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Title: Deficits in Temporal Order Memory Induced by Interferon-alpha (IFN-α) Treatment are Rescued by Aerobic Exercise.

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Abstract

Patients receiving cytokine immunotherapy with IFN-α frequently present with neuropsychiatric consequences and cognitive impairments, including a profound depressive-like symptomatology. While the neurobiological substrates of the dysfunction that leads to adverse events in IFN-α-treated patients remains ill-defined, dysfunctions of the hippocampus and prefrontal cortex (PFC) are strong possibilities. To date, hippocampal deficits have been well-characterised; there does however remain a lack of insight into the nature of prefrontal participation. Here, we used a PFC-supported temporal order memory paradigm to examine if IFN-α treatment induced deficits in performance; additionally, we used an object recognition task to assess the integrity of the perirhinal cortex (PRH). Finally, the utility of exercise as an ameliorative strategy to recover temporal order deficits in rats was also explored.

We found that IFN-α-treatment impaired temporal order memory discriminations, whereas recognition memory remained intact, reflecting a possible dissociation between recognition and temporal order memory processing. Further characterisation of temporal order memory impairments using a longitudinal design revealed that deficits persisted for 10 weeks following cessation of IFN-α-treatment. Finally, a 6 week forced exercise regime reversed IFN-α-induced deficits in temporal order memory.

These data provide further insight into the circuitry involved in cognitive impairments arising from IFN-α-treatment. Here we suggest that PFC (or the hippocampo-prefrontal pathway) may be compromised whilst the function of the PRH is preserved. Deficits may persist after cessation of IFN-α-treatment which suggests that extended patient monitoring is required. Aerobic exercise may be restorative and could prove beneficial for patients treated with IFN-α.

Keywords: IFN-α, Temporal Order Memory, Stress, Depression, Prefrontal Cortex, Exercise

1. Introduction

Interferon-alpha (IFN-α) is a clinically effective antiviral drug used in diseases associated with chronic inflammation such as rheumatoid arthritis, some cancers and viral disorders [1]. It has been most widely used in the treatment of chronic Hepatitis C virus (HCV) [2], a disease that poses global challenges to morbidity and mortality. It was estimated that 399,000 people died from HCV-related illness and 110 million people were living with chronic HCV infection in 2017 [3].

Despite its clear utility, IFN-α-treatment has been associated with neuropsychiatric and cognitive adverse events [1]. IFN-α treatment can induce severe depression in patients with HCV [4-6], deficits in working memory [7, 8] and attentional processing [8, 9] and general cognitive impairment [10-13]. The brain regions susceptible to dysfunction in patients treated with IFN-α remains ill-defined, however, two strong candidates are the hippocampus (HC) and prefrontal cortex (PFC). The role of the HC in IFN-α-induced deficits has been well characterised in previous studies [14], however, there remains a paucity of research investigating the contribution of the PFC. Studies have reported alterations in anterior cingulate processing [9], decreases in glucose metabolism [15] and prefrontal hypometabolism [16] in patients treated with IFN-α. A study in rats receiving IFN-α treatment has also shown decreases in the density of monoaminergic axons in the PFC [17].

The PFC has a pivotal role in the neural basis of higher cognitive functions but is particularly vulnerable to the effects of stress [18, 19]. In this regard, stress can produce pronounced structural remodelling of the PFC via dendrite retraction [20] and also disrupt long-term potentiation in the hippocampo-prefrontal (HC-PFC) pathway [21, 22]. Accordingly, IFN-α-treatment has been characterised as a systemic stress which consequently activates the hypothalamic-pituitary-adrenal (HPA) axis and pro-inflammatory cytokines [23].

A temporal order memory paradigm which utilises spontaneous exploration has been found to be useful in assessing prefrontal function in the rat [24]. Temporal order memory is sub-served by the PFC and is therefore classified as a higher-order mnemonic task [25]. Temporal order memory is the ability to temporally sequence past experiences in order to plan for prospective goals and actions [26]. The recollection of time-stamps that enable the discrimination of new experiences from previous ones in a chronological order. Evidence suggests that temporal order memory requires an intact PFC, perirhinal cortex (PRH) and the involvement of the ventral HC [25, 27]. Previous studies report that the PRH is required for performance of object recognition tasks. Lesion of the medial PFC or PRH impairs object-based temporal order memory in the rat [25]. Here, demonstration of intact object recognition memory, but not temporal order memory may indicate the functional impairment of the HC, the PFC and/or the HC-PFC pathway [28] following IFN-α treatment.

It is of added value to also focus on research into potential strategies to ameliorate cognitive and neuropsychiatric deficits. Several randomised controlled trials have examined the efficacy of antidepressant prophylaxis alongside IFN-α treatment [29], however, very little focus has been placed on behavioural modifications such as exercise. It is well-established that physical exercise has beneficial effects on stress, mood and learning and memory [30-32] and demonstrates positive effects on synaptic plasticity [33] and neurogenesis [31]. However, reports on the effects of exercise in counteracting cognitive decline following IFN-α treatment are limited [14] and have not focused on temporal order memory.

The present study examined temporal order memory versus object recognition memory in rats treated with IFN-α to advance our understanding of the circuitry involved in IFN-α–induced deficits. We validated a temporal order memory paradigm which we applied alongside an

object recognition task to measure cognitive deficits in IFN-α-treated rodents. Comparison of temporal order memory deficits and object recognition memory in the IFN-α treated rat provide some focus on the loci of dysfunction in IFN-α deficits. Further, a concurrent forced exercise regime was employed to recover IFN-α-induced temporal order memory deficits.

