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Publication Date	2016-01-13
Publication Information	Moriarty, Orla, Lang, Yvonne, Idrees, Zubair, McGuire, Brian E., & Finn, David P. (2016). Impaired cued and spatial learning performance and altered cannabinoid CB1 receptor functionality in the substantia nigra in a rat model of diabetic neuropathy. Behavioural Brain Research, 303, 61-70., doi: 10.1016/j.bbr.2016.01.027
Publisher	Elsevier
Link to publisher's version	https://doi.org/10.1016/j.bbr.2016.01.027
Item record	http://hdl.handle.net/10379/15088
DOI	http://dx.doi.org/10.1016/j.bbr.2016.01.027

Impaired cued and spatial learning performance and altered cannabinoid CB_1 receptor functionality in the substantia nigra in a rat model of diabetic neuropathy

Running title: Cognition and CB_1 receptor functionality in STZ rats

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Abstract

Diabetes, and associated diabetic neuropathic pain, impact negatively on cognitive function. However, the underlying mechanisms remain poorly understood. This study investigated neuropathic pain-related behaviour and cognitive function in the rat streptozotocin (STZ) model of diabetes, and assessed cannabinoid$_1$ (CB_1) receptor functionality in discrete brain regions. Male Lister-Hooded rats received STZ (60mg/kg s.c.) or vehicle. Sensory responses were assessed in von Frey and Hargreaves tests. Cognitive, motor and sensorimotor functions were assessed using novel object recognition and Morris water maze tasks. CB_1 receptor functionality was assessed by [^{35}S]GTPγS (guanosine 5'-O-[gamma-thio]triphosphate) autoradiography. STZ treatment was associated with mechanical allodynia and thermal hypoalgesia. Novel object recognition was unaltered in diabetic rats. STZ treatment was associated with impaired performance in the Morris water maze acquisition phase, but there were no differences in memory retrieval in the probe trial. Stimulus-response learning in the water maze cued trial was also disrupted in STZ-treated rats, possibly indicating sensorimotor deficits. CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding was attenuated in the substantia nigra of STZ-treated rats but unaltered in the hippocampus. In conclusion, STZ treatment as a model of diabetic neuropathy was associated with specific functional deficits in the MWM, effects which may be related to altered CB_1 receptor functionality in the substantia nigra.

Keywords: Diabetes; streptozotocin; neuropathic pain; Morris water maze; cannabinoid$_1$ (CB_1) receptor; [^{35}S]GTPγS binding

1. Introduction

Neuropathy is a common complication of diabetes. The incidence of diabetic neuropathy is correlated positively with diabetes duration [1], and the prevalence of painful neuropathic symptoms in diabetic patients has been estimated at ~ 34% [2].

Type 1 and type 2 diabetes are associated, to varying degrees, with cognitive deficits such as learning, memory and attentional impairments, slowing of psychomotor speed, and impaired mental flexibility [3-7]. Although these deficits have been well characterised, the mechanisms involved are poorly understood. Chronic pain, particularly neuropathic pain, is also associated with cognitive deficits [8, 9]. It has been proposed that pain-induced changes in neural resource utilisation, expression of neuromediators and neuroplasticity may affect cognition through a complex network of brain regions [8]. Given the prevalence of neuropathic pain in diabetes, it is possible that pain contributes to the cognitive deficits observed. This hypothesis is supported by studies reporting that a diagnosis of neuropathy was correlated with impaired cognitive performance in diabetic patients [3, 7]. A study by Ryan et al. [3] found a diagnosis of neuropathy to be the best predictor of cognitive test performance of all predictors investigated (including advanced background or proliferative retinopathy, overt nephropathy, or one or more episodes of severe hypoglycaemia). In addition, patients with diabetic neuropathy have been included in large scale studies demonstrating cognitive impairment associated with chronic pain [9].

The streptozotocin (STZ) rodent model of diabetes has been used extensively to investigate the pathophysiology of the disease. STZ is cytotoxic to pancreatic β-cells and administration of a single high dose most closely mimics Type 1 diabetes. This model is associated with symptoms mirroring those of clinical diabetes, including altered sensitivity to thermal, mechanical and chemical noxious stimuli [10, 11]. The STZ model has also been used to investigate learning

and memory impairments associated with diabetes. STZ treatment is associated with impairments in a variety of cognitive behavioural tasks such as spatial learning in the water maze [12-15], T-maze active avoidance [16], and object-placement learning tasks [17-19]. Learning impairments relate to the duration and severity of diabetes and to the complexity of the cognitive task [20].

The endocannabinoid system comprises endogenous ligands, including *N*-arachidonoyl ethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG), enzymes that biosynthesise or degrade the endocannabinoids, and two $G_{i/o}$-protein coupled receptors, cannabinoid$_1$ (CB_1) and cannabinoid$_2$ (CB_2) [21]. This system is involved in energy balance through regulation of lipid and glucose metabolism, and altered cannabinoid signalling has been associated with obesity, dyslipidaemia and type 2 diabetes [22]. A role for the endocannabinoid system in pain and analgesia is well established [23], and cannabinoid ligands have been shown to modulate neuropathic pain, including that induced by STZ [24]. Furthermore, the endocannabinoid system mediates cognitive functions such as memory and attention, and cannabinoids have been shown to affect cognitive performance in humans [25, 26] and rodents [27, 28]. Given the involvement of the endocannabinoid system in the triad of diabetes, neuropathic pain and cognition, it represents a logical substrate for investigation in the context of cognitive impairment associated with diabetic neuropathy. [^{35}S]GTPγS autoradiography has been used to assess both *ex vivo* function and neuroanatomical distribution of $G_{i/o}$-protein-coupled CB_1 receptors in discrete brain regions [29, 30].

The aims of the present study were: (a) to investigate the development of nociceptive behaviour and cognitive performance (recognition memory and spatial learning and memory) in STZ-diabetic rats and (b) to investigate the effect of STZ treatment on CB_1 receptor-mediated G-protein coupling in the hippocampus, a region classically associated with cognitive function

and also implicated in pain [31, 32], and in the substantia nigra, which is involved in movement, sensorimotor coordination, behavioural adaption based on reward or motivation, somatosensory discrimination and aspects of cognition [33-36].

2. Research Design and Methods

2.1 Experimental design

Male Lister-hooded rats (Charles River, UK, 225-250g) were singly-housed under standard laboratory conditions of temperature (20 ± 2°C), humidity (50-80%) and lighting (12:12 hour light/dark cycle, lights on at 08:00h), with food and water available *ad libitum*. Lister-hooded rats have been shown to perform well in a variety of standard tests of cognitive function including the Morris water maze [37]. Animals were singly-housed to accurately monitor food and water intake. Experiments were carried out in accordance with the guidelines and approval of the Animal Care and Research Ethics Committee of the National University of Ireland, Galway, under licence from the Irish Department for Health and Children and in compliance with European Communities Council directives 2010/63/EU and 86/609/EEC. The timeline for *in vivo* experimental procedures is outlined in Figure 1. Type 1-like diabetes was induced and blood-glucose concentrations were determined according to protocols described previously [38]. Rats received a single subcutaneous injection of 60mg/kg STZ (Sigma Aldrich Ireland) or citrate-buffer vehicle (STZ n=16, control n=16). Blood-glucose concentrations greater than 15mM were considered indicative of a diabetic phenotype.

