CHAPTER 6:
Characterisation of the Morris Water Maze and the Elevated Plus Maze

6.1. INTRODUCTION

The neurodegenerative pathology of Sanfilippo syndrome results in a host of behavioural changes, including developmental delay and cognitive decline (as discussed in **Chapter 1**). Prior to assessing the clinical effect of CAV-NS vector administration in MPS IIIA mice in the longer-term studies described in **Chapter 7**, it was necessary to validate several behavioural tests in the congenic C57Bl/6 strain of MPS IIIA mice. The Morris Water Maze, which evaluates memory and learning ability, has been used to assess cognitive function in a variety of LSD mouse models including MPS IIIA (Gliddon and Hopwood, 2004; Crawley *et al*, 2006a), MPS IIIB (Fu *et al*, 2007), MPS VII (Chang *et al*, 1993; Bastedo *et al*, 1994; O'Connor *et al*, 1998; Frisella *et al*, 2001; Sakurai *et al*, 2004), aspartylglucosaminuria (Jalanko *et al*, 1998) and cystinosis (Dr Eric Kremer, personal communication), and has also been evaluated in an α-mannosidosis guinea pig model (Robinson *et al*, submitted by LDRU). However, the performance of congenic MPS IIIA mice in the Morris Water Maze was in the process of being independently determined within the LDRU when this stage of the project was undertaken. Therefore, the first aim of the experiments described in this chapter was to contribute to the characterisation of the memory and spatial learning abilities of congenic unaffected and MPS IIIA mice at 16-wks of age using the Morris Water Maze.

The Elevated Plus Maze examines "emotionality" or anxiety-related behaviours by assessing the conflict between the desire to explore a novel environment and the anxiogenic elements such as elevation and an unfamiliar, brightly illuminated arena. This test has a predictive value for both anxiogenic (anxiety-inducing) and anxiolytic (anxiety-relieving) compounds in rats (Pellow *et al*, 1985) as well as mice (Lister, 1987). As the administration of anxiolytic agents induces increases in exploratory behaviour on the open arms, common indices of anxiety include the percentage of entries into the open arms, the percentage of time spent on the open arms and the percentage of the path length on the open arms. Parameters

such as total path length and arm entries into the open and closed arms also give an indication of total activity. Given that MPS IIIA mice have biphasic hyper-/hypo-active changes in activity compared to wild-type animals, as measured by the exploration of an open field arena (Hemsley and Hopwood, 2005), and that MPS IIIB mice are less anxious/fearful than unaffected controls in the Elevated Plus Maze (Cressant *et al*, 2004), the second objective was to investigate whether similar differences in anxiety could be detected in MPS IIIA mice using the Elevated Plus Maze.

6.2. SPECIFIC METHODS

Morris Water Maze testing of male C57Bl/6 mice was performed as previously described, with minor modifications to the published protocol to permit use of an automated HVS Image tracker system and changes to the layout of the CYWHS Animal House (Gliddon and Hopwood, 2004; Crawley *et al*, 2006a).

6.2.1. Morris Water Maze Equipment and Testing Room Layout

The testing room had dimensions of 3.0 x 2.5 m and contained a door with the glass pane obscured by black plastic sheets, a rubbish bin, covered mirror, sink and a stainless steel bench covered in a green drape on which a laptop containing HVS Image software was placed (**Fig. 6.1**). Two posters of abstract shapes and the operator acted as additional visual cues. Poster 1 was constructed from fluorescent yellow and pink cardboard in triangular and diamond shapes and Poster 2 was a dull brown, rectangular shape segmented with thick, black lines.

A light grey, plastic pool with a diameter of 1.2 m was positioned directly under a ceiling-mounted video camera. The video camera was connected to a laptop containing Water 2020 software (version 1/2003) provided by HVS Image (http://www.hvsimage.com). The platform was constructed from Perspex and was 29 cm high with a circular base and top, both 10 cm in diameter. The platform surface was roughened with the use of sand and glue to provide additional grip before the entire platform was painted white. The platform was placed 35 cm from the wall in the target quadrant and the pool filled until the platform was submerged approximately 1 cm below the surface of the water. The water was made opaque by the addition of approximately 500 mL non-toxic white paint.

