm, and center arm. For the test to commence, the animal is placed in the starting arm, and a dividing wall is removed to allow the access to the arms. The test consists of two phases, acquisition and reversal. The animal is trained to associate one of the arms with a reward in the acquisition phase, and in the reversal phase, the animal must learn to associate the other previously unrewarded arm with a reward. Studies have also utilized visual discrimination tasks, either in a maze, such as a T-maze, or with the use of a touchscreen. Here, animals are presented visual stimuli, such as vertical or horizontal stripes, and either of these stripes are associated with a food reward, regardless of spatial location in the case of a T-maze. Further, the reversal learning procedure used in a T-maze has also been modified for use in operant chambers. For example, levers have been associated with a reward, such that pressing the right lever results in a reward whereas pressing a left lever does not. In the reversal learning phase, these contingencies are reversed. This task can also be paired with a visual stimulus, in that pressing a lever below a hole with an illuminated light could be associated with a reward whereas pressing one without illuminated light could go unrewarded (Highgate and Schenk, 2021). Several studies have also used a digging task for food where the stimuli were either odors or tactile (McAlonan and Brown, 2003). With regard to the brain regions involved, the orbitofrontal cortex (OFC) has been associated with reversal learning and this has been shown using lesion studies in rats. Rats were first trained on a two-lever spatial discrimination task; only one of the levers was rewarded, following which the associations were reversed. They observed that the rats with OFC lesions could not easily inhibit previous responses, which led to perseveration in the reversal phase (Boulougouris et al., 2007).

1.2.2 Attentional set shifting

Attentional set formation and shifting are executive functions that are measures of cognitive and behavioral flexibility and are required for the performance of daily adaptive goal-directed activities (Tait et al., 2014). Set shifting assess the capacity to focus attention on relevant stimuli of a certain dimension in the presence of irrelevant stimuli and other dimensions and then switch rules to focus attention on the previously irrelevant stimuli and dimensions as required, thus allowing for goal-

directed behavior; the frontal cortex has been implicated in this function. A few protocols have been designed to test attentional set shifting and these have revealed a number of diseases, such as ADHD, schizophrenia, Alzheimer's disease, Parkinson’s disease, and Huntington's disease in which attentional set shifting has gone awry (Brown and Tait, 2016). Furthermore, these tests have been adapted to rodents, thus paving the way for further understanding the underlying neural mechanisms of attentional set shifting both in diseased and healthy individuals.

1.2.2.1 Tests for attentional set shifting in humans

The Wisconsin card sorting task (WCST) was the first to be used as an established method to measure attentional set shifting as it was first used to explore cognitive dysfunction in patients with frontal cortex damage (Milner, 1963). In the WCST, individuals are required to sort a deck of cards according to either the shape, color, or number of shapes on the cards, and must identify a change in rules without being instructed. After 10 consecutive trials, the irrelevant stimulus on the card becomes relevant: for example, instead of focusing on the shape, the individual must now focus on the color of the shapes on the card and sort the deck of cards accordingly. However, although the WCST provides useful information about executive functioning in a disorder, a more refined test, the Cambridge Neuropsychological Test Automated Battery (CANTAB), tests higher forms of cognitive flexibility and is therefore, of better use for testing attentional set shifting.

The CANTAB was developed to include more complexity than the WCST. It addresses three main forms of cognitive flexibility (Sharma, 2013). This test contains two dimensions: lines and shapes. The test starts with a simple discrimination phase in which the subject has to associate a cue from only one of the dimensions with a reward. In the next phase, the second dimension is superimposed on the first one; however, this is considered irrelevant and the subject has to till focus on the same dimension that was in the simple discrimination phase. The test also contains a reversal, in which a previously rewarded cue of one dimension goes unrewarded. The next phase, which is the intradimensional shift (IDS) phase, involves the same dimensions, but different cues. If the individual is still able to focus on the relevant dimension and ignore the irrelevant one, leading to improved task performance, this is an indication that the individual has formed an attentional set. The final phase is the extradimensional shift (EDS), in which the individual must associate a new dimension that was previously unrewarded, with a reward, thus requiring a shift in strategies. Therefore, if the number of trials taken to solve an EDS task is higher than those taken to solve an IDS task, this likely arises from

the cost of having to shift attention from one dimension to another. Thus, compared to the WCST, the CANTAB tests for more levels of cognitive flexibility, reversal, IDS, and EDS (Scarsi et al., 2020).

1.2.2.2 Tests for attentional set shifting in rodents

Cognitive flexibility has been investigated in rodents using a number of tests. The earliest experiments were two-choice discrimination tasks, and compared reversal learning to attentional shifting; one session consisted of one dimension with two cues and the rewarded and unrewarded dimensions were switched at reversal, whereas the second session had a dimension that differed from the first. However, as these experiments did not include an IDS phase set formation could not be tested (Kelleher, 1956). Thereafter, experiments were performed that compared IDS and EDS; however, one group of rats underwent IDS and the other, an EDS. Thus, animals that underwent the IDS, that is, a shift from one exemplar to another exemplar learned the task faster than those that underwent the EDS, that is a shift from one dimension to another dimension (Shepp and Eimas, 1964). Further, Joel et al. (1997) performed a task that consisted of a delayed non-match-to-sample rule and its reversal, which essentially is a simulation of the rule shifting feature of the WCST. Finally, Ragozzino (2007) employed a strategy where rats had to switch between spatial and response discriminations in a plus-maze. However, none of these tests was suitable enough as they either did not have animals perform both the intra- and extra-dimensional shifts in one test or did not perform the test under natural conditions.

Therefore, similar to the CANTAB ID/ED test, Birrell and Brown (2000) established the attentional set-shifting task (ASST) in rats. Here, the stimulus dimensions were odor (olfactory stimulus), digging medium (visual stimulus), or texture (tactile stimulus). The animals had to learn stimuli associations by retrieving reward by digging from either one of two bowls that contain the exemplars. As with the CANTAB, the reversal and the ID/ED shifts on the ASST are measures of cognitive flexibility. In the shift from ID to ED, the previously irrelevant and unrewarded dimension becomes the relevant and rewarded dimension and hence, a high number of trials or errors on the ED in comparison to the ID task, taking into account the controls, indicates an impairment in cognitive flexibility. Furthermore, a high shift cost (ID/ED ratio) within a subject group compared among groups also reflects a change in attentional set shifting performance and therefore cognitive flexibility (Brown and Tait, 2016).

Although rats learn the ASST relatively quickly, in order to successfully implement the ASST in mice, several aspects need to be considered. The first ASST for mice was developed by Bissonette et al.

(2008) prior to which, there had been significant debate as to whether mice could form attentional sets. Studies using a digging task adapted from Birrell and Brown (2000) for rats as well as a visual task showed that mice cannot form attentional sets (Colacicco et al., 2002; Brigman et al., 2005). However, subsequent studies (Garner et al., 2006; Bissonette et al., 2008) showed that mice could form attentional sets by using a task design that was slightly different from the initial ASSTs in mice (Garner et al., 2006; Bissonette et al., 2008). Garner et al. (2006) showed that overtraining in mice helps form attentional sets; in their digging task, they added an extra overtraining phase, which had the same dimensions and stimuli as the ID reversal phase. Bissonette et al. (2008) also incorporated the overtraining concept; however, their task had four ID phases and one ID reversal phase, with each of the ID phases consisting of different stimuli of the same dimension and the ID reversal phase consisting of same stimuli as the last ID phase. Thus, these studies observed formation of attentional sets and therefore, a shift. Owing to the high number of exemplars that can be used, the phases in the task may not be limited, that is, for example, more ID shifts can be introduced, and hence the formation of attentional sets can be used as an internal task control.

1.3 Neural correlates of cognitive flexibility in humans and rodents

The neural correlates of cognitive flexibility have been elucidated using task switching or set shifting paradigms, in combination with functional MRI (fMRI). Cognitive flexibility is a top-down EF that involves several components, such as salience detection, inhibition, working memory, and inhibitory control. Therefore, identifying brain areas associated with specific components of cognitive flexibility poses a challenge (Uddin, 2021). Therefore, several studies have shown overall that the lateral frontoparietal cortex and the midcingulo-insular network are involved in supporting executive function and cognitive flexibility overall. For example, the salience network, particularly the anterior insula or dorsal ACC, plays a role in detecting salience of stimuli. Furthermore, the dorsal attention network, consisting of the intraparietal sulcus and frontal eye fields, is thought to underlie top-down processing and the ventral attention network, comprised of right tempo-parietal junction and ventrolateral prefrontal cortex (vlPFC), underlies bottom-up attention. Additionally, studies that implemented paradigms that required use of working memory where participants had to utilize two or more rule representations also implicated the executive control network, which is the dorsolateral prefrontal cortex (PFC), vlPFC, premotor, and parietal cortices. Furthermore, studies have shown the right anterior insula, the right inferior frontal junction, and the right vlPFC in inhibitory control (Dajani and Uddin, 2015).