All behavioural testing was carried out during the light phase by an experimenter blind to treatment condition and the order of testing was randomised to minimise confounding effects of time of day.

2.2 Assessment of mechanical and thermal sensitivity

Mechanical paw-withdrawal thresholds were assessed using the von Frey method described previously [39]. Testing was carried out in a six-chambered Perspex arena and animals were acclimatised to the arena prior to testing. Measurement of withdrawal thresholds involved mechanical stimulation of the hind paws with von Frey filaments (Touch-Test ™ Sensory Evaluators, North Coast Medical, Inc.) of differing weights (1.4g to 12g), and a positive response was recorded if flinching, licking or withdrawal of the paw was observed. The 50% response threshold was calculated for each hind paw and a mean response threshold for each rat was computed as the mean of the two hind paws.

Thermal nociceptive responses were assessed using a protocol similar to that described previously [39]. A commercial instrument (Plantar/tail flick combination system, model 336, IITC Life Science Inc., USA) was used, and animals were habituated to the arena prior to testing. The thermal stimulus was applied until a positive response (criteria similar to von Frey testing) was recorded or until a cut-off time of 20s was reached. The mean withdrawal latency for each rat was computed from the applications to both hind paws (four/paw).

2.3 Assessment of cognitive function

2.3.1 Novel-object recognition

The procedure used for the novel-object recognition test was similar to that previously described by King et al. [40], with some modifications. The apparatus was constructed of Perspex and melamine-coated chipboard (60cm x 30cm x 40cm). The “familiar” objects used were two plastic bottles covered with white masking tape. A third bottle, the “novel object” had three strips of black tape, making it visually distinct. Animals were habituated to the arena in the absence of objects for 30 minutes on the day before the test day. The test day comprised

three stages: habituation (3min exposure to arena in absence of objects), exposure 1 (3min familiarisation to identical objects) and exposure 2 (3min exposure to one familiar and one novel object). Habituation and exposure 1 were separated by a 7min inter-trial interval and exposures 1 and 2 by a further 2min interval. Exploration of an object was defined as described previously [41]. The three test stages were recorded to videotape for subsequent analysis. Object exploration and general behaviours (sniffing, rearing, walking) were manually rated using Ethovision® software (Noldus, The Netherlands), and the discrimination ratio for each object was calculated as (time spent exploring either object)/(time spent exploring both objects).

2.3.2 Morris Water Maze (MWM)

Procedures for water-maze acquisition training, probe trial and cued test were similar to those described previously [42]. The apparatus consisted of a circular black plastic pool (95cm diameter) with a white plastic platform (11cm x 43cm). Visual cues were placed around the outside of the maze during acquisition training and during probe trials. For acquisition training, the pool was filled to 2cm above the level of the platform and the water was made opaque (by addition of white paint, Icon Apprentice, Ireland), such that the platform was hidden. The training consisted of four trials per day for five days, throughout which the platform was positioned in the southwest quadrant of the pool. Animals were released from one of four release points in a quasi-random order, and were given 120s to locate the hidden platform. Once the platform was successfully located, the rat was allowed to remain on the platform for 10s. If the rat did not locate the platform within 120s, it was guided towards it by the experimenter. The animal was then removed from the pool, dried and placed in a heated recovery cage for an inter-trial period (5-15min). A probe trial was carried out on the day after acquisition training was completed. The platform was removed from the maze, and rats were released from the northeast quadrant. The probe trial consisted of a single 60s trial for each rat.

The cued test was conducted the day after probe trial and each rat underwent six trials. The water level was reduced to 2cm below the height of the platform so that the platform was visible. The procedure was similar to that for acquisition, except the platform was moved to a different quadrant between trials. All trials were recorded to videotape; post testing, Ethovision® software was used to measure the following parameters: distance moved (path length) to locate the platform for acquisition training and cued test, distance moved in each of the arena zones (platform, annulus, and southeast, southwest, northeast and northwest quadrants) and swim speed for the probe trial.

2.4 [^{35}S]GTPγS autoradiography

Animals were killed by decapitation on day 46 post-STZ injection. Brains were removed, snap-frozen on dry ice and stored at -80°C until sectioning. A subset of the brains from control and STZ-treated rats were chosen randomly for ^{35}S]GTPγS autoradiography to give final n numbers of 6 (control) and 5 (STZ). 20µm sections containing the dorsal hippocampus (Bregma -2.8mm) and substantia nigra (Bregma -5.2mm) were cut on a cryostat and thaw-mounted onto gelatin-coated glass slides (4 sections per slide and a minimum of 3 slides per brain). The protocol for [^{35}S]GTPγS autoradiography was as described previously [30]. Slides were thawed at room temperature for 30min, then washed 3 x 20min in TRIS buffer (50mM TRIS, 100mM NaCl, 10mM $MgCl_2$, 0.2mM EGTA, pH 7.4). For each rat brain, slides were assigned to the following *ex vivo* treatment groups: (A) basal, (B) CB_1 receptor agonist HU210 (5µM), (C) HU210 (5µM) + CB_1 receptor antagonist SR141716A (5µM). Drugs were obtained from Tocris Bioscience, UK. Each slide (*n*=3/group/brain region) was pre-incubated in 1ml of GDP assay solution (TRIS buffer, 2mM GDP (Fluka, UK), 0.5% w\v BSA) for 20min in a humidity chamber at 25°C. The 1ml GDP assay solution used to incubate Group C also contained the antagonist SR141716A (5µM). The pre-incubation GDP solution was then removed and 1ml

of a "hot" GDP assay solution, containing [^{35}S]GTPγS (0.04nM; Sigma-Aldrich Ireland) and the relevant drug(s), was applied to each slide for 90min in the humidity chamber at 25^{o}C. To evaluate non-specific binding, separate sections were incubated in "hot" GDP assay solution containing an excess of non-[^{35}S]-labelled GTPγS (10μM). Following the incubation period, slides were removed and rinsed twice in ice-cold TRIS buffer (pH 7.4) followed by a further 3 x 2min washes in ice-cold TRIS buffer. Slides were then dipped in distilled, deionised H_2O and dried in a warm air stream before exposure to Hyperfilm-β max autoradiographic film (GE Healthcare, UK) for a minimum of 48 hours. Films were developed by hand using Kodak GBX developer and fixer (Kodak, UK). Autoradiograms were digitised using an Epson Perfection 4870 Photo scanner, and resulting images were analysed densitometrically using ImageJ software for PC. A standard curve relating greyscale density to disintegrations per minute (DPM) was constructed using ^{14}C microscale standards which had been exposed alongside the samples. The dorsal hippocampus (CA1-3 and dentate gyrus) and substantia nigra were then outlined with reference to the rat brain atlas [43], and measured greyscale values were converted to DPM.

2.5 Statistical Analysis

Behavioural data were analysed by repeated-measures or one-way analyses of variance (ANOVA), with the exception of probe-trial data which were analysed by Student's unpaired two-tailed *t* tests (for individual arena zones and distance moved). For analysis of autoradiography data, *ex vivo* [^{35}S]GTPγS drug treatment groups within the control and STZ-induced diabetic groups were expressed as a percentage of basal DPMs per region per brain, and these data were then expressed as mean ± SEM for each treatment group. Data were analysed using two-way ANOVA (with STZ treatment and *ex vivo* [^{35}S]GTPγS drug treatment

as factors). ANOVA was followed by Fisher's LSD *post hoc* tests where appropriate. The level of statistical significance was set at $p<0.05$.