2. Results

Experiment 1: *Temporal order memory is delay-dependant in the rat*

During the test phase the animals explored the old familiar object more than the recent familiar object when a 1 hour or a 6 hour delay period was imposed. In contrast, when a 24 hour delay elapsed the exploration was comparable between old and recent familiar objects, therefore no discrimination was present. To verify if animals were able to discriminate between old and recent familiar objects, one sample t tests compared to the hypothetical value of 0 (chance level of Discrimination Index DI) were conducted. Animals could identify the old familiar object at 1 hour and 6 hour delay but not at the 24 hour delay (one sample t test: 1 hour: $t = 2.56$, $p = 0.0002$; 6 hour: $t = 4.4$, $p = 0.0045$; 24 hour: $t = 0.25$, $p = 0.08$). The performance of the animals at the three test delays can be seen in Figure 1B. Statistical analysis of rat exploration preference demonstrated significantly higher levels of discrimination for the old familiar object at 1 hour and 6 hour compared to 24 hour delay ($F_{(2, 18)} = 8.16$, $p = 0.003$). Tukey's *post hoc* analyses revealed DI at 1 hour and 6 hour were significantly different to DI at 24 hour test, $p<0.05$ and $p<0.01$ respectively. Animals explored objects equally at each sample phase (Mean exploration Sample 1; 1 hour group: 34.3 ± 6.8; 6 hours group: 38.2 ± 6.6; 24 hours group: 42.0 ± 8.1; Sample 2; 1 hour group: 31.6 ± 4.7; 6 hours group: 30.1 ± 6.6; 24 hours group: 47.8 ± 8.1). There was no significant differences in total exploration at the different test time points one-way ANOVA, $F_{(2, 21)} = 3.109$, $p = 0.0657$ (Mean exploration; 1 hour: 36.82 ± 5.45; 6 hours: 35.6 ± 3.9; 24 hours: 43.8 ± 11.4), therefore the result at 24 hour delay is not due to lack of exploration. Together these data indicate that temporal order memory is intact at 1 hour and 6 hour tests but not at 24 hour test.

Experiment 2: *Temporal order memory but not object recognition memory is impaired in IFN-α treated animals*

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Rats were treated with saline or IFN-α for four weeks. Following treatment, temporal order memory was tested at the cessation of treatment (T0), one week following treatment (T1), two weeks following treatment (T2) and 10 weeks following treatment (T10). A test time delay of 6 hour following the sample phase was used in this experiment. One sample t-tests were conducted to determine the discrimination of the old and recent familiar objects. At T0 IFN-α animals were impaired in the temporal order memory task (T0: Sal: t = 2.7, p = 0.02; IFN: t = 1.8, p = 0.11). Unexpectedly, at T1 and T2 neither group identified the old familiar object (T1: Sal: t = 0.05, p = 0.9; IFN: t = 2.1, p = 0.06; T2: Sal: t = 1.06, p = 0.3; IFN: t = 1.4, p = 0.19). At T10 IFN-α animals were impaired in the temporal order memory task (T10: Sal: t = 2.5, p = 0.03; IFN: t = 0.5, p = 0.6). Figure 2B illustrates that overall IFN-α treated rats were impaired in temporal order memory compared to saline (Sal) treated counterparts and this persisted for 10 weeks following cessation of treatment. Statistical analysis revealed IFN-α treatment had a significant effect on temporal order memory (Multi-factorial repeated measures ANOVA: There was an effect of treatment (IFN-α Vs Sal), $F_{(1, 14)}$ = 9.6, p = 0.007; There was no global effect of time/repeat testing following cessation of treatment, $F_{(3, 42)}$ = 0.73, p>0.05; and there was no interaction between treatment and time following cessation of treatment $F_{(3, 42)}$ = 2.65, p>0.05). Tukey's *post hoc* analyses revealed the DI of saline and IFN-α treated animals was significantly different at T10 (p = 0.012), and very close to significance at T0, p = 0.051. The result observed at T1 was an unexpected observation, however, it is important to note the ANOVA demonstrates a significant main effect of IFN-α treatment on temporal order memory over the 10 weeks post treatment. No differences in total exploration of objects were observed between saline and IFN-α treated rats (two-way ANOVA: IFN: $F_{(1, 56)}$ = 0.005093, p = 0.9434; Time: $F_{(3, 56)}$ = 2.516, p = 0.0675; Interaction: $F_{(3, 56)}$ = 0.3632, p = 0.7798), (Mean exploration; T0: Sal: 36.38 ± 3.7; IFN: 30.45 ± 5.09; T1: Sal: 37.67 ± 6.6; IFN: 42.89 ± 6.6; T2: Sal: 29.13 ± 4.2; IFN: 30.49 ± 4.2; T10: Sal: 44.3 ± 7.3; IFN: 42.53 ± 4.7). Together these

data support the hypothesis that IFN-α treatment interferes with temporal order memory ability in the rat.