However, although fMRI studies in humans have implicated several brain regions in cognitive flexibility, rodents and non-human primates are a useful animal model that can be used to elucidate the role of specific brain regions associated with different components of cognitive flexibility. For example, Robbins (2007) showed that lateral PFC lesions result in impaired extradimensional set shifting; however, OFC lesions result in reversal learning deficits. Similarly, OFC lesions in rats resulted in deficits in reversal learning as indicted by increased perseverative responses in the reversal phase, that is, animal failed to apply the new rules (McAlonan and Brown, 2003). Additionally, lesions of the ACC and medial PFC (mPFC) resulted in impairments in intradimensional set shifting and extradimensional set shifting, respectively (Ng et al., 2007). Furthermore, dorsomedial striatal lesions impaired both reversal learning and strategy switching, such that the animals could not maintain a new choice pattern once they had already made the selection, pointing to its role in regressive errors (Ragozzino, 2007).

1.4 Cognitive flexibility and chronic jet lag

Several studies have shown cognitive flexibility deficits in neuropsychiatric disorders, such as schizophrenia, ASD, or ADHD. Furthermore, several studies have shown an association between chronic sleep deprivation and decreased cognitive performance, such as impairments in working memory, attention, and decision making in humans (Alhola and Polo-Kantola, 2007). Cho (2001) showed that airline crew who experienced repeated jetlag, i.e., traveled frequently across time zones and had a shorter time to recover showed poorer cognitive performance on a visual spatial task. An explanation for these deficits is that during jetlag, a shifted light dark (LD) cycle results in a desynchronization of internal physiological processes among themselves as well as with the external environment. After travel across multiple time zones, circadian rhythms do not set immediately and remain more closely entrained to the LD cycle in the original time zone than to the destination time zone (Walker et al., 2020). However, although a traveler's circadian rhythm eventually adapts to the LD cycle of their destination, airline crew, such has pilots who are required to travel frequently across multiple time zones may experience symptoms of chronic jet lag (CJL). Thus, this frequent circadian desynchronization has many effects, such as daytime sleepiness, fatigue, and impaired alertness, and initiation and maintaining of sleep, which are thought to be a result of the failure of the internal circadian rhythm to adjust to the shift in LD cycle at the destination of the individual (Suhner et al., 1998; Vosko et al., 2010). Additionally, other effects of chronic jet lag including decreased hippocampal neurogenesis, cognitive deficits, such as object recognition memory, and depressive

behaviors were observed to be worse when conditions of eastward travel were simulated rather than westward travel in rats. Furthermore, Iggena et al. (2017) showed that melatonin restored hippocampal neural precursor cell proliferation and prevented cognitive deficits, which was spatial memory, when administered in the final week of a shift in the LD cycle.

2 OBJECTIVES

Although there exist studies in humans and mice showing the cognitive deficits due to chronic jetlag, there are no studies in mice addressing the effects of CJL on cognitive flexibility, particularly, attentional set shifting. Therefore, the current study aimed to use an established CJL paradigm to assess the effect of chronic advances of the dark phase by 6 hours (eastward travel) on cognitive flexibility as measured using the ASST in mice. Thus, the aims were to identify the effects of CJL specifically on reversal learning and attentional set shifting, whether these effects are sex specific, and whether the duration of CJL, either 1 or 2 months has effects. Furthermore, the objective of this study was also to develop a technical protocol and a CJL mouse model that can be used to test pharmacological interventions and elucidate the neural mechanisms underlying the effects of chronic jetlag on cognitive flexibility. We hypothesized that CJL would have effects on cognitive flexibility as indicated by increased number of trials required to complete the task in the reversal and EDS phases.

3 METHODS

3.1 Animals

C57BL/6J mice (N: male, 18; female, 16) aged 10–14 weeks were used for the experiment. Mice were from the breeding colony of the Institute for Pharmacology and Toxicology (Origin: Charles River, Sulzfeld, Germany) and housed in groups of approximately four per cage under controlled humidity (50-55%) and temperature conditions (22±2°C). Food and water were provided *ad libitum* except 1 week prior to and during the ASST. During this period, mice were food restricted (2 g/mouse) and a basal weight of 85-90% was maintained. A standard LD cycle (12:12 h) was followed for the control animals whereas a chronic LD shift (LDS) paradigm was followed for the test animals as described in section 3.3. All the experiments were conducted during the light phase and were performed in compliance with European guidelines regarding the care and use of animals for experimental procedures (2010/63/EU) as well as ethical approval of local authorities (Landesverwaltungsamt Sachsen-Anhalt; Az. 42502-2-1618 Uni MD).

3.2 Attentional set-shifting task

3.2.1 Experimental setup

The custom-made ASST apparatus (41 cm × 22 cm × 24 cm) consisted of a waiting area with two transparent doors leading to two separate identical choice compartments (see Figure 1). Three bowls were placed in the setup: one was placed in the waiting compartment and contained water and the other two were placed separately in each choice compartment, and contained the digging medium to be used for testing. Additionally, a filter paper with odorant was stuck to the outside of the bowls in the relevant trials; the digging medium and the odors (Table 1; Sigma-Aldrich Chemie GmbH, Germany) were the two-dimensional cues. Further, the reward, which was half a chocolate rice pellet (Nordgetreide GmbH & Co. KG, Lübeck, Germany), was placed in either one of the bowls. Additionally, the powdered form of this reward was sprinkled at the bottom of the bowls to ensure that both the bowls smelled like chocolate rice and that the mice select the correct bowl based on the odorant or medium used in the task rather than the odor of the reward. During the habituation and testing phases, the mouse was kept in the waiting compartment. As pre-planned in a pseudo-randomized manner, on some trials, either the left door to the choice compartment was opened first followed by the right door once the mouse entered through the left door, or vice versa, or both doors were opened simultaneously to allow the mouse to enter the choice compartments and make a decision.

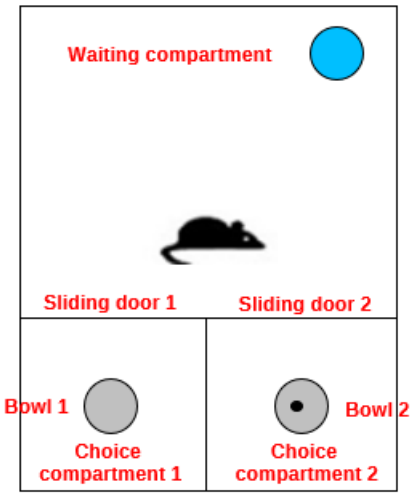

Figure 1: *ASST apparatus used in the study*. The apparatus consisted of a waiting compartment with two choice compartments. The mice could access the choice compartments once the sliding doors were opened. Of the three bowls, one of the bowls in the choice compartments was rewarded.

3.2.2 Habituation phase

The aim of this phase was to habituate the mice to experimenter handling, the apparatus, and the test protocol. Mice were first habituated to handling for 1 week. Thereafter, during the week of food restriction, they were habituated only to the reward, the apparatus, and the reward and water bowls used in the apparatus. To habituate the mice to the reward, bowls used in the task were placed in the home cage and the reward was added throughout both these bowls containing bedding and additionally sprinkled in the cage. On the next day, the entire cage of mice together was habituated to both the apparatus (group habituation) and bowls containing home cage bedding placed in each compartment, which both contained the reward sprinkled throughout the bowls; this session lasted 45 minutes. On the following day, a single mouse was trained to dig out the reward from the bottom of either one of the bowls containing home cage bedding (single habituation). The mice were first trained to retrieve the reward from the top of either one of the bowls and the reward was placed at increasing depths depending on how successfully the mice retrieved the reward in preceding trials. The training session was terminated when the mouse successfully retrieved the reward from the bottom of the bowl.

3.2.3 Testing

3.2.3.1 Two-dimensional cues used in the task

Olfactory (odor) and visuo-tactile stimuli (digging medium) were used as the two-dimensional cues. Exemplars of the odors and digging media used are shown in Table 1. Odors were diluted 1:20 in paraffin oil (PanReac AppliChem, IL, USA) and 20 µl of this solution was used. Depending on the testing phase, the mice had to differentiate either between two odors or between two media (see below).

Table 1. Exemplars of odors and digging media used in the ASST

Odor ID	Odor	Digging medium ID	Digging medium
O1	Valeric acid	M1	Big sand ball
O2	S-(+)-carvone	M2	Small sand ball
O3	Eucalyptol	M3	Big white grains
O4	2-Phenylethanol	M4	Small white grains
O5	Citral	M5	Big green pearl
O6	R-(+)-carvone	M6	Small green pearl

3.2.3.2 ASST phases

During the test days, the bowls contained the dimensional cues relevant for the task, and only one of the bowls had the reward. The rewarded and unrewarded bowls were placed in the choice compartments and for each trial, either one door or both the doors to the choice compartments were opened to allow the mice access. This was determined a priori in a pseudorandomized manner. The crux of the task is that the mice had to learn which dimensional cue of the bowl was associated with the reward. The test started with odor as the relevant dimension for half of the mice and digging medium as the relevant dimension for the other half, and these including the exemplars were balanced across the groups of mice tested for both males and females. The first four trials were free trials for all phases unless mentioned otherwise, where the reward was placed at the top of the medium, and these were not included in final result; both the doors were opened to allow the mice to properly explore both the compartments. For the subsequent trials, when mice had made their bowl choice, as indicated by the mouse digging the bowl, the door to the bowl in the other compartment was closed. A successful trial was considered when the mouse dug and retrieved the reward and an error trial was considered when

the mouse dug the unrewarded bowl. Six consecutive successful trials indicated the end of a phase and the final trial was noted as the trial to criterion.