3. Results

3.1 Induction of diabetes following STZ injection

The effects of STZ administration on body weight, water intake and blood glucose are shown in Figure 2. Two-way repeated-measures ANOVA revealed significant effects of time ($F_{(50,1400)} = 253.3$, $p < 0.0001$) and treatment ($F_{(1,1400)} = 279.7$, $p < 0.0001$), and a treatment x time interaction ($F_{(50,1400)} = 333.5$, $p < 0.0001$) on body weight. Compared with vehicle-treated controls, STZ-treated animals displayed reduced body weight gain. Two-way repeated-measures ANOVA revealed significant effects of time ($F_{(40,1120)} = 7.937$, $p < 0.0001$) and treatment ($F_{(1,1120)} = 226.9$, $p < 0.0001$), and a treatment x time interaction ($F_{(40,1120)} = 6.282$, $p < 0.0001$) on water intake. Post-hoc analysis revealed that STZ-treated animals had a marked increase in water intake compared to vehicle-treated controls. Two-way repeated measures ANOVA revealed significant effects of time ($F_{(4,112)} = 491.2$, $p < 0.0001$) and treatment ($F_{(1,112)} = 8502$, $p < 0.0001$), and a treatment x time interaction ($F_{(4,112)} = 484.6$, $p < 0.0001$) on blood glucose concentrations. Blood glucose concentrations were significantly increased from 3 days post-STZ injection and remained elevated throughout the experiment, confirming development of a diabetic phenotype. All STZ-treated rats developed hyperglycaemia and two animals were excluded due to ill health.

3.2 Development of mechanical allodynia following STZ injection

von Frey testing for mechanical allodynia was performed on days 4, 6, 10, 17, 19, 23 and 26 post-STZ injection and baseline measures were taken two days pre-STZ injection. There were no differences between the two groups at baseline (Figure 3). Two-way repeated-measures

ANOVA revealed a significant main effect of time ($F_{(7,175)} = 9.124$, $p < 0.0001$) and an interaction effect (treatment x time; $F_{(7,175)} = 2.500$, $p = 0.0180$). In the STZ-treated group, hind paw 50% withdrawal threshold was significantly decreased at all post-injection time points compared with baseline (Figure 3). There was also a decrease in threshold in the control group relative to baseline at a number of discrete post-injection time points (days 4, 10, 17 and 26, Figure 3). The 50% withdrawal threshold was significantly lower in STZ-diabetic animals compared with controls on days 19 and 23 post-STZ injection, indicating development of mechanical allodynia in the STZ-treated rats (Figure 3). Three animals were excluded for von Frey analysis due to missing values in the timecourse of mechanical threshold measurements.

3.3 Hargreaves test for thermal sensitivity

Hargreaves testing was carried out one day before STZ or control injection (baseline), and on days 5, 9, 11, 18, 20, 24 and 27 post injection. There were no differences between the two groups at baseline. Two-way repeated-measures ANOVA revealed a significant effect of treatment ($F_{(1,196)} = 22.46$, $p < 0.0001$) but not time. The paw-withdrawal latency was significantly longer in STZ-treated rats compared with control rats on days 9, 18, 20, 24 and 27 post injection, indicating expression of thermal hypoalgesia in these rats (Figure 4).

3.4 Novel object recognition (NOR)

STZ treatment had no effect on general exploratory behaviours (sniffing and walking) during the habituation period on the test day (Figure 5(a)), but STZ-treated animals did show significantly reduced rearing compared with control animals. Discrimination ratios were calculated both for the identical objects in exposure 1 and for the novel and familiar objects in exposure 2. During exposure 1, both objects were explored equally, and there were no between-group differences (data not shown). During exposure 2, the discrimination ratio for the novel object was significantly greater than that for the familiar object in both control and STZ-treated

animals, indicating a preference for the novel object in both groups (Figure 5(b)). Data were analysed by a one-way ANOVA ($F_{(3.51)} = 10.57$, $p<0.001$) followed by Fisher's LSD *post hoc* tests. There were no significant between-group differences.

3.5 Morris water maze (MWM)

Two-way repeated-measures ANOVA revealed significant effects of time ($F_{(4,92)} = 40.68$, $p<0.0001$) and treatment ($F_{(1,23)} = 10.20$, $p< 0.01$), and a treatment x time interaction ($F_{(4, 92)} = 3.20$, $p<0.05$) on path length to platform. Fisher's LSD *post hoc* test revealed that the performance of both STZ-treated and control rats improved over the acquisition phase as indicated by a significant effect of time on path length to platform (Figure 6(a)). The STZ-treated rats showed a reduced learning rate, and travelled further to reach the platform than controls on days 2-5 of acquisition. Both groups appeared to reach a steady-state path length as there was no significant difference in performance on day 4 vs. day 5 within either group (Figure 6(a)).

Treatment had no significant effect on distance moved in the zones of interest (platform, annulus, southwest quadrant) during the probe trial (Figure 5(b)). Student's unpaired two-tailed *t* tests did not reveal any significant between-group differences for any of the arena zones of interest. STZ-treated rats did have a significantly lower percentage distance moved in the northeast quadrant; however, this quadrant had no task-specific relevance. STZ-treated rats were found to have a significantly reduced swim speed compared with controls in the probe trial, suggesting a global motor deficit in the STZ-treated rats compared with controls (Figure 6(c)).

The cued test measured the path length to the visible platform. It is considered a test of sensorimotor function and of the ability to recognise the platform as an escape route. Animals that failed to recognise the platform as an escape route in the cued test were excluded from all

other MWM analyses (i.e. acquisition and probe trial, n=5 STZ-treated animals). Two-way repeated-measures ANOVA revealed a significant effect of time ($F_{(5,115)} = 7.79$, $p< 0.0001$) and treatment ($F_{(1,28)} = 9.34$, $p=0.005$) on path length. The path length to the platform decreased over time in the control group (Figure 6(d) $p<0.05$ trials 4-6 vs. trial 1), suggesting improved understanding of the sensorimotor task. However, in the STZ-treated group, path length was only reduced relative to trial 1 in trial 4. STZ-treated rats travelled further before locating the platform than controls in trials 2, 3, 5 and 6 (Figure 6(d)).

Together, these results suggest that STZ treatment is associated with a specific deficit in MWM acquisition learning, but not memory retrieval, and with motor and sensorimotor impairments.

3.6 [^{35}S]GTPγS binding autoradiography

Two-way ANOVA with *ex vivo* drug treatment and treatment (STZ or control) as factors revealed a significant effect of drug treatment ($F_{(2,28)}=12.91$, $p<0.001$) on [^{35}S]GTPγS binding in the hippocampus. Fisher's LSD *post hoc* tests showed that Treatment of hippocampal brain sections with the cannabinoid receptor agonist HU210 significantly stimulated [^{35}S]GTPγS binding in the hippocampus of both control and STZ-treated rats, an effect that was significantly attenuated by the CB_1 receptor antagonist/inverse agonist SR141716A in both groups. There were no significant differences in hippocampal [^{35}S]GTPγS binding between control and STZ-treated animals in either drug condition (Figure 7(a)). Dorsal and ventral hippocampal regions and dentate gyrus were also analysed separately and no significant between-group differences were observed (data not shown).