In a separate cohort of animals object recognition memory was tested following four weeks of IFN-α or saline treatment. Statistical analysis revealed IFN-α treatment did not affect performance in the object recognition memory task when tested at a 5 min delay or a 6 hour delay compared to saline treated controls (Figure 2C: Multi-factorial repeated measures ANOVA: There was no effect of treatment (IFN-α Vs Sal), $F_{(1, 10)} = 0.8$, $p = 0.38$ and no effect of delay time (5 min Vs 6 hour): $F_{(1, 10)} = 0.001$, $p = 0.96$). All groups learned the task by successfully identifying the novel object, one sample t tests compared to the hypothetical value of 0.5, chance level on DI (5min test delay: Sal: $t = 9.8$, $p = 0.0002$; IFN: $t = 10.82$, $p = 0.0001$; 6 hour test delay: Sal: $t = 18.38$, $p < 0.0001$; IFN: $t = 70.18$, $p < 0.0001$). No differences in total exploration of objects were observed between saline and IFN-α treated rats (two-way ANOVA: IFN: $F (1, 20) = 0.1316$, $p = 0.7206$; Time: $F (1, 20) = 1.433$, $p = 0.2453$; Interaction: $F (1, 20) = 0.4625$, $p = 0.5042$) (Mean exploration; 5min: Sal: 41.6 ± 5.1; IFN: 34.6 ± 3.8; 6 hour: Sal: 45.07 ± 9.6; IFN: 47.2 ± 6.9). Overall, no differences were observed between saline treated or IFN-α treated animals, demonstrating object recognition memory was preserved in rats treated with IFN-α.

As different objects were utilized for the object recognition task and the temporal order memory task it is important to note that animals in both experiments had explored objects to an equivalent amount, with no effect of experiment or treatment on exploration (two-way ANOVA: IFN: $F (1, 84) = 0.084$, $p = 0.7725$; Experiment: $F (5, 84) = 2.106$, $p = 0.0726$; Interaction: $F (5, 84) = 0.3234$, $p = 0.8976$). This demonstrates object selection did not impede on task performance.

Experiment 3 *Exercise recovers the temporal order memory deficit in IFN-α-treated animals*

In this experiment the effect of exercise and IFN-α treatment were assessed on temporal order memory. A test time delay of 6 hours following the sample phase was used in this experiment. Temporal order memory was tested at four weeks and six weeks of exercise following two weeks and four weeks of IFN-α or saline treatment respectively. One sample t-tests were conducted to determine the discrimination of the old and recent familiar objects. Only saline sedentary animals could identify the old familiar object at the 2 week test (one sample t test: Sal + Sed: t = 2.86, p = 0.03; Sal + Ex: t = 1.4, p = 0.19; 24; IFN + Sed: t = 0.5, p = 0.62; IFN + Ex: t = 1.009, p = 0.34). At the 4 week test time only the sedentary IFN-α treated rats could not identify the old familiar object (one sample t test: Sal + Sed : t = 3.3, p = 0.04; Sal + Ex: t = 8.1, p = 0.0002; 24; IFN + Sed: t = 1.6, p = 0.15; IFN + Ex: t = 4.8, p = 0.008). Further statistical analysis revealed IFN-α treatment had a significant effect on temporal order memory and this was prevented by exercise (Multi-factorial repeated measures ANOVA: There is an effect of treatment (IFN-α Vs Sal): $F_{(1, 25)}$ = 12.8, p=0.001; There is no overall effect of exercise: $F_{(1, 25)}$ = 0.16, p=0.68; but there is an interaction between treatment and exercise: $F_{(1, 25)}$ = 6.5, p=0.01. There was no overall within subjects effects of repeated testing (two weeks Vs four weeks): $F_{(1, 25)}$ = 1.26, p=0.27; no effect of repeated testing by treatment (IFN-α Vs Sal): $F_{(1, 25)}$ = 0.63, p=0.43; no effect of repeat testing by exercise: $F_{(1, 25)}$ = 2.5, p=0.12; and no effect of repeat testing by IFN-α x Exercise: $F_{(1, 25)}$ = 1.69, p=0.20). Although IFN-α treatment had a main effect, exercise alone did not have a significant main effect, but there was a significant interaction of the two. This may be accounted for by the lack of effect of exercise on IFN-α-induced deficits following two weeks of treatment. Tukey's *post hoc* analysis revealed a significant difference in the performance of Sal+Sed and Sal+Ex groups compared to IFN-α group on the temporal order memory test after two weeks of treatment (p < 0.05 for both). Further, Tukey's *post hoc* analysis of the temporal order memory test at 4 weeks revealed

Sal+Sed, Sal+Ex and IFN+Ex groups were significantly different to IFN-α group (p<0.05 for all). No differences in total exploration of objects were observed between exercised or sedentary, saline and IFN-α treated rats (Mean Exploration; 2 Week: Sal + Sed: 38.4 ± 6.7; Sal + Ex: 47.6 ± 4.9; IFN + Sed: 44.0 ± 7.8; IFN + Ex: 45.2 ± 7.6; 4 Week: Sal + Sed: 35 ± 5.8; Sal + Ex: 42.5 ± 4.5; IFN + Sed: 33.5 ± 4.8; IFN + Ex: 33.8 ± 3.1). Statistical analysis by two-way ANOVA revealed there were no main effects of exercise or treatment on total exploration in the temporal order memory task (ANOVA: IFN: $F_{(3, 50)} = 1.325$, $p = 0.2768$; Exercise: $F_{(1, 50)} = 1.142$, $p = 0.2904$; Interaction: $F_{(3, 50)} = 0.2740$, $p = 0.8439$). Together these data suggest that exercise can prevent the IFN-α-induced impairment in temporal order memory.