The ASST consists of seven phases (see Table 2): simple discrimination (SD), compound discrimination (CD), IDS and EDS with the latter three phases also including reversal phases (R1, R2, R3).

Each phase and their reversal were performed on the same day and therefore the test lasted 4 days. On testing Day 1, in the simple discrimination phase, mice had to learn to differentiate between two different types of odor or digging media. If odor was used, the mice had to dig out the reward from a bowl containing bedding. On testing Day 2, CD and its reversal, each bowl had both a relevant (e.g. odor) and irrelevant dimension (e.g. digging medium), and mice had to focus on the odor type, the relevant dimension, which was associated with a reward. For example, if odor was the relevant starting cue, the associated bowl additionally contained a digging medium as the irrelevant cue. The reversal phase was then performed on the same day, where the type of odor not previously associated with a reward was now rewarded. None of the reversal phases had free trials. Further, the type of odor or digging medium used in the SD phase was also used as the relevant cue in the CD phase. The ID phase and its reversal (R2) were performed on testing Day 3. Here, although the relevant dimension and irrelevant dimensions were the same as those in the previous phase, the exemplars of odor and digging medium changed. On Day 4, the ED phase and its reversal was performed. Here, in addition to the change in exemplars of odor or digging medium, the previously irrelevant dimension was now associated with the reward whereas the previously relevant dimension was unrewarded. For example, if odor was the previously rewarded dimension, digging medium was now the relevant dimension. The ED phase did not include a free trial. For all phases, if the mouse did not dig the bowl for 10 minutes, the experiment was paused and resumed after not more than an hour.

Table 2. Example of an ASST protocol				
ASST phase	Relevant dimension	Irrelevant dimension	Rewarded combination	Unrewarded combination
Simple discrimination (SD)	Odor		**O5**	O6
Compound discrimination (CD)	Odor	Medium	**O5**M1/**O5**M2	O6M1/O6M2
Compound discrimination reversal (R1)	Odor	Medium	**O6**M1/**O6**M2	O5M1/O5M2
Intra-dimensional shift (ID)	Odor	Medium	**O1**M5/**O1**M6	O2M5/O2M6
Intra-dimensional shift reversal (R2)	Odor	Medium	**O2**M5/**O2**M6	O1M5/O1M6
Extra-dimensional shift (ED)	Odor	Medium	O3**M3**/O4**M3**	O3M4/O4M4
Extra-dimensional shift (R3)	Medium	Odor	O3**M4**/O4**M4**	O3M3/O4M3

Table 2: *Example of an ASST protocol*. Cues of two dimensions were used in the ASST, one odor (O, olfactory cue) and the other, digging medium (M, visuo-tactile cue). The protocol shown here has odor as the starting dimension and bedding as the digging medium. The protocol shows the seven phases of the ASST and the different materials used through the phases. The relevant dimensions are in bold.

3.3 Chronic jet lag paradigm

The chronic LD shift paradigm from Horsey et al. (2019) was used to simulate CJL (CJL) in this experiment. In this LDS protocol, the light period was shortened by 6 h to simulate the effects of eastward travel (phase advance), yielding four shifts that were each maintained for a 1-week duration. The shift always occurred at the beginning of the week (Monday). The experimental groups were control (CON), 1-month CJL (1MCJL), and 2-month CJL (2MCJL). The 1MCJL mice underwent 6-h shifts that lasted 4 weeks whereas the 2MCJL mice underwent a 6-h shifts that lasted 8 weeks over the course of the study. The standard and shifted LD cycles are shown in Fig. 2. Habituation to handling and the apparatus was always performed during the light phase during the 3rd and 4th LD shift respectively. The ASST test was conducted on week 5 and week 9, after the shifted LD cycle returned to the duration of the standard LD cycle. Furthermore, the LDB test was performed on week 6 and week 10 of the shifted LD cycle, during the light phase.

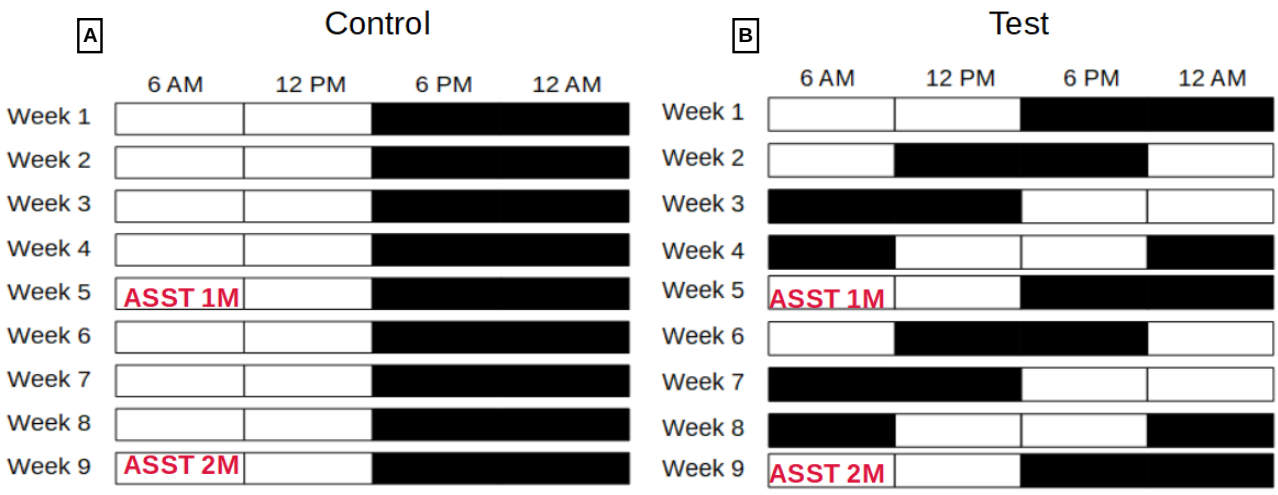

Figure 2: *LD cycles used in the experiment*. A standard LD cycle **(A)** was used as a control and a shifted LD cycle **(B)** was used to simulate CJL. Animals underwent simulated CJL for either 4 weeks or 8 weeks. In week 1, the animals were allowed to habituate to the room and the light. The ASST test was performed in week 5 and week 9 during the light phase (symbol) and the LD box test was performed during week 6 and week 10 (not shown) during the light phase for 1MCJL and 2MCJL animals, respectively.

3.4 Light-dark box test for anxiety

The LD box (LDB) test for anxiety was conducted on the first day of the week following the ASST test (week 6 or 10, respectively), during the light phase, to test the effect of a shifted LD cycle on anxiety. The LDB (49.5 cm × 49.5 cm × 41.5 cm) consisted of a light compartment and a dark compartment, with a door separating them to allow the mice to pass through. Mice were placed in the dark compartment and were allowed to explore the entire box for 10 minutes. The test was recorded using a camera and the videos were analyzed using Ethovision XL 12 software (Noldus, Wageningen, The Netherlands). The percentage total time spent in the light box of the total time spent in the entire box as well as the frequency of crossings was used as the outcome measure.

3.5 Statistical analysis

For statistical analysis, Prism 9.0 (GraphPad Software Inc. LaJolla, USA) and Systat 13.0 (Systat software GmbH, Düsseldorf, Germany) were used. All statistical tests except for three-way ANOVA were conducted using GraphPad Prism. The measures of cognitive flexibility were number of trials to criterion, total number of errors made until the criterion was reached, number of perseverative errors,

and number of regressive errors. Additionally, the measures of anxiety in the LDB test were time spent in the light compartment and frequency of crossings. Total errors in the reversal phases and extra-dimensional shift phase were categorized as perseverative and regressive and were calculated using logistic regression in GraphPad Prism. Briefly, every trial attempted by a particular animal was assigned a value of "1" for a successful trial or "0" for an unsuccessful trial and regressed by trial number. A logistic curve of best fit was generated, which represented the probability of a correct response with respect to trial number, and the trial number after which the value of this curve became greater than or equal to chance performance value of 50% was noted. All the errors that on or before this trial were characterized as perseverative errors, as they occurred because the animal followed the old rule with greater than chance probability. Further, regressive errors were those that occurred after this trial, as these errors were made after the animal had disengaged from the previous rule and was in the process of acquiring the new rule.

Normality of the data were tested using the Kolmogorov Smirnov test. Some of the data were not normally distributed and all attempts to transform the data were made, but failed. Hence, all the results from the post-hoc tests were confirmed using Kruskal-Wallis tests. To assess for sex differences, that is interaction between sex, group, and phase, a three-way ANOVA was used. To assess for interactions between group and phase, two-way ANOVAs were used. Ordinary one-way ANOVAs were used to assess for differences among the groups irrespective of sex and phase. Post-hoc Dunnett's tests were used for the two-way ANOVAs. A value of $p < 0.05$ was considered significant.

4 RESULTS

The effect of CJL on cognitive flexibility was assessed using the number of trials required to complete an ASST phase (trials to criterion) and number of errors made until the trials to criterion was reached. Furthermore, the errors made on the task were categorized into perseverative errors and regressive errors. In total, 11 mice (males = 6, females = 5) were used for the experiment in each group, namely the CON, 1MCJL, and 2MCJL.