In the substantia nigra, two-way ANOVA revealed significant main effects of drug ($F_{(2,26)}$=35.05, p<0.001) and treatment ($F_{(1,26)}$=5.46, p<0.05) on [^{35}S]GTPγS binding. HU210 significantly stimulated [^{35}S]GTPγS binding in both control and STZ-treated animals and this stimulation was again significantly attenuated by SR141716A in both groups. The magnitude of the HU210-induced [^{35}S]GTPγS stimulation was significantly smaller in the STZ-treated group compared with the control group (Figure 7(b)). Representative autoradiograms from sections containing the substantia nigra are presented in Figure 8.

4. Discussion

The results presented herein confirm that administration of streptozotocin induces a diabetic phenotype, characterised by increased blood-glucose concentration, reduced weight gain and increased water intake. Mechanical allodynia developed over time relative to baseline, and mechanical threshold was significantly lower in STZ-treated rats compared with controls on days 19 and 23 post treatment. Thermal hypoalgesia was also observed in STZ-diabetic animals. Recognition memory in the novel object recognition paradigm was not impaired in STZ-treated animals. Furthermore, non-object-directed exploratory behaviour (sniffing) was not affected by STZ treatment. There was, however, a significant reduction in rearing in STZ-treated rats compared with controls. In MWM acquisition, STZ-treated rats showed a deficit in spatial learning. STZ treatment was also associated with motor and sensorimotor impairments, but not with adverse effects on memory-retrieval. CB_1 receptor functionality in the hippocampus was not altered in STZ-diabetic animals, but was significantly lower in the substantia nigra of STZ-treated compared with controls.

The development of tactile allodynia/mechanical hyperalgesia in the STZ model of diabetes is well documented in the literature [10, 11, 38] and is consistent with the present results. Studies

show signs of reduced mechanical/tactile thresholds within 1-2 weeks post induction of diabetes that further reduce with time [10, 44, 45]. Changes in mechanical threshold compared with baseline were also observed in the saline-treated animals at discrete time points, possibly due to sensitisation of the hind paws over time. This is consistent with the observation by Chaplan at al. [46] that repetitive low-intensity stimulation with von Frey filaments of the hind paw of naïve rats was associated with a gradual decrease in mechanical response threshold. This is presumed to be adaption of behaviour in response to "annoyance or nuisance in the absence of nociception". Nevertheless, the STZ-treated group showed greater sensitivity to mechanical stimuli than the saline-treated group overall; confirming that induction of diabetes produced the expected neuropathic pain-like phenotype. STZ-treated rodents show varying responses to thermal stimuli, including hypoalgesia, hyperalgesia, no alteration of response, and initial hyperalgesia followed by hypoalgesia or a return to control levels [38]. The reasons for these conflicting results are unclear but possible explanations include methodological differences e.g. the specific location of the heat-stimulus application, the time of testing post STZ administration, and species and strain differences in behavioural pain testing [38]. It is important to note that the between-group difference in paw-withdrawal latency is relatively small (mean difference < 2s). Hypoalgesia reported by Rutledge et al. [47] represented an average change in response latency of up to 7.6s. However, STZ-induced hyperalgesia with an average change in response latency of approximately 2s has been reported previously [38, 48] under similar experimental conditions to the present study. No significant time effect was observed in the present study, suggesting that thermal sensitivity was not altered post STZ compared with baseline.

We found no effect of STZ treatment on recognition memory in the novel-object recognition task. Interestingly, some previous findings of impaired recognition memory in the STZ model were based on object-placement/spatial-recognition paradigms, rather than on the commonly

used object-recognition paradigm employed in the present study [49-51]. This methodological difference may explain the lack of any STZ-induced effect in the present study. In a spatial/object learning and memory paradigm, Popovic et al. [17] found that increasing task complexity was negatively correlated with task performance. Therefore, the novel-object paradigm used herein may not have been sufficiently complex to detect differences between STZ-treated and control rats.

We found that STZ treatment was associated with a deficit in MWM acquisition, as path length to the hidden platform was longer for STZ-treated animals than for control rats. This is in line with previous studies investigating water-maze performance in STZ-diabetic rats [12-14, 52]. Measurement of path length (distance swam to get onto the platform) controls for the potential confound of motor impairment in the MWM, and therefore the present findings suggest that the deficits observed in STZ-treated rats are specifically related to cognitive impairment. The impairment also appears to be specific to spatial learning, as there were no between-group differences in the probe trial which predominately assesses memory retrieval.

Biessels et al. [12] demonstrated that deficits in water-maze performance were not observed if animals are given non-spatial pre-training, and suggested that STZ-induced effects did not equate to impaired spatial learning but rather to impaired learning of the maze procedure itself, i.e., diabetic animals fail to learn that the platform is the quickest route of escape. In the present study, animals that failed to learn to use the platform as an escape route (based on cued-trial performance) were excluded from the MWM acquisition and probe-trial results. Notably, this exclusion criterion applied to five STZ-treated rats (36% of subjects) and to no control rats. In the cued trial itself, the STZ-treated group took a significantly longer path to the visible platform than did the control group. This suggests that even though they associated the platform with escape, STZ-treated animals still failed to grasp the primary goal of the cued task, which

may be due to a deficit in sensorimotor integration, i.e., coordinating visualisation of the platform and the objective of swimming to it.

We hypothesised that impaired cognitive function in STZ-treated rats may be associated with reduced functionality of cannabinoid receptors in the hippocampus. This was based on the involvement of the endogenous cannabinoid system in pain, particularly neuropathic pain [22, 23], and in cognition [25-28]. It has been suggested that pain-related alterations in neurotransmitter systems, including the endocannabinoid system, may mediate the cognitive deficits observed in neuropathic pain in animals and humans [8], and this may extend to deficits associated with painful diabetic neuropathy. To our knowledge, cannabinoid-induced G-protein coupling has not been investigated previously in an animal model of diabetic neuropathy.

Our results suggest that cannabinoid agonist-stimulated [^{35}S]GTPγS binding in the hippocampus is unaffected by STZ administration. This finding does not preclude the involvement of the hippocampal cannabinoid system in STZ-related deficits in the water maze, but may be due to methodological issues such as the time post STZ at which animals were sacrificed. It is also possible that cannabinoid receptor functionality was altered in other cognition-related brain regions that were not investigated in the present study. Neuropathic pain is believed to negatively affect executive functions mediated by the prefrontal cortex [8]. Reversal tasks in the Morris water maze assess cognitive flexibility (analogous to human executive functions) and future studies should investigate the effects of STZ treatment on reversal learning and the cannabinoid system in the prefrontal cortex.

There was a significant difference between STZ and control rats for HU210-stimulated [^{35}S]GTPγS binding in the substantia nigra. Given the behavioural results observed, the altered cannabinoid functionality in this region may be of particular interest. The substantia nigra is a

midbrain structure and a component of the basal ganglia. The role of the substantia nigra in movement is well established, and it is also associated with sensorimotor coordination, behavioural adaption based on reward or motivation, somatosensory discrimination, visual perception, spatial working memory and habit learning [33-36]. The substantia nigra pars reticulata contains spontaneously active GABAergic neurons [53] which tonically inhibit movement. Cannabinoid-mediated disinhibition of GABAergic tone would therefore facilitate movement, and a decrease in CB_1 receptor functionality could be associated with impaired motor function. Such a mechanism may underlie the decrease in distance moved in the probe trial, and the reduced rearing behaviour observed in the present study. While the standard place-learning MWM task is thought to be hippocampal-dependent, the cued test requires stimulus-response learning and recent evidence has shown that cued-test performance is disrupted by lesion of the substantia nigra [54, 55]. Thus, the finding of impaired functionality of cannabinoid receptors in the substantia nigra in this study may be a contributory factor in the impaired cued-test learning in the STZ-treated animals. Future studies should involve manipulation of the cannabinoid system in the substantia nigra in the STZ model to determine whether deficits in cued learning can be reversed.