3. Discussion

The present study examined temporal order memory and object recognition memory in rats treated with IFN-α in order to provide further insights into the possible neural circuitry involved in IFN-α–induced cognitive deficits. An initial validation experiment demonstrated that there are delay-dependent effects when making temporal order memory judgements (*experiment 1*): naive Han-Wistar rats were able to discriminate the temporal order of objects after a delay period of 1 hour and 6 hour but not after 24 hour. We also established that the temporal order memory task is sensitive to assess amnestic (IFN-α) (*experiment 2*) and a promnestic manipulation (exercise) (*experiment 3*).

Our data show that there is a deficit in temporal order discriminations in rats treated with IFN-α. IFN-α-treated animals could not discriminate between objects based on the sequence of presentation and were therefore unable to reconcile events that occur remotely compared to those that occurred more recently, this deficit persisted for up to 10 weeks post IFN-α treatment. Despite impairments in the temporal order memory task, IFN-α-treated animals retained recognition memory in the week following cessation of treatment. The basis for impaired temporal order proficiency cannot be ascribed to an inability to make familiarity judgments because object recognition remained intact. To further verify that the difference in the performance on the two memory tasks is an effect of IFN-α-treatment we discuss interpretations of the data in relation to the design of the study. In the object recognition task we used spaced familiarisation sessions, we therefore cannot exclude the possibility that a more challenging object recognition task (e.g. using a one sample phase) may produce deficits. However, these data are in line with previous experiments [25, 34, 35], which demonstrate dissociation between the object recognition task and the temporal order memory task with prefrontal impairments.

Object recognition memory was only measured once following cessation of IFN-α treatment: the result was sufficiently robust to lead us to believe that recognition memory was not altered by IFN-α treatment and would not change in the following weeks. There is a factor of time to consider, animals in the temporal order task were examined for up to 10 weeks post treatment and were therefore older than those in the object recognition task. It is well established that older animals have deficits in hippocampal function and object recognition function is sensitive to hippocampal damage. Age-related deficits in hippocampal function appear in middle aged rodents, approximately 14 months [36] [37] [38]. Animals compared here are 3 months and 6 months which are both considered young. Previous studies using up to 12 month old control rats found no difference in performance of the object discrimination task with a 1hour retention interval [39]. Another study investigating object recognition memory in mice with a 1hour and 24 hour test interval found no differences between 3month and 6month old control mice [40]. Importantly, comparable total exploration time between the animals in the tasks further indicate that age differences during early adulthood do not influence the results in our setting. Earlier studies within the laboratory investigating cognitive and exploratory deficits in hippocampal or prefrontal memory systems observed significant differences only with more advanced age-groups, see [36] & [37]. The objects used in the two memory tasks were different, however it is unlikely that this has impacted on performance on the tasks. Examination of total exploration scores showed the animals explored the objects equally in the two different tasks.

Extensive disconnection studies completed to investigate the neural correlates of temporal order memory have precluded the need for lesion studies in this series of experiments. The anatomical basis for deficits in the animals in the present study may therefore be deconstructed using a systems approach. The data presented here are consistent with results produced in studies where either reversible [34] or permanent [24, 25] lesions of the PFC have been utilised.

Our results suggest that the integrity of the PFC is required for the completion of temporal order memory tasks, although there is evidence that this region may not be the sole neural correlate [25, 41]. Lesion studies conducted by Barker et al (2007) demonstrated the PRH and the PFC are required for temporal order memory [25] whilst only the PRH is required for object recognition memory tasks. The sparing of recognition memory in this study lends support that the PRH are preserved in IFN-α-treated animals. This suggests that impairment in temporal order memory occurring in IFN-α treatment may reside in impairments in PFC function or the pathway between the PRH-PFC. The HC has also been implicated in object recognition tasks with spatial or temporal components [42]. Lesion studies reveal significant impairments in temporal order memory when there is a disconnection of the HC with the PFC or PRH. However, standard object recognition memory remains intact if there is a disconnection of these pathways or an ablation of the HC alone. The neural circuit required for temporal order memory therefore involves the HC, PRH and PFC [42], which indicates a double dissociation between the effects of HC and PRH on object recognition and temporal order memory. Taken together, we suggest that deficits in temporal order memory but not object recognition memory observed in IFN-α-treated rats implicates impairment in the PFC or HC-PFC pathways. Further interconnected regions may be involved in temporal order memory. The regions other than the ventral HC and the PFC that have been implicated thus far are the dorsal CA1 [43], retrosplenial cortex [44] the ventral subiculum and the lateral entorhinal cortex [45] . These are brain regions that convey information via direct and indirect pathways between the HC and PFC [46]. Anatomical regions involved in both direct (ventral HC to PFC) and indirect bidirectional pathways (via the thalamic nucleus reuniens to hippocampal area CA1 or via the cortical pathway and the lateral entorhinal cortex) are implicated in the functional circuitry of temporal order memory. It has been speculated that the cortical pathway maybe the better suited pathway for the processing of information about objects and events [46]. It is plausible that if one

pathway is disconnected alternative pathways can be utilised to retain this core link between the HC and PFC.