4.1 Trials to criterion

The criterion to complete an ASST phase was defined as six consecutive correct responses. The mean number of trials to criterion for all mice, and male and female mice are depicted in Figure 3. CJL impaired ASST performance as shown by a generally increased number of trials to criterion ($F_{(2,30)}$ = 8.78, p = 0.001; Fig 3A). Post-hoc comparisons revealed that the main effect of group was based on effects in the 2MCJL group ($F_{(2, 30)}$ = 8.783, p = 0.0010). As shown in figure 3B, mice in this group took significantly more trials to reach the criterion in the IDS (t(11) = 3.25; p = 0.01) and R3 (t(11) = 2.50; p = 0.03) phases. Further, a trend was observed for CD (t(11) = 2.34; p = 0.07) and R1 (t(11) = 2.17; p = 0.08) phases. As expected, the performance of the mice differed among the ASST phases ($F_{(2.38,71.35)}$ = 5.49, p = 0.004). CJL effects were not specific to particular ASST phases (group × phase: $F_{(12,180)}$ = 0.74, p = n.s.). A three-way ANOVA using phase as a within-subject factor and sex and group as between-subject factors did not reveal any significant sex-specific effects of CJL (sex: $F_{(1, 27)}$ = 0.214, p = n.s; sex × group × phase: $F_{(12, 162)}$ = 0.449, p = n.s).

Figures 3C-F illustrate the trials to criterion separately for male and female mice. These data indicate that the effects of CJL are more robust in female mice, since 2MCJL resulted in a generally increased number of trials to criterion ($F_{(12, 162)}$ = 6.522 p = 0.0121), thus indicating impaired ASST performance (Fig. 3E, F).

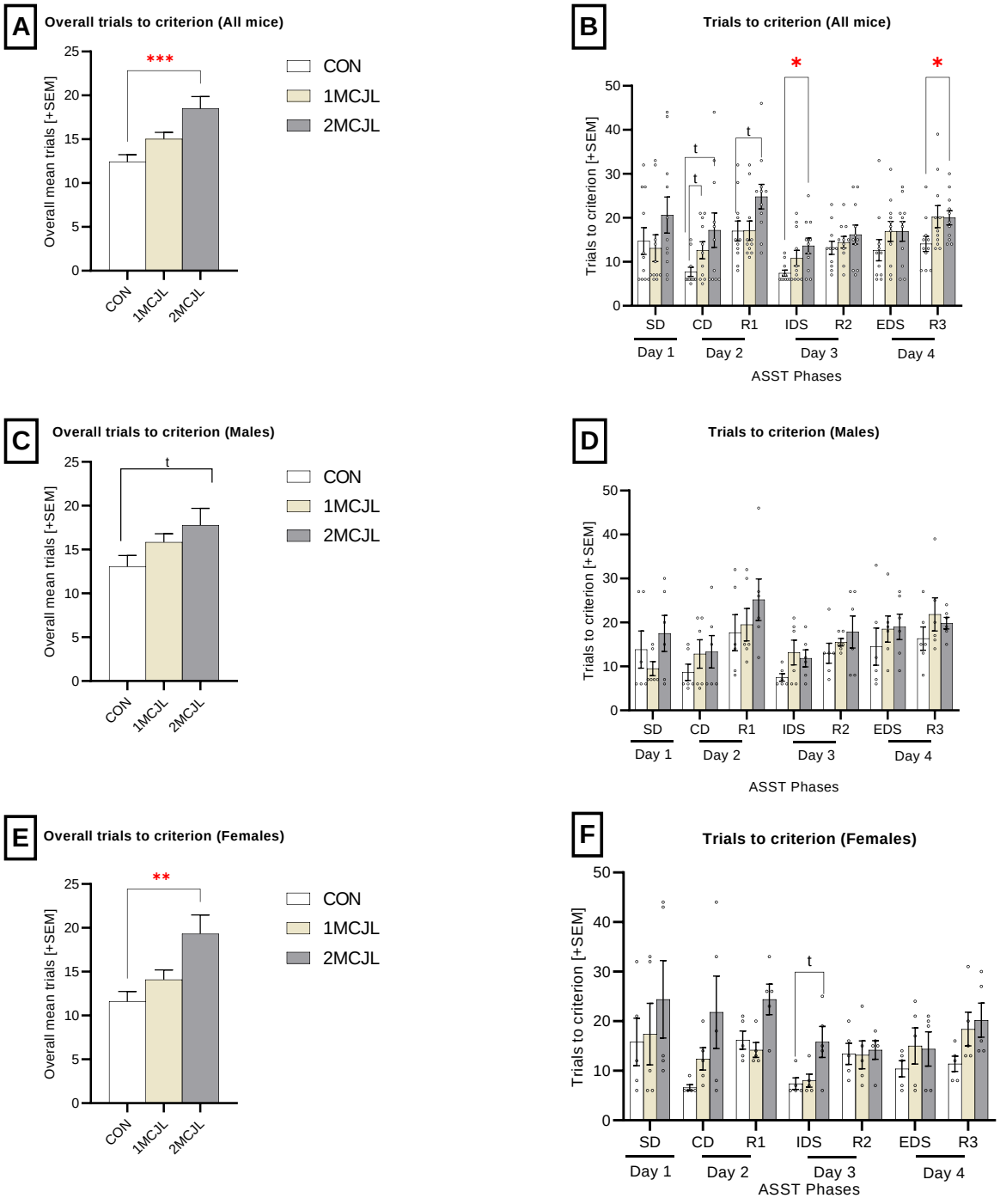

Figure 3: *Effect of CJL on trials required to reach criterion in the ASST*. Data are represented for all mice (A, B), male (C, D) and female mice (E, F). Panels A, C, and E represent the effects of CJL on the mean number of trials required to reach criterion in all ASST phases. Data are represented as mean + SEM; the dots represent the data from individual animals. *p < 0.05, ** p < 0.01, ***p < 0.001, t < 0.10 (post-hoc Dunnett's test after significant effects in ANOVA).

4.2 Errors to criterion

Total errors to criterion were defined as the number of errors that the animals made until they reached six correct consecutive responses. The mean errors to criterion for all mice, as well as for male and female mice are depicted in Figure 4. CJL impaired ASST performance as shown by generally increased errors to reach trials to criterion ($F_{(2,30)} = 6.847$, $p = 0.0036$). As expected, the performance of the mice differed among the ASST phases ($F_{(2.634,\ 79.02)} = 11.50$, $p < 0.0001$). However, the effects of CJL were not specific to particular ASST phases (group × phase: $F_{(12,180)} = 0.9916$, p = n.s.). Post-hoc comparisons (ordinary one-way ANOVA followed by post-hoc comparisons) revealed that the main effect of group was based on effects in the 2MCJL group ($t(11) = 1.512$; $p = 0.0018$; Fig. 4A). As shown in figure 4B, mice in this group took significantly more trials to reach the criterion in the ID ($t(11) = 3.504$, $p = 0.0061$) and R1 ($t(11) = 2.493$; $p = 0.0401$) phases. Further, a trend was observed for CD ($t(11) = 2.185$; $p = 0.0902$). A three-way ANOVA using phase as a within-subject factor and sex and group as between-subject factors did not reveal any significant sex-specific effects of CJL (sex: $F_{(1,\ 27)} = 1.539$, p = n.s; sex × group × phase: $F_{(12,\ 162)} = 0.469$, p = n.s).

Figure 4C-F illustrate the errors to reach trials to criterion separately for male and female mice, respectively. An ordinary one-way ANOVA followed by a post-hoc test revealed that the effects of CJL are more robust in female mice, since 2MCJL resulted in a generally increased errors to trial ($t(5) = 3.989$, $p = 0.0034$), thus indicating impaired ASST performance (Fig. 4E, F).