In conclusion, STZ-diabetic rats showed impairments in cognitive function in both the cued- and spatial-learning tasks of the MWM. These behavioural observations were accompanied by altered cannabinoid receptor functionality in the substantia nigra. Further investigation is warranted to determine whether and how the endocannabinoid system in the substantia nigra mediates STZ-induced alterations in water-maze behaviour.

Acknowledgements

This work was supported financially by grants from Science Foundation Ireland (05/Y12/B686) and by the Irish Higher Education Authority's Programme for Research in Third Level Institutions Cycle 4 (National Development Plan 2007-2013) through the National Biophotonics and Imaging Platform. OM, YL, ZI, BEM and DPF contributed substantially to study conception and design, and/or to acquisition, analysis and interpretation of data. OM, YL, BEM and BPF drafted the article or revised it critically for important intellectual content. DPF is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors have no conflicts of interest to declare.

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Figure Legends

Figure 1. *In vivo* experimental design

Figure 2. Administration of STZ leads to the development of a diabetic phenotype as demonstrated by reduced body weight, increased water intake and elevated blood glucose concentration.

(a) Animal body weight. (b) Water intake. (c) Blood glucose concentration. Data are expressed as mean ± SEM, n = 14-16 animals per group.

Figure 3. STZ treatment is associated with the development of mechanical allodynia. Hind paw withdrawal threshold measured by von Frey testing. Data are expressed as mean ± SEM for average of right and left hind paw, n = 11 – 16 animals per group. Fisher's LSD *post hoc* tests showed that the average paw withdrawal threshold decreased post-STZ injection and was significantly lower at all post-injection time points compared with baseline ($^{*}p<0.05$). Withdrawal threshold was also lower in the control group at days 4, 10, 17 and 26 compared with values collected pre-administration of STZ vehicle control ($^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$). STZ-treated animals had a significantly lower mechanical threshold than the control animals on days 19 and 23 post-STZ administration ($^{\Phi}p<0.05$, $^{\Phi\Phi}p<0.01$).

Figure 4. STZ treatment is associated with the development of thermal hypoalgesia. Hargreaves test for thermal sensitivity. Mean paw-withdrawal latency of right and left hind paws. Data are expressed as mean ± SEM, n = 14-16 animals per group. STZ-treated rats had significantly longer withdrawal latencies than control rats on days 9, 18, 20, 24 and 27 post-

STZ administration ($^{\Phi}p<0.05$, $^{\Phi\Phi}p<0.01$ STZ vs. control), indicating expression of thermal hypoalgesia.

Figure 5. STZ treatment does not alter recognition memory in the novel object recognition task. (a) General behaviours during the habituation phase on test day. Data are expressed as mean ± SEM, n = 14 -16 per group. There were no differences between STZ and control groups on measures of sniffing and walking. Duration of rearing was significantly reduced in the STZ group compared with the control group (**$p<0.01$ vs Control, Student's unpaired two-tailed t tests). (b) Discrimination ratios for novel and familiar objects during exposure 2. Data are expressed as mean ± SEM (n = 14 – 16 per group). Both groups spent a significantly greater proportion of the trial exploring the novel object vs. the familiar object (**$p < 0.01$). There was no significant difference between STZ-treated and control animals.

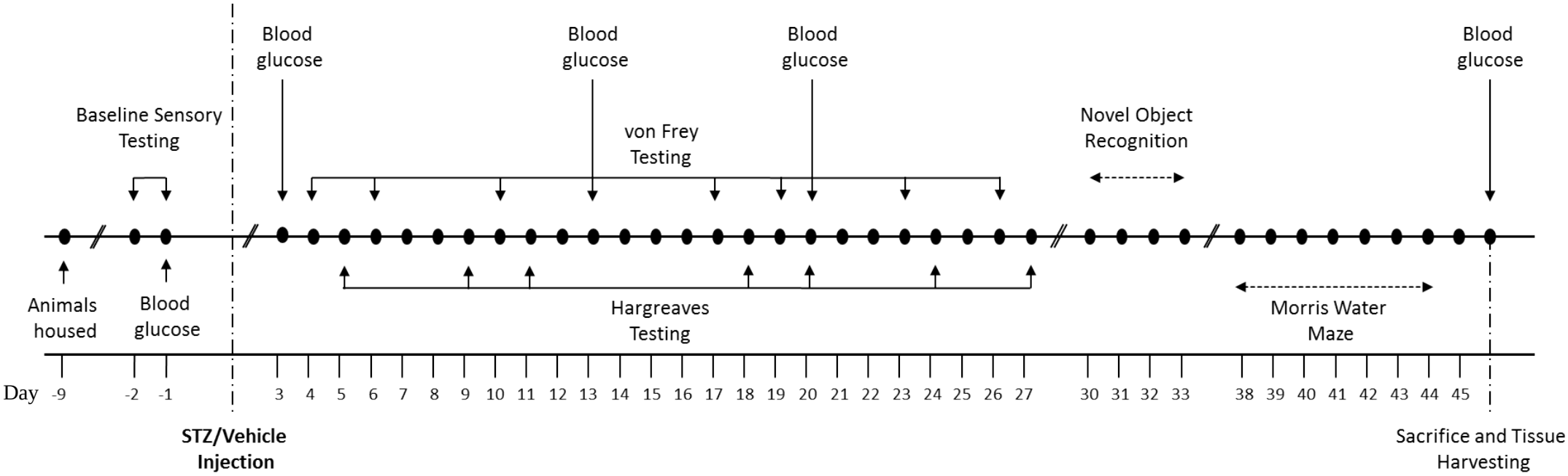
Figure 6. Morris water maze acquisition, probe trial and cued test performance.