The activation of inflammatory cascades have been associated with impaired cognitive function arising from IFN-α-treatment. In particular, the interleukin-6 (IL-6) gene has been linked to risk of neuropsychological symptoms in patients receiving IFN-α treatment and may therefore be used as a predictor [47]. Prather et al., [47] found associations between plasma IL-6 levels and depressive symptoms measured using the Beck Depression Inventory-II (BDI) in patients treated with IFN-α. Here, a longitudinal examination of temporal order memory was conducted after the cessation of treatment to determine the persistence of cognitive deficits. The data presented here supports a persistence of deficits in temporal order memory and the neural correlates associated with this type of memory following IFN-α treatment, IFN-α-induced deficit for up to 10weeks post treatment, perhaps from structural reorganisation or a specific prefrontal hypometabolism [16]. The implication of this persistence in cognitive deficits is important and suggested recommendations would favour extended patient monitoring with regular follow-ups after the cessation of treatment.

The final experiment (*experiment 3*) investigated the potential for aerobic exercise to ameliorate the negative effects of IFN-α-treatment. Exercise, when implemented prior to the initiation and throughout the course of IFN-α-treatment, restores the ability to differentiate the serial order of object presentation (Figure 3). The recovery was dependent on the time points that were used regarding length of drug treatment. IFN-α treated animals were impaired in the temporal memory task when tested following two weeks of treatment, and exercise did not prevent the impairment. This indicates that four weeks of exercise is not sufficient to protect against IFN-α-induced cognitive dysfunction. Animals were exercised for two weeks prior to IFN-α treatment to prevent the formation of a negative association between IFN-α administration and exercise. An exercise regime appears to act as a positive modulator for

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temporal order memory and restores its function. Hence, implementing an exercise routine may be of considerable value for the treatment of memory deficits via several possible mechanisms: exercise increases neurogenesis and neurotrophin expression in the HC [31] [48] [49]. There are currently to our knowledge no studies published investigating exercise-induced memory improvements in humans taking immunotherapy. Although, it is suggested exercise in combination with immunotherapy may in fact be therapeutic in cancer treatment [50]. Exercise has also been shown to be a broadly anti-inflammatory agent by leading to increased levels of Il-4, IL-1ra and IL-10, and also by suppression of TNF-α production in vivo [51]. More recently levels of pro-inflammatory cytokines TNF-α and IL-1β have been identified as predictors of antidepressant effects of exercise in major depressive disorder [52]. These data indicate regulation of inflammatory factors may be a key area of focus in development of future treatments for symptoms of affective disorders.

In conclusion, our data suggest that IFN-α treatment compromises temporal order memory, a form of mnemonic processing supported by PFC, and PFC - HC connectivity. Importantly, these deficits are reversed using a course of aerobic exercise, suggesting that exercise supports the recovery of cognitive function in brain regions affected by IFN-α. These data therefore suggest a potential therapeutic pathway worthy of further clinical investigation in humans undergoing IFN-α treatment.

4. Methods and Materials:

4.1 Experimental Design

Experiment 1: Validation of a temporal order memory task in male Han-Wistar rats. See Figure 1A for a schematic of the behavioural testing protocol.

Experiment 2: The effect of IFN-α or saline treatment on temporal order memory and object recognition memory. See Figure 2A for an outline of the treatment and behavioural regime.

Experiment 3: Amelioration of temporal order memory deficits in IFN-α treated rats by aerobic exercise. See Figure 3A for a schematic of the treatment, exercise and behavioural testing regime.

4.2 Animals

In total 68 male Han-Wistar rats (BioResources Unit, Trinity College Dublin) weighing between 215-350g were used (Experiment 1: n = 8; Experiment 2: n = 28; Experiment 3: n = 32). However, in *experiment 3* two animals were excluded from the saline sedentary group due to unexplained weight loss within the first week of experimental procedures. Animals were housed two/three per cage within a controlled environment (Laminar airflow unit, 12h light/dark schedule with lights on at 08:00-20.00). Rats received food and water *ad libitum*. Behavioral testing was conducted during the light phase of the schedule. Animals were naïve to experimental procedures and had not previously been used in any other experiments. All rats were naïve to injection stress.

All experiments were carried out under regulations laid out by the Health Products Regulatory Authority (HPRA) Ireland in accordance with the European Union Directive 2010/63/EU for animal experiments.

4.3 Experimental Procedures

Temporal Order Memory Task: This task was carried out as per Mitchell and Laiacona (1998) [24]. Prior to behavioural testing all rats were handled at regular intervals Animals were handled for 5 days for approximately 15 minutes each day to acclimatise them to the experimenter. In experiment 2 and 3 animals were also handled three times a week for four weeks during drug treatment. In experiment 2 and 3 animals were also handled three times a week for four weeks during drug treatment.

Rats were tested in an arena consisting of an open grey plastic packing crate of the following dimensions L69 x W45 x H70 cm (length x width x height). The arena was located within a testing room which was dimly lit with four anglepoise lights pointing downwards and placed at the corner of the arena, approximately 10-15 lux at the center of the arena. The room and lighting conditions were controlled for in both tasks, to avoid influencing task performance. The arena was surrounded by floor to ceiling length black curtains to prevent any extrinsic cues. The objects used for exploration consisted of different conformations of children's Duplo® lego bricks (Lego, Nyíregyháza, Hungary). The objects were affixed to the floor of the arena with Blu-tack® (Bostik, La Défense, Paris, France) to prevent displacement during the exploratory phases. Prior to the commencement of behavioural testing rats were habituated to the empty arena on four consecutive days for three minutes per day. On the fifth day, behavioural testing began. The task procedure consisted of three distinct elements; two sample phases (S1 and S2) and a test phase. The duration of each phase was five minutes. In sample phase 1 (S1) rats were placed in the arena with two identical copies of objects ('Old' objects) located 10cm away from the walls in the middle of the arena. Following S1 the animal was removed from the arena and placed in the home cage for a 1 hour inter-trial interval before re-entry back into the arena for the second sample phase (S2). In S2, the rat was placed in the company of two new identical objects ('New' objects). Following a delay period (1 hour, 6