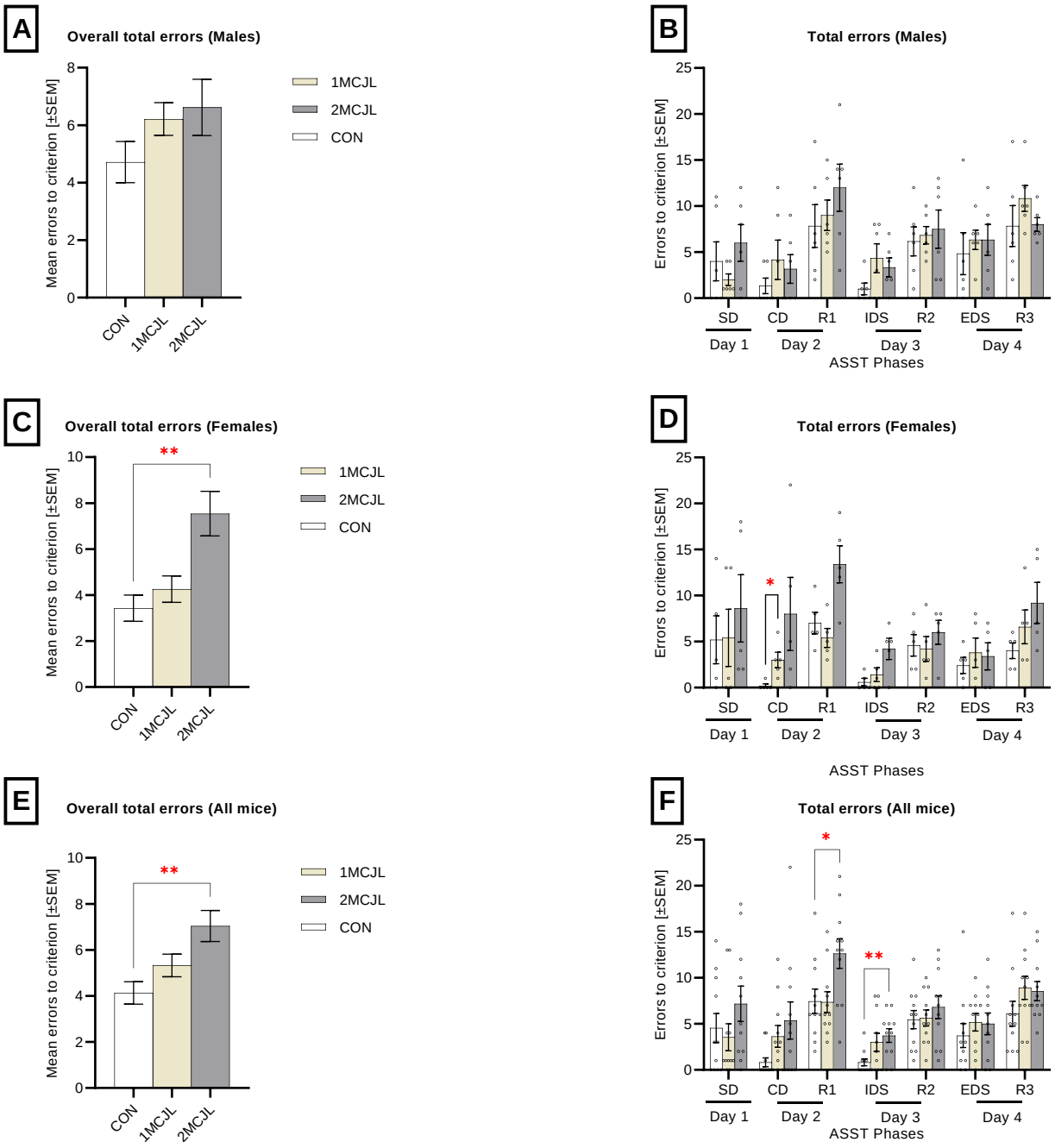

Figure 4: *Effect of CJL on total errors made until trials to reach criterion in the ASST*. Data are represented for all mice (A, B), male (C, D) and female mice (E, F). Panels A, C, and E represent the effects of CJL on the mean number of errors made until the criterion was reached in all ASST phases. Data are represented as mean ± SEM; the dots represent the data from individual animals. *$p < 0.05$, ** $p < 0.01$, (post-hoc Dunnett's test after significant effects in ANOVA).

4.3 Perseverative errors

Perseverative errors were assessed for the R1, R2, and R3 phases, and the ED phase. The number of perseverative errors made for all mice, as well as for male and female mice are depicted in Figure 5. CJL did not have any effects on the number of perseverative errors made ($F_{(2, 30)} = 1.924$, p = n.s.; Fig. 5A), but there was a trend to interaction between group and phase (group × phase: $F_{(6, 90)} = 2.151$, $p = 0.0552$). The number of perseverative errors that the mice made differed among the ASST phases (phase: $F_{(2.232, 66.96)} = 16.19$, $p < 0.001$). Ordinary one-way ANOVA did not reveal any effects of CJL on the number of perseverative errors overall ($F_{(2, 30)} = 1.924$, p = n.s.; Fig. 5A). A three-way ANOVA using phase as a within-subject factor and sex and group as between-subject factors did not reveal any significant sex-specific effects of CJL (sex × group × phase: $F_{(6, 81)} = 0.971$, p = n.s.). Of note, there was a main effect of sex, i.e. female mice had less perseverative errors than male mice ($F_{(1, 27)} = 5.412$, $p = 0.023$). However, there was no interaction between group and sex (group × sex: $F_{(6,81)}$, p = n.s.).

Further, an analysis of the females separately revealed that CJL generally resulted in higher perseverative errors and that this effect was significantly more pronounced in some ASST phases (group × phase: $F_{(6,36)} = 2.834$, $p = 0.0483$; phase, $F_{(2.204, 26.44)} = 13.26$, $p < 0.001$; group, $F_{(2,12)} = 8.039$, $p = 0.0061$; Fig. 5D). Specifically, post-hoc multiple comparisons revealed more perseverative errors in the R1 phase in the 2MCJL group ($t(5) = 3.303$, $p = 0.0268$). Figure 5C and 3D illustrate the effects of CJL on perseverative errors separately for male and female mice.

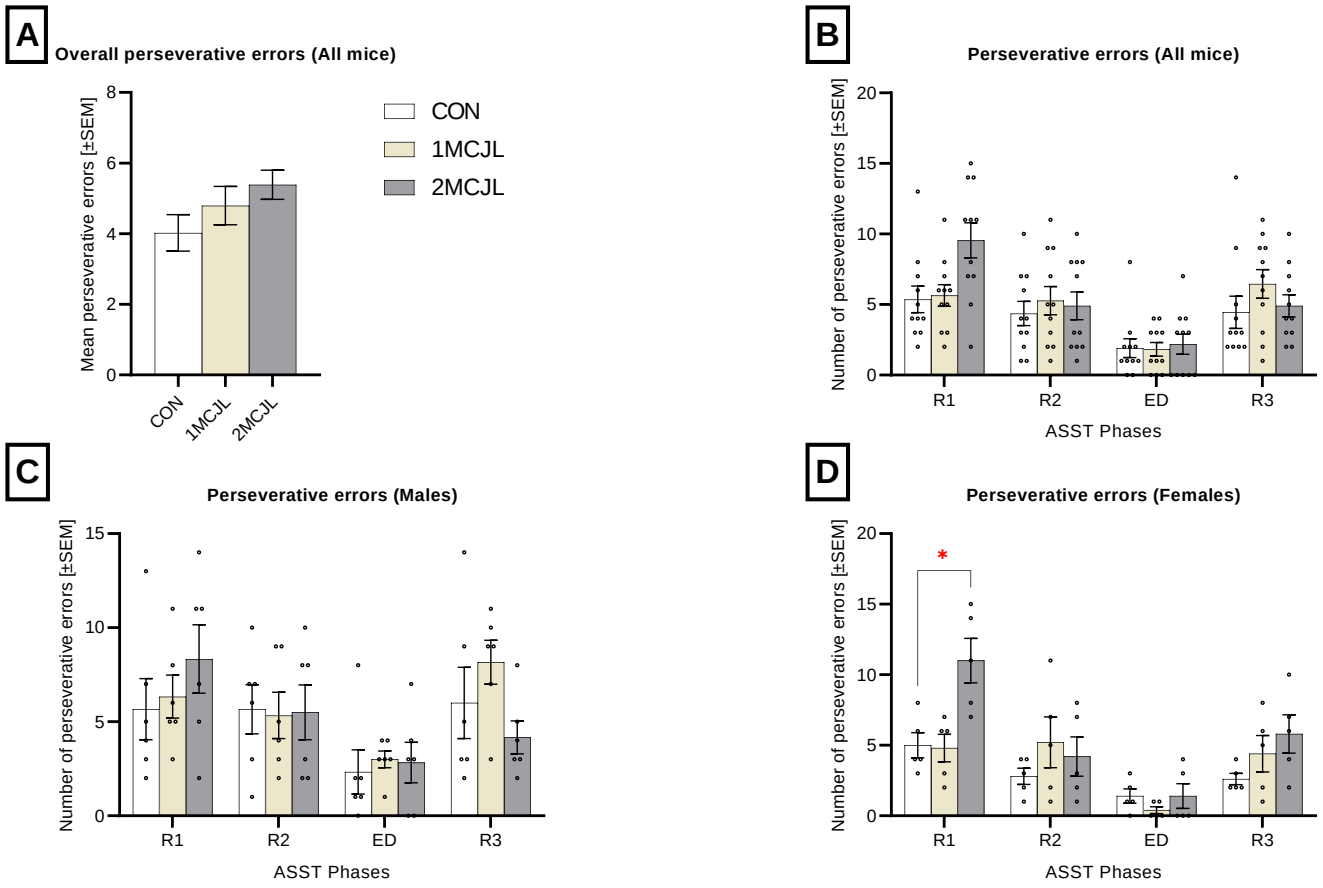

Figure 5: *Effect of CJL on perseverative errors in the R1, R2, R3, and ED phases of the ASST* for all mice (B), males (C) and females (D). Panel A represents the effect of CJL on the mean number of perseverative errors. Data are represented as mean ± SEM *p < 0.05 (post-hoc Dunnett's test for multiple comparisons was conducted after significant main effects were obtained in two-way ANOVA).