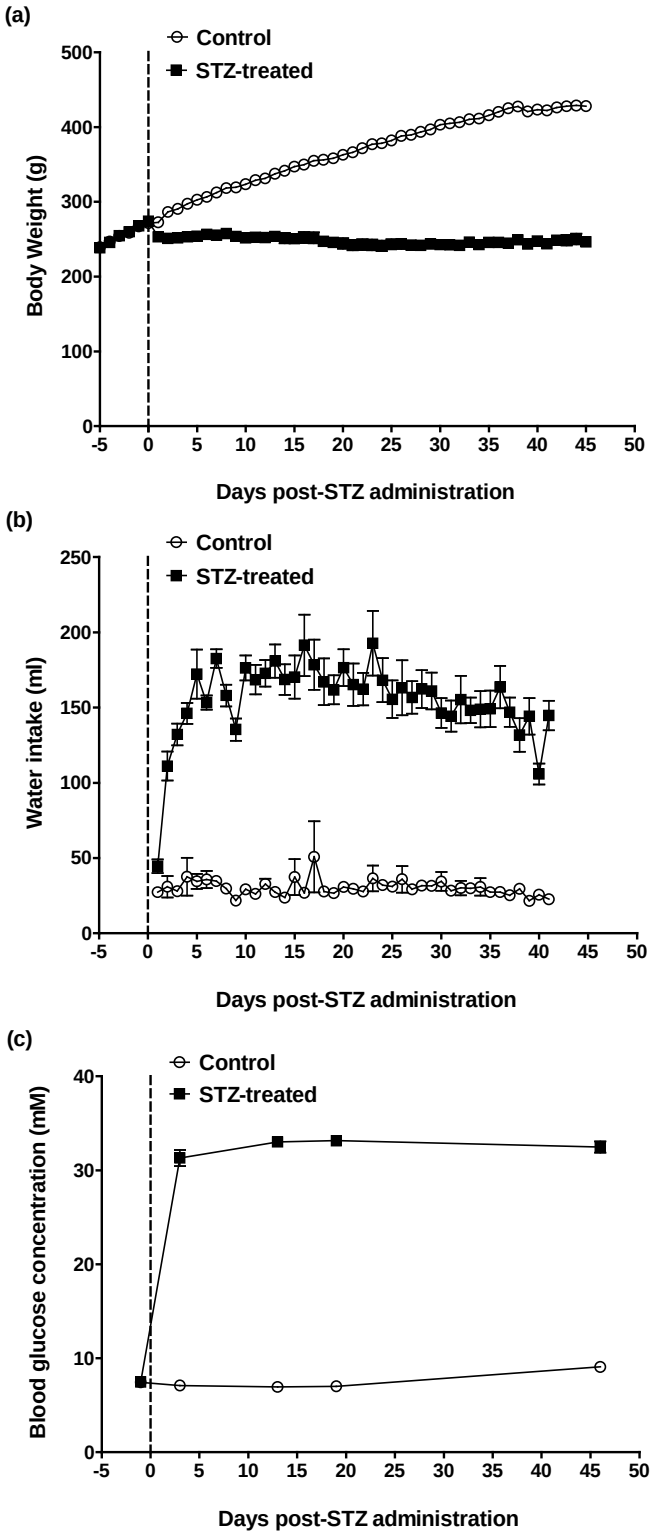
(a) Distance moved to get onto the platform during acquisition training (path length). Data are expressed as the mean path length to the platform per day (that is, the mean of four trials) ± SEM (n = 9-16 animals per group). Fisher's LSD *post hoc* tests showed that the path length to get onto the platform decreased over time in both groups. Control $^{\dagger\dagger\dagger}p<0.001$ days 2-5 vs. day 1, STZ *$p<0.05$ days 2-5 vs. day 1. Control vs STZ $^{\Phi\Phi\Phi}p<0.001$, $^{\Phi\Phi}p<0.01$, $\Phi p<0.05$. (b) % distance moved in arena zones during the probe trial. Data are expressed as mean ± SEM (n = 9-16 animals per group). The % distance moved in the northeast quadrant was significantly lower in the STZ group compared with the control group (*$p<0.05$). Dashed lines represent the expected % times that the animal would spend in a specific zone without any previous training; 1% platform area, 4% annulus area, 25% quadrant (inset: schematic depicting platform position

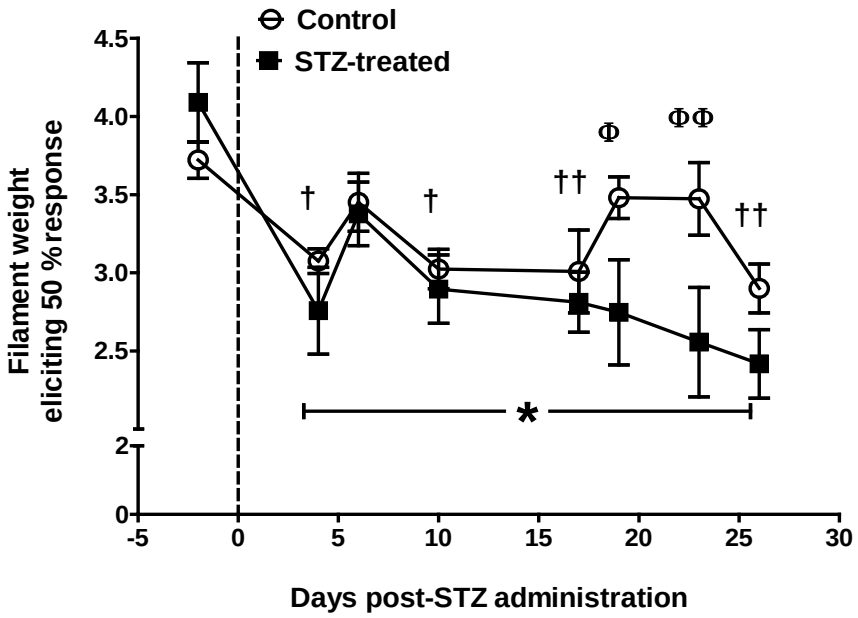
during acquisition training). (c) Swim speed during the probe trial. Data are expressed as mean ± SEM (*n*= 9-16 animals per group). A Student's unpaired two-tailed *t* test revealed that the STZ-treated rats moved significantly slower during the probe trial (**$p< 0.01$). (d) Path length to the *visible* platform during the cued test. Data are expressed as mean ± SEM (*n* = 14-16 animals per group). There was a significant decrease in the path length to get onto the platform over time in both control and STZ groups (control $^{\dagger}p<0.05$ trials 4-6 vs. trial 1, STZ *$p<0.05$ trial 4 vs. trial 1). STZ-treated rats travelled further to locate the visible platform than did control rats in trials 2, 3, 5 and 6 ($^{\Phi}p<0.05$, $^{\Phi\Phi}p<0.01$).

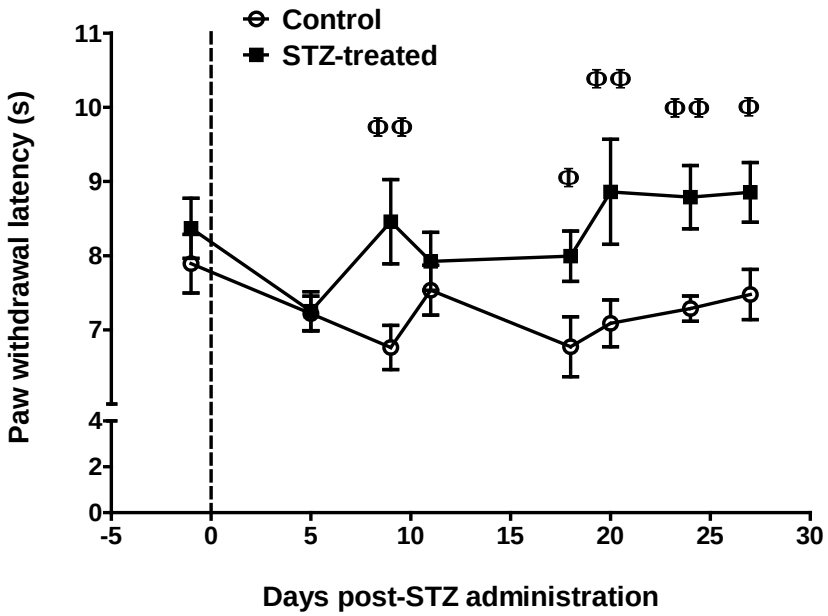
Figure 7. CB_1 receptor agonist-stimulated [^{35}S]GTPγS binding is attenuated in the substantia nigra of STZ-treated rats but unaltered in the hippocampus. The effect of the cannabinoid receptor agonist HU210, in the presence or absence of the CB_1 receptor antagonist/inverse agonist SR141716A, on [^{35}S]GTPγS binding in the (a) Hippocampus and (b) Substantia nigra of control and STZ-treated rats. Data are expressed as a % of basal [^{35}S]GTPγS binding ± SEM (*n* = 5-6 rats per group). (*$p<0.05$, **$p<0.01$, ***$p<0.001$ HU210 vs. basal, ††$p<0.01$, †††$p<0.001$ HU210 vs. HU210 + SR141716A). The HU210-stimulated increase was significantly greater in control rats than in STZ-treated rats (##$p<0.01$).