hour or 24 hour in *experiment 1*; 6 hr in *experiment 2* and *3*) the rat was reintroduced to the arena for the test phase. In the test phase the objects presented consisted of a third (new) copy of each object from S1 and S2. The location of the object in the arena was counterbalanced in order to account for any bias towards exploration of the left or right portion of the arena. If temporal order was considered intact the animal would spend more time in the test phase exploring the object they had previously explored from S1 ('Old' object) than the object from S2 (the 'New' object). This distinction in exploration is based on a recency judgement i.e. the object from S2 was seen more recently and is familiar, however, the object in S1 regains an element of novelty. After all exposures to exploration the arena and objects were cleaned thoroughly with Savlon® (Johnson & Johnson, One Johnson & Johnson Plaza, New Brunswick, New Jersey, U.S) to prevent the detection of odour traces. Duplicate copy objects were interchanged to avoid spontaneous object preferences developing through scent-marking.

Experiments were recorded using the Canopus Mediacruise® (Canopus Corporation Ltd, Kobe, Japan) system and later scored by an observer masked to treatment. The observer had previous training and experience in scoring object exploration videos within the laboratory. The operational definition for exploration was defined as active exploration consisting of head directed movements towards the object and direct sniffing or snout within $\leq$ 2cm away from the object face (for full review of these parameters please see [53]). Time spent exploring each of the objects was recorded using two stopwatches. Climbing was discounted in this case, as it was a means of reaching a higher level to sniff the area above the arena and was not active exploration of the objects. Data are presented as a discrimination index (DI) which allows for discrimination between the old object (T_O) and "new" object (T_N), using the difference in exploration time for the old object, but then dividing this value by the total amount of exploration of the old object and "new" object [DI = ($T_O - T_N$)/($T_O + T_N$)] [53].

For *experiment 1* three delay periods were chosen for inter-trial intervals between S2 and the test phase; 1 hour, 6 hour or 24 hour interval to allow for comparison to Mitchell and Laiacona [24]. Replication of results between the two studies was of importance as Han-Wistar rats were used here and Long-Evans rats of similar age were used by Mitchell and Laiacona [24]. These intervals were counterbalanced for repeated measures so that all the rats were exposed to the three different intervals in a different order. Animals were given a seven day break between each testing session and new sets of objects were used for each week. For *experiment 2* and *experiment 3* only the 6 hour test interval was implemented. This was based on the intact temporal order memory observed in experiment 1.

In *experiment 2* the temporal order memory test included a repeated measures protocol with temporal order testing performed at a four week time point (T0) and three further time points (T1, T2, T10), indicating one, two and 10 weeks after cessation of treatment (Figure 2A). Eight different sets of objects were used for this experiment with three copies in each set. The time points post-treatment were chosen to determine the persistence of IFN-α-induced cognitive dysfunction following cessation of treatment administration.

In *experiment 3* the temporal order memory test was conducted at 2 weeks and 4 weeks post treatment. The reasons for this were two-fold; firstly, it would give an indication of how quickly into treatment, IFN-α can impact on cognitive functioning and secondly, how effective exercise would be at preventing any impairments observed at this time point.

Object Recognition Memory Task: The object recognition task was conducted in a naive group of animals (n=12) not exposed to the temporal order memory task. The recognition memory task was performed after four weeks of IFN-α treatment (see Figure 2C). The four trial (distributed) object recognition task was utilised in this study instead of the mainstay procedure of a one-trial massed approach [54]. The recognition memory task that was used in this

experiment utilised a spaced initial stimulus familiarisation as opposed to a massed familiarisation. There is evidence that distributing the familiarisation sessions over a spaced procedure may enhance the recognition ability [55]. Rats were tested in the same arena used in the temporal order memory task (dimensions: L69 x W45 x H70 cm) which was located in a dimly lit room, surrounded by floor to ceiling length black curtains, as described above. The objects used for exploration were constructed from general laboratory equipment (1Litre glass bottles, microcentrifuge tube racks, plastic beakers (Sigma-Aldrich) of similar dimensions (approximately 8cm x 20cm). In the object recognition task, general laboratory equipment items were used whereas in the temporal order memory task, objects were constructed from Lego® blocks. Every effort was made to ensure objects of equal size and dimensions were used in both experiments to eliminate abnormally high levels of spontaneous investigation. Duplicate copy objects were interchanged to avoid spontaneous object preferences developing through scent-marking. The object recognition test took place five days following cessation of four weeks of IFN-α or saline treatment. The animals were exposed to the arena on four consecutive days for three minute habituation sessions. The test day consisted of exposure to 3 x 5 minute sample trials with two different objects (objects A and B) with an inter-trial interval of five minutes. After each exposure, the objects and the arena were cleaned with Savlon® to remove any odour cues. The test phase (choice phase) of the session involved the substitution of one object with a novel object (object C) and a duplicate of object A or B. Recognition memory was defined as more exploration to the novel object (object C). Two delay periods (5 min or 6 hour) were used between the third sample phase and the test phase. These time periods were chosen to reflect the time period used in the temporal order task and to determine that there was no delay-dependent deficit in object recognition memory at 6 hour in the animals. Experiments were recorded using the Canopus Mediacruise® system and later scored by an experimenter masked to treatments as described above. Data are presented as a discrimination

index (DI) which allows for discrimination between the familiar object (T_F) and novel object (T_N), using the difference in exploration time for the novel object, but then dividing this value by the total amount of exploration [$DI = (T_N - T_F)/(T_F + T_N)$].