4.4 Regressive errors

Regressive errors were assessed for the R1, R2, and R3 phases, and the ED phase. The effect of CJL on regressive errors made in the ASST for all mice, as well as for male and female mice are depicted in Figure 6. An ordinary one-way ANOVA revealed a trend in the effects of CJL overall ($F_{(2, 30)}$, p = 0.0691), and a post-hoc analysis revealed that the 2MCJL group contributed to these effects (t (11) = 2.332, p = 0.0489; Fig. 6A). The effects of CJL were not specific to a particular phase (group × phase: $F_{(6, 90)}$ = 0.5580, p = n.s.). Further, the number of regressive errors made did not differ significantly among the phases ($F_{(2.258, 67.75)}$ = 1.857, p = n.s.; Fig. 6B). A three-way ANOVA using phase as a within-subject factor and sex and group as between-subject factors did not reveal any significant sex-specific effects of CJL on regressive errors made (sex: $F_{(6, 81)}$ = 2.632, p = n.s.; sex ×

group × phase: $F_{(6,81)} = 0.529$; p = n.s.). Figure 6C and 6D illustrate the effects of CJL on perseverative errors separately for male and female mice.

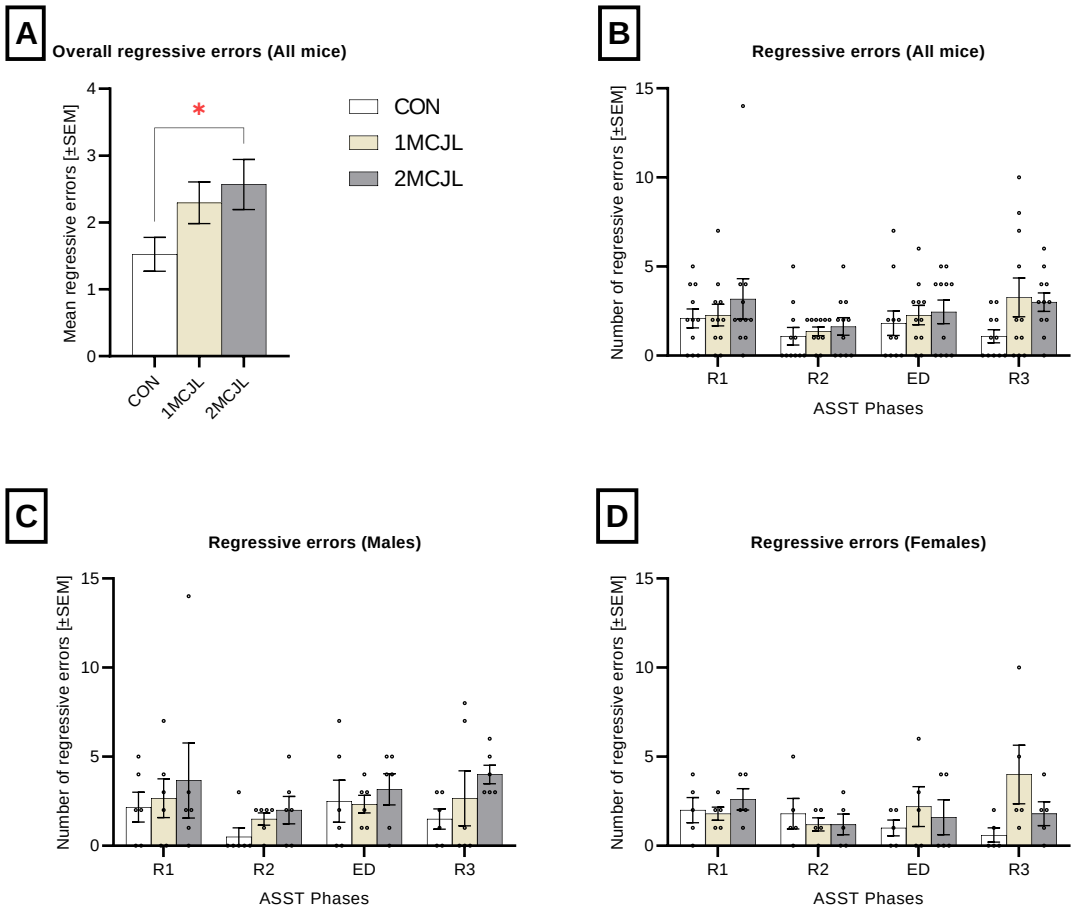

Figure 6: *Effect of CJL on regressive errors in the R1, R2, R3, and ED phases of the ASST* for all mice (B), males (C) and females (D). Panel A represents the effect of CJL on the mean number of regressive errors. Data are represented as mean ± SEM *p < 0.05 (post-hoc Dunnett's test for multiple comparisons was conducted after significant main effects were obtained in two-way ANOVA).

4.5 Light-dark box test

The light box test was used to assess for anxiety-like behavior in the control, 1MCJL, and 2MCJL groups. The test was conducted during the light phase on Day 1 and Day 4 of the CJL paradigm, in the week following the ASST after a 6-h advance of the dark phase. These days coincided with the SD and ID phases of the ASST. The parameters assessed were percentage of time spent in the light compartment and frequency of crossings from the dark to the light compartment and vice versa.

The percentage time spent in the light compartment for all mice, as well as for male and female mice are depicted in Figure 7. CJL did not have any effects on the percentage time spent in the light compartment either on Day 1 or Day 4 (day × group: $F_{(2, 30)} = 1.710$, p = n.s.; day: $F_{(1,30)} = 0.1516$, p = n.s., group: $F_{(2, 30)} = 1.212$, p = n.s.; Fig. 7A). Further, a three-way ANOVA using day as a within-subject factor and sex and group as between-subject factors did not reveal any significant sex-specific effects of CJL (sex: $F_{(1, 27)} = 0.446$, p = n.s; sex × group × phase: $F_{(1, 27)} = 1.086$, p = n.s). Figure 7B and 7C illustrate the percentage time spent in the light compartment separately for male and female mice.

The frequency of crossings from the light compartment to the dark compartment and vice versa for all mice, as well as for male and female mice are depicted in Figure 8. An interaction between day and group was observed for the frequency of crossings (group: $F_{(2, 30)} = 4.620$, p = 0.0178; day: $F_{(1,30)} = 7.707$, p = 0.0094; day × group: $F_{(2, 30)} = 5.035$, p = n.s; Fig. 8A). A post-hoc comparisons test revealed that 2MCJL resulted in an increase in frequency of crossings on Day 4 ($t(11) = 4.181$, p = 0.0002). A three-way ANOVA using day as a within-subject factor and sex and group as between-subject factors did not reveal any significant sex-specific effects of CJL (sex: $F_{(1, 27)} = 0.932$, p = n.s; sex × group × phase: $F_{(1, 27)} = 3.189$, p = n.s). Figure 8B and 8C illustrate the frequency of crossings from light compartment to the dark compartment and vice versa separately for male and female mice.

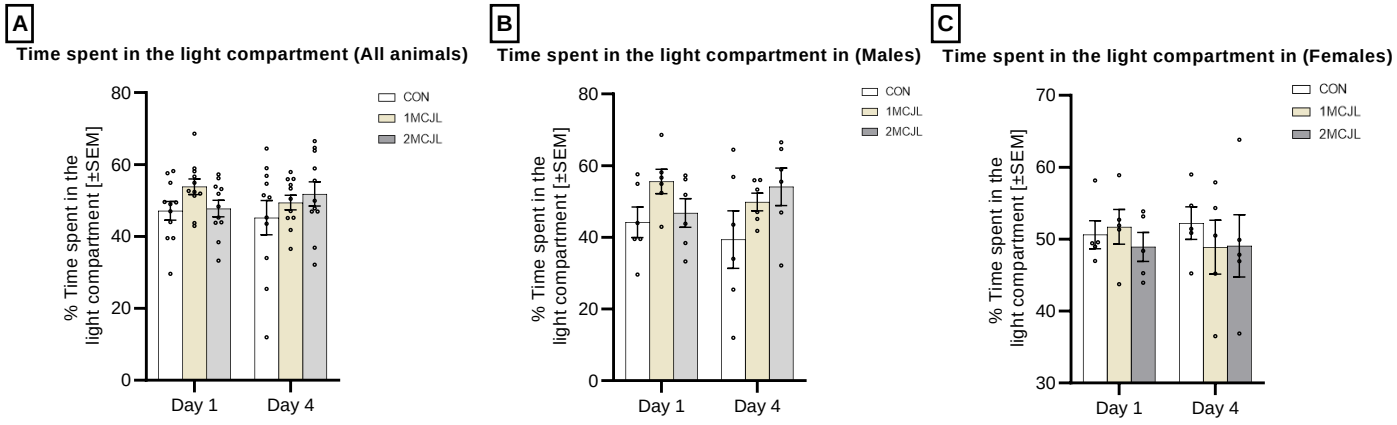

Figure 7: *Effect of CJL on time spent in the light compartment* for all mice (A), males (B) and females (C). Data are represented as mean ± SEM (post-hoc Dunnett's test for multiple comparisons was conducted after significant main effects were obtained in two-way ANOVA).