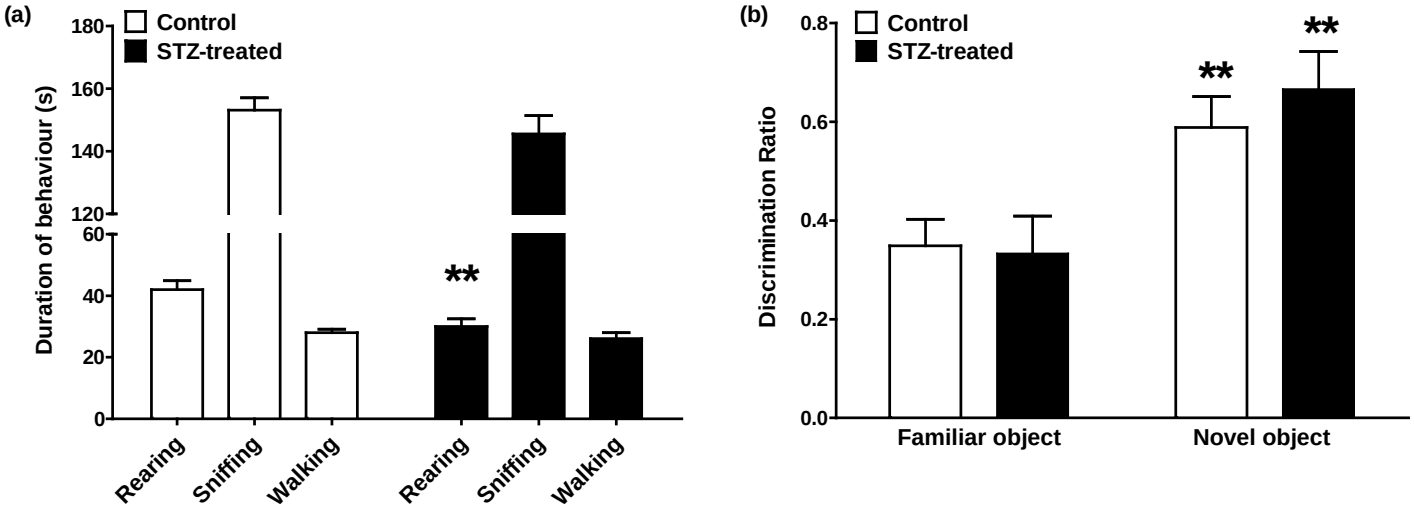
Figure 8. Representative autoradiograms of [^{35}S]GTPγS binding in control and STZ-treated rat brain sections (20 µm) containing the substantia nigra (SN). (A) and (D) represent basal [^{35}S]GTPγS binding, (B) and (E) represent HU210-stimulated [^{35}S]GTPγS binding, and (C) and (F) represent HU210-stimulated [^{35}S]GTPγS binding in the presence of the CB_1 receptor antagonist/inverse agonist SR141716A. Scale bar = 5mm.

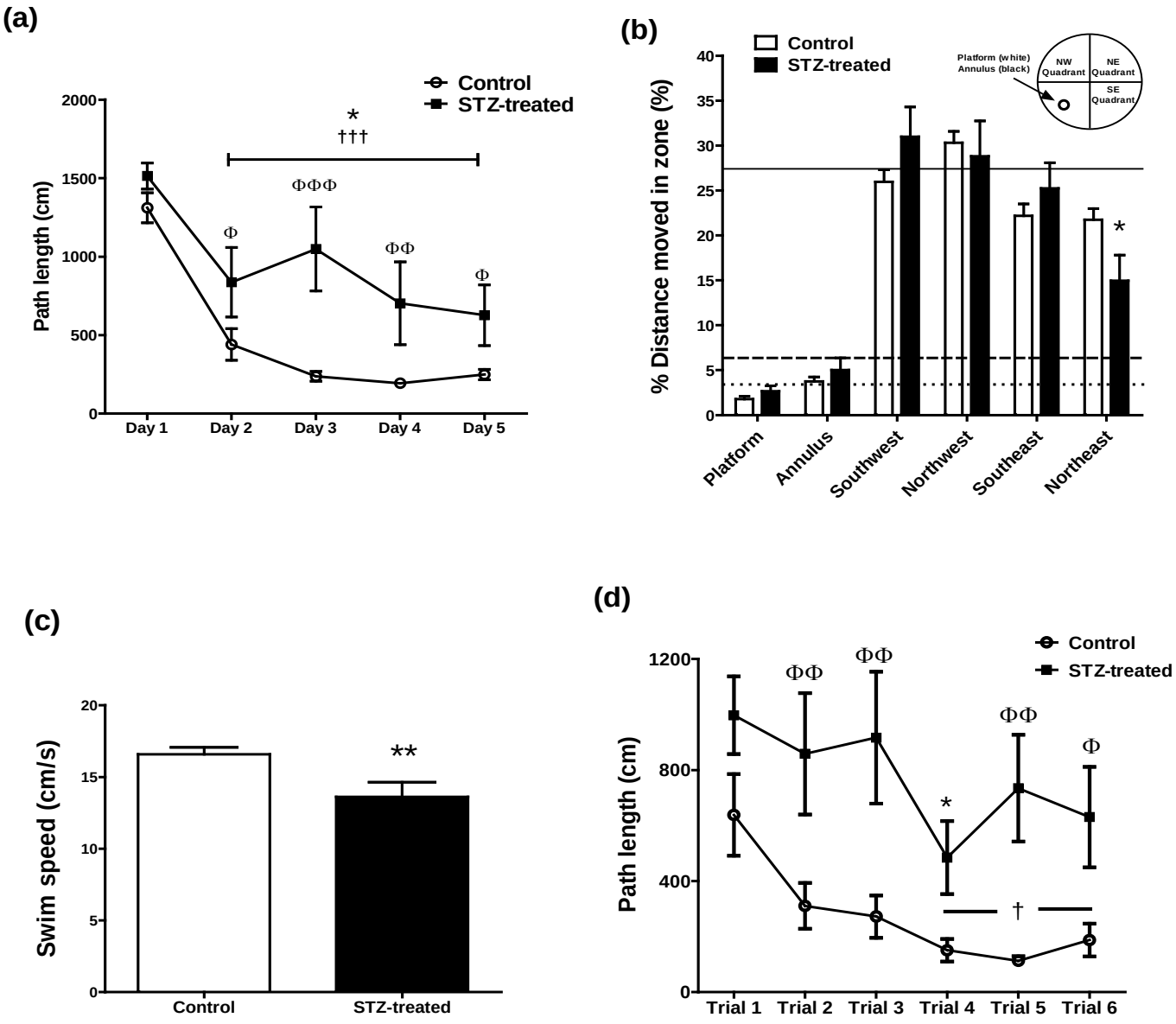

Blood glucose
Blood glucose
Blood glucose
Blood glucose
Baseline Sensory Testing
von Frey Testing
Novel Object Recognition
Animals housed
Blood glucose
Hargreaves Testing
Morris Water Maze
Day -9 -2 -1 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 30 31 32 33 38 39 40 41 42 43 44 45
STZ/Vehicle Injection
Sacrifice and Tissue Harvesting