In *experiment 3* rats were tested at two weeks and four weeks after the commencement of IFN-α or saline treatment, reflecting four weeks and six weeks of exercise respectively. Time points were chosen to examine how quickly IFN-α treatment may impact on cognitive function and to examine how effective physical exercise is at preventing cognitive dysfunction.

Exercise protocol: The exercise component is the same as that described in [14] and a modified version of that used in [33]. Rats were habituated to the motorized treadmills (Exer 3/6 treadmill, Columbus instruments) as previously described [14, 33, 56] for a two day period. Animals were exercised for two weeks prior to commencement of IFN-α treatment to prevent negative associations between exercise and IFN-α administration. Animals were placed on the belt of the treadmill running at a slow moving pace (0.78 km/h), the belt speed was increased to 1.02 km/h over a 15-30 min period. The treadmills are equipped with wire loops at one end of the belt through which a mild electric shock can be delivered; these act to motivate the rats to run continuously and were activated at low levels (on average an intensity of two on a scale of 0–10; this represents a current of 0.7 mA with an interpulse interval of 2 seconds) throughout all exercise sessions. Animals were monitored for signs of fatigue and distress throughout the exercise protocol. During habituation animals were encouraged to run by dangling pieces of string at the top of the treadmill, encouraging rats to chase the string. Observations during this initial introduction to the treadmills determined that the rats tired after 20 minutes running. Therefore, rats were trained every other day for a period of six weeks, with each training day consisting of two 20-min running sessions (belt speed 1.02km/h), with a 20-min rest interval between run sessions to avoid fatigue, as in [14]. The exercise sessions were conducted at the

end of the light period (18.30-20.00), in order to reduce interruptions to the sleep pattern of the animals. Animals were randomly assigned to the four treatment groups: Saline sedentary (Sal), Saline exercised (Sal+Ex), IFN-α sedentary (IFN), IFN-α exercised (IFN+Ex). Sedentary rats were places on a stationary treadmill for the same duration, with shock loops activated.

4.4 Drug Treatments

IFN-α or Saline Treatment: Roferon-A (human recombinant interferon-alpha 2a, Roche Pharmaceuticals, USA) (~170,000 IU / kg, diluted in saline, s.c.), or saline as vehicle (0.9% NaCl, s.c.) was administered once a day, three times per week for four weeks, with each rat receiving 12 injections prior to the commencement of behavioural testing. The IFN-α dose was adjusted once a week to compensate for any increase in weight gain. The dose used parallels a mid-range human dose (12 MIU); IFN-α doses can range from, for example, 3 MIU, s.c. three times per week for chronic Hepatitis C virus to 30 MIU/m2 body surface area for AIDS-related Kaposi's sarcoma [57, 58]. Administration of 170,000IU/kg to rats has previously given reliable and reproducible behavioural deficits [14, 59].

4.5 Statistical Analysis

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) version 23 and graphs were constructed using GraphPad Prism version 7 and Coral Draw X6. Data analysis was as follows; Behavioural parameters of *Experiment 1* were analysed by a repeated measures one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* comparisons where appropriate. Significance was set at $p < 0.05$. Behavioural parameters of *Experiment 2* and *3* were analysed by multi-factorial repeated measures ANOVA, followed, when appropriate by Tukey's *post hoc* comparisons. In *Experiment 3* one animal was excluded from the Sal+Ex group based on low levels of total exploration (less than 10 seconds) in the

sample phases of the temporal order memory task, and as such, was not used during the testing

stage.