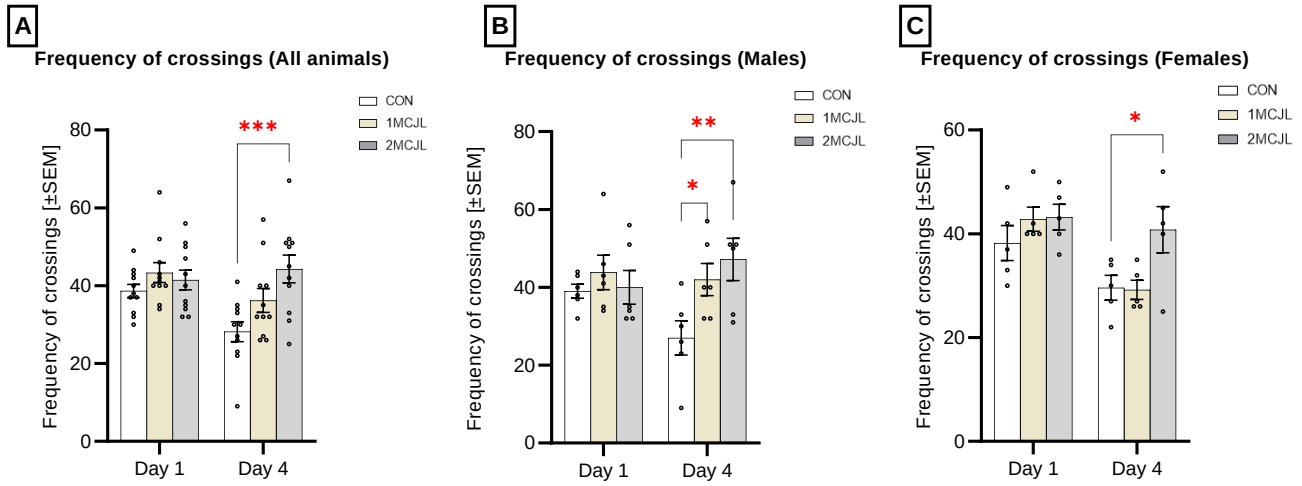

Figure 8: *Effect of CJL on frequency of crossings from the light compartment to the dark compartment and vice versa* for all mice (A), males (B) and females (C). Data are represented as mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05 (post-hoc Dunnett's test for multiple comparisons was conducted after significant main effects were obtained in two-way ANOVA).

5 DISCUSSION

In this study, we adapted a CJL paradigm to study its effects on cognitive flexibility, measured using the ASST, in mice (Horsey et al., 2019). Data were compared among control, 1MCJL, and 2MCJL groups. Overall, 2MCJL increased the number of trials taken to complete the ASST and additionally, the number of errors made. Furthermore, 2MCJL increased the number of perseverative errors that the females made in the R1 phase; the number of regressive errors overall in the reversal and EDS phases combined were also higher in the 2MCJL group. Further, the light dark box test did not reveal effects of CJL on anxiety levels; however, the frequency of crossings was higher on Day 4 of the paradigm in the 2MCJL group. Thus, overall, 2MCJL had negative effects on performance in the ASST. However, in general, across all the parameters examined, no sex-specific effects were observed likely owing to the low statistical power.

5.1 Effects of chronic jet lag on cognitive flexibility

The current study revealed that compared with the control and 1MCJL, 2MCJL had a negative effect on overall performance in the ASST, in that, the number of trials taken to complete the task and the number of errors made were higher. These results are in line with those of the study by Cho et al. (2000) in humans. In this study, airline crew divided into groups based on their experience between 1-4 years of transmeridian flying were tested on a delayed match-to-sample memory visual task, and this revealed that airline crew with 4 years of flying experience had the least number of correct responses. Thus, a longer duration of CJL has negative effects on cognitive tasks.

In the ASST, in addition to the increased number of trials taken to complete a task and number of errors made, perseverative and regressive errors are considered an indication of cognitive inflexibility. Perseverative errors occur when humans and animals become stuck in set, such that they cannot abandon a previously learned response strategy and switch to a new one in response to negative feedback in either the reversal phases or the EDS phase. Such errors are generally observed in individuals with ASD and ADHD (Lawrence et al., 2004; Landry and Mitchell, 2021). Additionally, a study on human participants also showed that compared with day workers, shift workers who also had poorer sleep quality made more perseverative errors on the Berg's card sorting task (Elhami Athar et al., 2020). In this study, the number of perseverative errors did not differ significantly among groups when the data were analyzed for all animals together. However, when the data from females were analyzed separately, a significant increase in the number of perseverative errors was observed in the R1 phase in the 2MCJL group than in the control and 1MCJL groups. This is in line with a study that

showed that following a LD cycle shift, female rats made more perseverative errors; however, these errors were observed in the EDS phase in that experiment because the set-shifting experiment only consisted of an acquisition phase and an EDS phase (Robertson et al., 2017). However, a three-way ANOVA comparing male and female mice did not reveal that 2MCJL had sex-specific effects on perseverative errors likely because of the low sample size of 5 female and 6 male mice. It is likely that in line with the results of previous studies, an increase in sample size will yield sex-specific effects of CJL on perseverative errors, following a three-way ANOVA analysis.

Additionally, the results in this study show that 2MCJL resulted in increased regressive errors overall in the reversal phases and the EDS phase combined. This indicates that the animals in the 2MCJL group, after initially shifting to a new response strategy, failed to maintain the new strategy and instead reverted to the previously learned response generally over all the phases (Miller et al., 2015). However, the effects of CJL were not specific to any one of the three reversal phases or the extra-dimensional shift phase likely owing to low statistical power. To the best of my knowledge, no previous studies assessed the effects of sleep deprivation on regressive errors. Therefore, the implications of the effects of regressive errors in individuals with sleep deprivation need to be assessed.

Previous studies have shown that a circadian disruption or even exposure to dim light during the nocturnal phase increases anxiety-like behaviors in mice (Horsey et al., 2019). However, the LDB test conducted in the current study did not reveal effects of CJL on anxiety because the mice did not spend significantly higher duration in the light compartment than in the dark compartment. However, animals who underwent 2MCJL had increased frequency of crossings from the dark compartment to the light compartment and vice versa. This could be an indication of hyperactivity or impulsivity (Zaichenko et al., 2012). Considering that impulsivity is also an executive function mediated by the PFC and has implications in cognitive flexibility and attention and therefore performance of aircrew, further tests such as the 5-Choice Serial Reaction Time Task (5-CSRTT) should be conducted to assess for this (Kim and Lee, 2011).

5.2 Potential neural correlates of chronic jet lag and cognitive flexibility

The current study shows that chronic shifts in LD cycle, particularly for 2 months, influence cognitive flexibility as indicated by an increased number of trials required to complete a task, increased number of errors made, and higher number of regressive errors in all animals, and higher perseverative errors in female animals. Specifically, 2MCJL consistently resulted in an increased number of trials to reach the criteria for completing the ASST and number of errors until criterion in the ID phase. Thus it

could be tempting to speculate that the ACC, which has been associated with intradimensional set shifting, could be affected by CJL here (Ng et al., 2007). However, considering that there was an overall effect of 2MCJL on cognitive flexibility, one cannot rule out the fact that other brain regions, including the OFC, mPFC, and dorsomedial striatum, which are all involved in reversal learning and/or strategy shifting are also involved (Ragozzino, 2007).

5.3 Suitability of the chronic jet lag paradigm to study its effects on cognitive flexibility

The CJL paradigm used in our study simulated eastward travel as this is shown to have more negative effects both in humans and mice (Kott et al., 2012; Leota et al., 2022). The CJL paradigm in our study was selected, such that the duration of the ASST, which is 4 days, could match the duration of the shifted LD cycle; hence, a LD schedule that lasted 6 days with phase advances by 6 hours at the end of each week was used (Horsey et al., 2019). We tested two groups, one with consecutive CJL shifts lasting 1 month and another with consecutive shifts lasting 2 months; although a study has shown that 2MCJL has negative effects, we chose to also test 1-month duration of CJL because this study assessed the effect of CJL on hippocampal neurogenesis and not cognitive flexibility. Although we did see effects of 2MCJL and not 1MCJL, the paradigm could be further modified in our study to a more high-throughput approach. Therefore, as in other previous studies, this paradigm could be altered to include more frequent shifts lasting 1 month, for example, a schedule of 4 days with phase advances of 6 hours (Iggena et al., 2017). However, the possibility that mice could adapt their activity to the LD cycle should be considered as discussed previously.

5.4 Validation of the ASST used in our study

The ASST is designed such that in the SD phase, the animals learn to associate a cue of a specific dimension with a reward followed by the CD phase where another irrelevant dimension is introduced and therefore tests and trains the mouse to focus on the relevant dimension, which was learned in the previous phase. The next phase, which is the ID phase, is thus introduced to act as an internal construct to validate the task; here, the relevant dimension remains the same, but the exemplars are modified. Therefore, it is expected that the number of trials that the animals take to solve the ID phase will be lower than those in the CD phase, thus indicating that the animal has formed an attentional set and has learned the task (Scheggia et al., 2014). However, some studies have shown that mice do not form attentional sets as easily as do marmosets or rats; in fact, an absence of an ID-ED set has previously been reported in mice (Garner et al., 2006; Janitzky et al., 2015). Thus, later studies

have attempted to circumvent this issue by introducing several IDS phases or reversal phases before the EDS shift (Bissonette et al., 2008; Broberg et al., 2009; Janitzky et al., 2015). Similarly, our study also included a reversal phase in addition to the IDS phase prior to the ED phase. Overall, our results show that the number of trials taken to complete the ED phase is similar or slightly higher than the number of trials taken to complete the ID phase. However, the best measure of attentional set shifting is the ID/ED ratio, also known as the switch cost, which would need to be analyzed once the sample size has been increased in future studies. Additionally, this would need to be compared between sexes and among test groups. Furthermore, the number of trials taken to complete the ID phase are overall similar or slightly lower than the number of trials taken to complete the CD phase, pointing to formation of an attentional set in the current study.