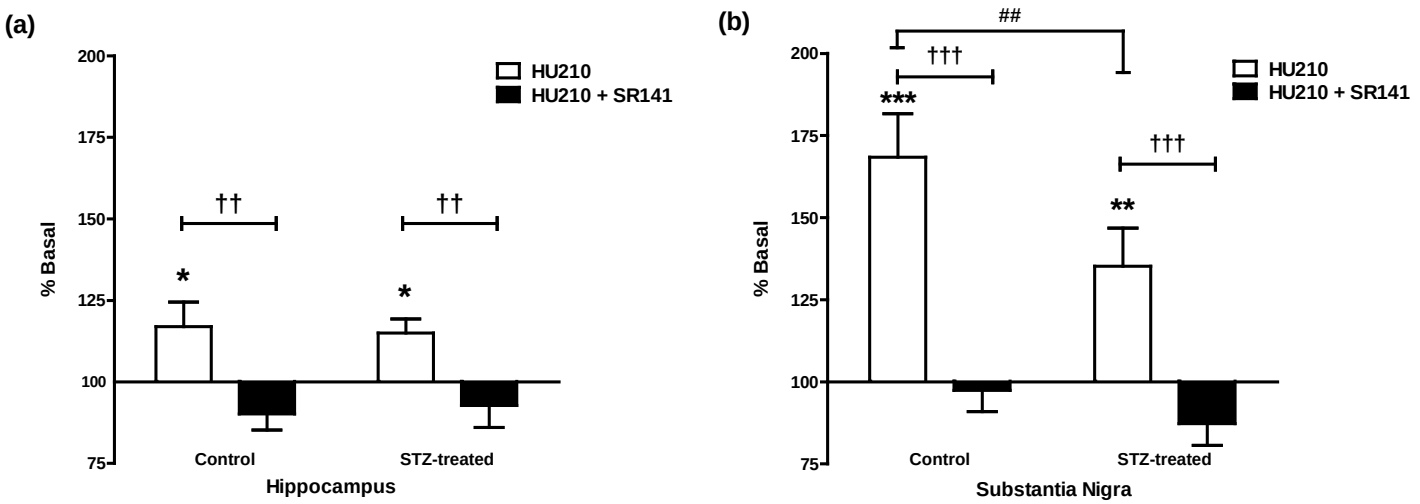

(a)
Control
STZ-treated
Body Weight (g)
500
400
300
200
100
0
-5
0
5
10
15
20
25
30
35
40
45
50
Days post-STZ administration
(b)
Control
STZ-treated
Water intake (ml)
250
200
150
100
50
0
-5
0
5
10
15
20
25
30
35
40
45
50
Days post-STZ administration
(c)
Control
STZ-treated
Blood glucose concentration (mM)
40
30
20
10
0
-5
0
5
10
15
20
25
30
35
40
45
50
Days post-STZ administration

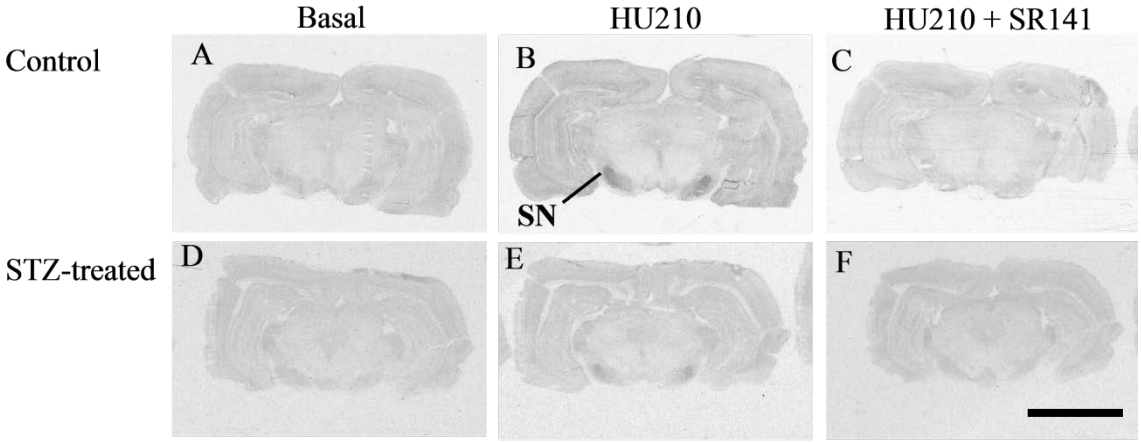

Control
STZ-treated
Filament weight eliciting 50 %response
4.5
4.0
3.5
3.0
2.5
2
0
-5
0
5
10
15
20
25
30
†
†
††
Φ
ΦΦ
††
*
Days post-STZ administration


Control
STZ-treated
Paw withdrawal latency (s)
11
10
9
8
7
4
2
0
-5
0
5
10
15
20
25
30
ΦΦ
ΦΦ
ΦΦ
Φ
Φ
Days post-STZ administration


(a)
Control
STZ-treated
Duration of behaviour (s)
180
160
140
120
60
40
20
0
**
Rearing
Sniffing
Walking
Rearing
Sniffing
Walking
(b)
Control
STZ-treated
Discrimination Ratio
0.8
0.6
0.4
0.2
0.0
**
**
Familiar object
Novel object


(a)
Control
STZ-treated
Path length (cm)
2000
1500
1000
500
0
Day 1
Day 2
Day 3
Day 4
Day 5
*
†††
ΦΦΦ
Φ
ΦΦ
Φ
(b)
Control
STZ-treated
Platform (white)
Annulus (black)
NW Quadrant
NE Quadrant
SE Quadrant
% Distance moved in zone (%)
40
35
30
25
20
15
10
5
0
*
Platform
Annulus
Southwest
Northwest
Southeast
Northeast
(c)
Swim speed (cm/s)
20
15
10
5
0
**
Control
STZ-treated
(d)
Control
STZ-treated
Path length (cm)
1200
800
400
0
ΦΦ
ΦΦ
ΦΦ
Φ
*
†
Trial 1
Trial 2
Trial 3
Trial 4
Trial 5
Trial 6


(a)
200
175
150
125
100
75
% Basal
HU210
HU210 + SR141
††
††
*
*
Control
STZ-treated
Hippocampus
(b)
##
†††
†††
***
**
200
175
150
125
100
75
% Basal
HU210
HU210 + SR141
Control
STZ-treated
Substantia Nigra


Basal
HU210
HU210 + SR141
Control
STZ-treated
A
B
C
D
E
F
SN