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Figure 1
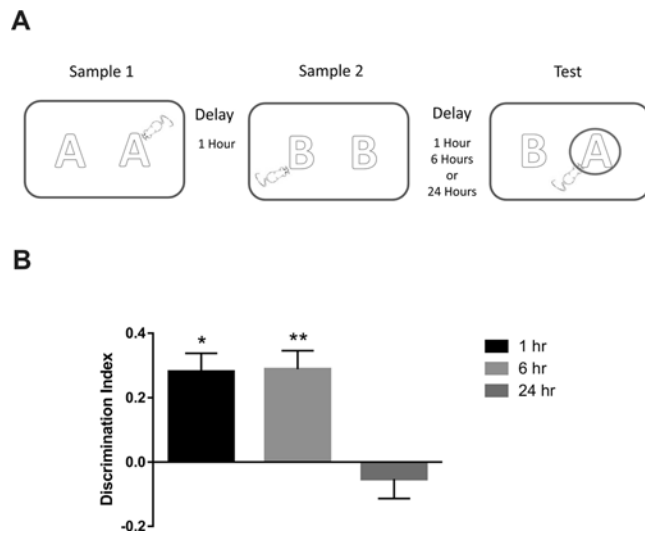
Figure 1: Temporal order memory is delay-dependant in the rat. Schematic representation of the temporal order memory task (A). Rats were required to differentiate between two objects which were presented in two sample phases with a 1 hour inter-trial interval and differing test trial delays of 1 hour, 6 hour and 24 hour between sample two and the test trial. Animals were able to differentiate the old familiar object after a 1 hour and 6 hour delay but not after 24 hour (B). Data are expressed as mean discrimination index (DI) ± SEM, n=8, asterisk represents significant difference to indicated group. Repeated measures ANOVA with Tukey's *post hoc* analysis *p<0.05, **p<0.01.
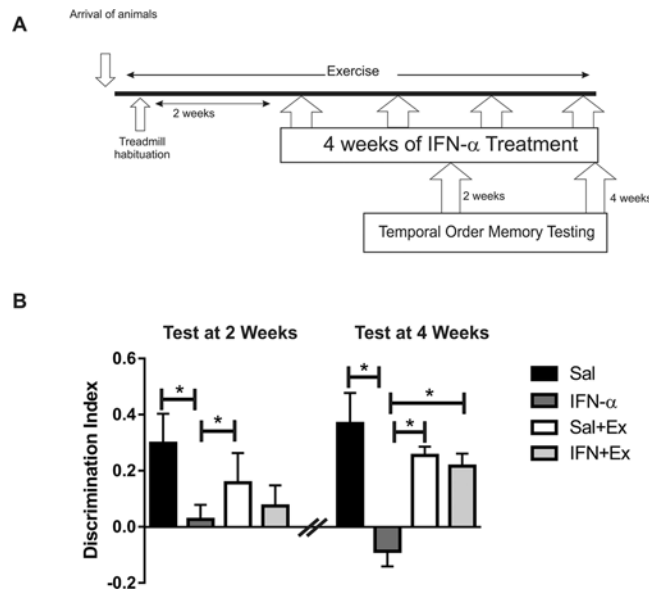
Figure 2
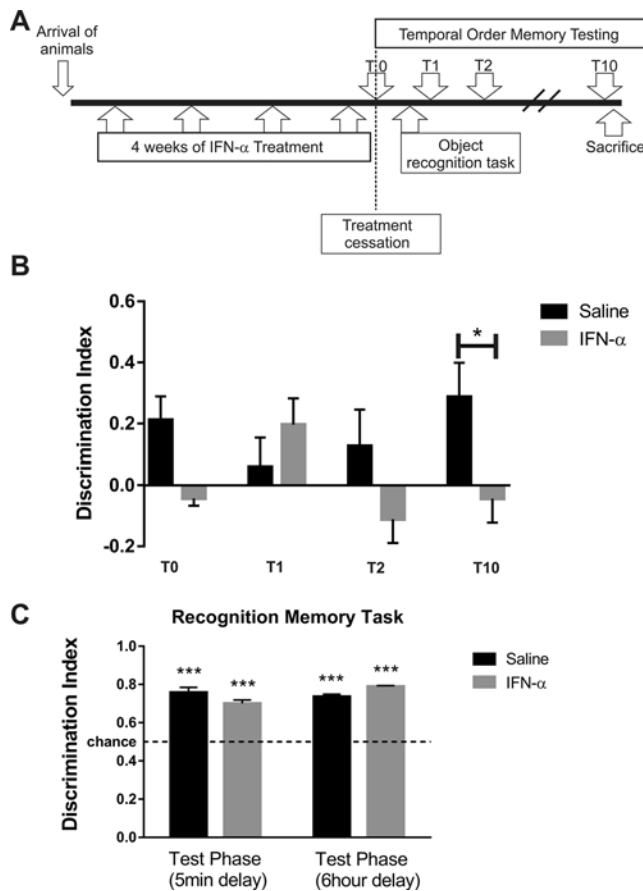
Figure 2: Temporal order memory but not object recognition memory is impaired in IFN-α treated rats. Schematic representation of experimental 2 design (A). Following 4 weeks of IFN-α treatment, rats were tested in the temporal order memory test (4 tests up to 10 weeks following treatment) or the object recognition memory test (1 test day). IFN-α treated rats were significantly impaired in the temporal order memory task compared to saline treated controls (B) up to 10 weeks post treatment. IFN-α treated animals had no deficit in recognition memory compared to saline treated controls (C). The dashed lines indicate an equivalent exploration time for the two objects (chance level). Data are expressed as mean discrimination index (DI) ± SEM, n=8 per group for temporal order memory test, n=6 per group for recognition memory test, asterisk represents significant difference to indicated group. Multi factorial repeated

measures ANOVA, with Tukey's *post hoc* analysis or one sample t test to chance, *p<0.05 ***p<0.0001.

Figure 3

Figure 3: Aerobic exercise recovers the temporal order memory deficit observed in IFN-α treated animals. Schematic representation of the design of Experiment 3 (A). Rats received IFN-α for 4 weeks thrice weekly. Two testing sessions were conducted, 2 weeks and 4 weeks after the beginning of IFN-α treatment. Rats were on the exercise regime for the duration of the experiment. Aerobic exercise prevented IFN-α-induced deficits in temporal order memory (B). Data are expressed as mean discrimination index (DI) ± SEM, n=8 for IFN and IFN+Ex groups, n=6 for Sal and n=7 for Sal+Ex, asterisk represents significant difference to indicated group. Multi-factorial repeated measures ANOVA, with Tukey's *post hoc* analysis *p<0.05.