5.5 Comparison of the ASST design used in this study with those in previous studies

The digging task and the odor and visuo-tactile dimensions are generally used to study cognitive flexibility and attentional set shifting owing to their similarity to the natural environment of mice and therefore, shorter training times; however, these tasks, such as the one used in our study could suffer from limitations. In the ASST digging task used in this study, the food reward is present in the digging medium, which is one of the dimensions used. Thus, mice could potentially make choices by directly smelling the food pellet. Additionally, this task is more manually intensive and response latencies cannot be accurately recorded. Furthermore, mice require more IDS phases than rats to form an attentional set. Therefore, several studies have also successfully implemented other semi-automated or automated task designs, similar to the CANTAB used in humans and non-human primates. For example, Scheggia et al. (2014) designed an automated novel two-chamber operant-based task to test attentional set shifting in mice using tactile, odor, and visual dimensions. Here, infrared beams tracked animal movements and the reward was delivered automatically. This task could also accurately record response latencies. Further, another study used head-fixed mice and had different vibration frequencies and odors delivered through spouts as dimensions (Spellman et al., 2021). Both these studies reliably show a set formations in the ID phase and a set-shift in the ED phase; however, in the study by Scheggia et al., (2014) mice took 5–9 days to complete the task and the study by Spellman et al. (2021) reveals that the mice require a high number of trials to complete the task. Thus, considering that the CJL paradigm involves shifts in LD cycle between 3 days to 1 week, such set shifting protocols that require days for habituation and training are not feasible (Iggena et al., 2017; Horsey et al., 2019). Heisler et al. (2015) attempted to design an ASST that lasted 2 testing days, with SD, CD, R1, and IDS

phases performed on Day 1 and IDS phases 2 and 3 and R2 and EDS performed on Day 2. However, our study follows a 4-day protocol as in Colacicco et al. (2002), where each phase is performed on separate days. This ensures that the state of the mice, such as fatigue and food motivation remains constant at the start of each phase; especially considering that the number of trials required to learn odor is high, the risk of animals achieving satiety and therefore losing motivation to access the food is higher. Additionally, the 4-day protocol is more suitable to administer pharmacological agents. Each phase of the ASST is associated with different brain regions, and therefore, performing each phase on separate days ensures that the pharmacological agent acts uniformly on each phase, as otherwise, the effects would not be uniform on each phase and brain area owing to the mouse metabolizing the compound. Finally, our protocol also attempts to overcome the limitations listed above: in our protocol, the powdered form of the reward is sprinkled throughout the bowls containing the reward to ensure that they both had the same odor, thereby reducing biases. Further, in the current study, an attentional set was formed as an analysis of the number of trials show that mostly, the number of trials required to complete the ED phase was slightly higher than the number of trials required to complete the ID phase. This is in contrast to some other previous studies that needed to incorporate additional IDS phases to obtain attentional set formation in mice (Heisler et al., 2015). Thus, our ASST protocol is more suitable to test the effect of CJL on cognitive flexibility.

5.6 Limitations of this study

Although the results of this study look promising and several future studies could be conducted based on this study, the limitations must be considered. First, in the ASST digging task, identifying whether and confirming that the animal has made a choice via nosepoke could be subjective with regard to the experimenter. However, this identification by the experimenter improves with experience and therefore, the control and test groups should be properly balanced; for example, both males and females should be tested in the same session. Second, in general, mice needed more trials to learn odor cues than to learn visuo-tactile cues. Therefore, since only three cues each for odor and medium were tested, a higher variation was observed, and further addition of combinations of odors and media would be required to ensure randomization to reduce this variation; this would also help obtain normalized data, which was difficult to obtain in this study even after transforming the data. Furthermore, other different combinations of odors and media could be tested, for example, identifying odors that mice could easily learn. Third, considering that the ASST was performed for 4 days during the standard LD cycle following the shifts in LD cycle, the possibility that the animals could have adapted to the change

in LD cycle over the testing days cannot be ruled out. Previously, Robertson et al. (2017) showed that compared with female rats, male rats adapted faster to the LD cycle over 3 months as indicated by change in activity, when behavioral testing for attention was performed during the light phase. However, whether the activity patterns of the rats change over 4 days is currently unclear. Furthermore, the same study showed that adaptation of activity patterns to the reverse LD cycle was sex-specific; therefore, this should also be considered when comparing males and females in future studies.

Finally, although the literature shows that CJL has effects on sleep and deficits in attention and other cognitive abilities could be a result of sleep deprivation, sleep monitoring could help confirm that the effects on ASST performance here in this study are mediated by a lack of sleep. Although implanting of EEG electrodes is less feasible considering the experimental setup used in this study, sleep cycle monitoring or behavioral tracking software could be used to monitor sleep (Fisher et al., 2012). Additionally, the activity of the mice could be monitored during the entire duration of the shifts in LD cycle by placing a running wheel in the cage to examine the effect of CJL on activity both during the inactive (light) phases and active (dark) phases (Robertson et al., 2017). However, another study included a running wheel in the rat cage to assess whether physical exercise helps reverse deficits induced by chronic LD cycle shifts and noted declines in memory retention in male rats in the absence of a running wheel (Zelinski et al., 2013). Therefore, the inclusion of a running wheel to assess the effects of CJL on activity and therefore sleep patterns should be carefully considered, perhaps with the use of appropriate controls.

5.7 Pharmacological interventions for the effects of CJL on cognitive flexibility

Several studies point to the orexin system as a potential therapeutic target to treat the effects of CJL on cognitive flexibility. Previous studies have shown sex-specific effects of orexin on cognitive flexibility (Zelinski et al., 2013; Durairaja and Fendt, 2021; Durairaja et al., 2022). Further, the orexin system appears to modulate attentional function via glutamatergic and cholinergic inputs to the PFC (Calva et al., 2019). Additionally, the orexin system regulates the sleep-wake cycle via dense projections from orexin neurons in the lateral hypothalamus that innervate and activate noradrenergic neurons in the locus coerulus, which is also required for attentional processes and is involved in cognitive flexibility (Janitzky et al., 2015; Toor et al., 2021; McBurney-Lin et al., 2022). Bidirectional neural connections exist between the circadian and orexin systems; circadian rhythm disruption may disturb the function of orexin neurons and inversely, orexin system activity may influence circadian rhythm (Kantor et al., 2009; Blasiak et al., 2017). Orexin neurons receive direct and indirect inputs

from the superchiasmatic nucleus; hence, SCN-dependent circadian patterns are observed orexin levels in the brain and orexin neuron activation. Further, a previous study has discussed that orexin, when administered in a timed manner, could improve the quality of life in patients with sleep disturbances (Tsuneki et al., 2018). In fact, there is also evidence that intranasal orexin peptide administration improves performance on a delayed match-to-sample short term memory task in non-human primates that were sleep deprived; therefore, nasal orexin administration could be a useful therapy (Deadwyler et al., 2007). Further studies in mice are required to determine the optimal dosage, duration of administration, and the time point at which nasal orexin should be administered during the course of the CJL paradigm.

6 CONCLUSION

Overall, this study shows that 2MCJL has effects on cognitive flexibility because the mice in this group took longer to complete the ASST overall, made more errors in completing the task, including regressive errors. Additionally, female mice who underwent 2MCJL made more perseverative errors. Further, the mice showed increased number of frequency of crossings between the light and dark compartments on the light dark box test, which warrants further studies on the effects of CJL on impulsivity. These results have implications for aircrew and shift workers, who experience chronic disruptions in their circadian cycle. Our study shows effects of a chronic shift in LD cycle on executive functions, which are mediated by the PFC and are essential for accurate execution of tasks. Thus, these groups of people require particular attention owing to the requirement to perform at peak cognitive ability to avoid life-threatening errors. However, in order to realize the translational potential of these results, further studies are required. The number of male and female animals should be increased to minimize variation in the data and confirm the presence or absence of sex-specific effects. Additionally, switch cost should also be assessed as one of the measures of cognitive flexibility. Finally, taking together the association among orexin, the circadian cycle, and cognitive flexibility, nasal orexin should be administered to mice to test its effects on cognitive flexibility. This pharmacological intervention will pave the way to further understand the underlying circuit mechanisms of the effects of CJL on cognitive flexibility and additionally serve as a useful therapeutic intervention for aircrew and shift workers to either prevent or reverse cognitive flexibility deficits.

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LIST OF ABBREVIATIONS

1MCJL	1-month chronic jet lag
2MCJL	2-month chronic jet lag
ACC	Anterior cingulate cortex
ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorder
ASST	Attentional set-shifting task
CANTAB	Cambridge Neuropsychological Test Automated Battery
CD	Compound discrimination
CJL	Chronic jet lag
CON	Control
EDS	Extradimensional set shifting
EF	Executive function
fMRI	Functional MRI
IDS	Intradimensional shift
LD	Light-dark
LDB	Light-dark box
LDS	Light-dark shift
mPFC	Medial prefrontal cortex
OFC	Orbitofrontal cortex
PFC	Prefrontal cortex
R1	Reversal 1
R2	Reversal 2
R3	Reversal 3
SD	Simple discrimination
vlPFC	Ventrolateral prefrontal cortex
WCST	Wisconsin card sorting task