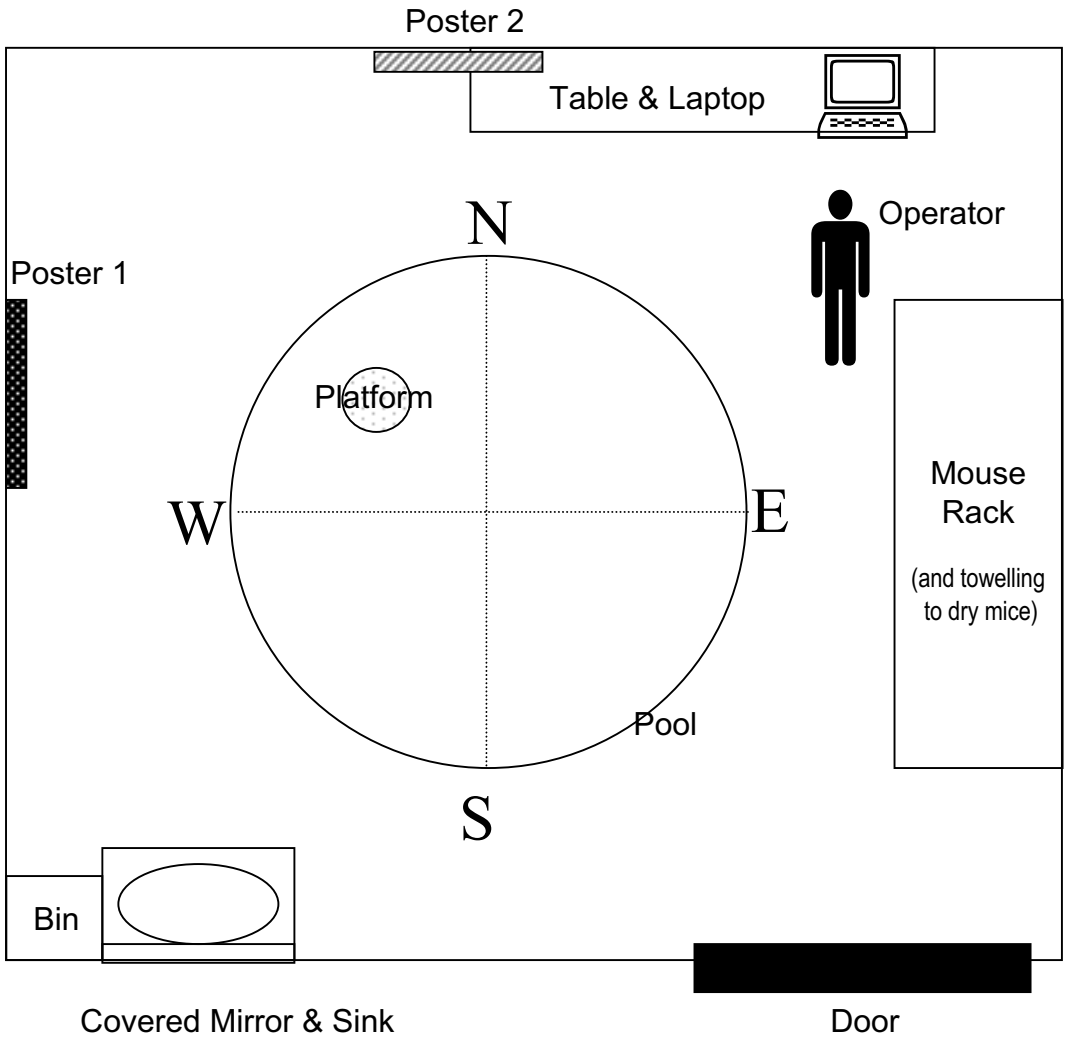


Figure 6.1: Schematic layout of the Morris Water Maze room. A 2.5 x 3.0 m room within the CYWHS Animal House was set up for Morris Water Maze testing as displayed. Testing was performed in a 1.2 m diameter plastic pool by placing the mouse at each cardinal point (north N, south S, east E or west W) in succession. The operator then returned to the position indicated near the north-east quadrant. A ceiling-mounted video camera connected to a laptop running Water 2020 software was used to record each trial.

Each morning approximately 12 L of water was removed by bucket from the pool and replaced with warm water until the water temperature was 25°C ± 1°C (to prevent hypothermia in the mice) prior to their transportation to the testing room on a trolley. Additional paint was added as required to maintain the opacity of the water.

6.2.2. Morris Water Maze Testing Procedure

Naïve male, congenic mice at 16-wks of age were separated into individual cages 2-days prior to habituation and the eyes were checked for cataracts, anophthalmia and microphthalmia. Mice found to have imperfect eyesight would have been excluded from the study, however, no eye problems were detected.

Habituation Phase

The day prior to testing (day 0), mice were habituated to the pool, platform and testing room. The habituation was performed on the same day as cage-cleaning in the PC2 holding room (located directly across the corridor from the behavioural testing room) which generated noise and foot traffic in the adjacent corridor. This start day was selected to minimise disturbance during the subsequent acquisition and probe phases. For the habituation phase, the water was clear and the platform was made visible by placing a black sink plug on the centre of the platform. Spatial cues, such as surrounding posters in the room, were visible during habituation. Each mouse was removed from the home cage and gently placed into the water at the eastern cardinal point facing the wall. The platform was located in the south-west, north-west and north-east quadrants, respectively (i.e. three trials). The latency to find the platform was recorded and the mice were guided to the platform after 90 secs.

Acquisition Phase

All testing was performed between 7 am and 1 pm. Each mouse underwent 4 trials each day over a 5-day acquisition period (days 1-5) with the submerged platform located in the north-west quadrant. Mice were placed in the pool facing the wall at each of the cardinal points in succession and the tracker system was remotely-initiated using a blue-tooth computer mouse. The time taken (latency) for the mouse to reach the platform was recorded manually and by Water 2020 software. If the mouse failed to find the platform within 90 sec, the operator guided the mouse to the platform before returning to the operator position indicated in **Fig. 6.1** and allowed the mouse to assess the surroundings for 10 sec. The mouse

was then removed from the pool by hand, dried in cloth towelling (fresh towels each day) and returned to its home cage between trials. At the end of each day, the rack of mice was transferred to the PC2 holding room overnight.

Mice were tested in randomly-allocated groups of 5-6 so that the inter-trial interval for each mouse was approximately 10 min, with a minimum inter-trial interval of 5 min. The order of each group of mice was rotated on each day of testing so that group 1 swam first on day 1, second on day 2 and so on. The rack holding the mouse cages was covered with white towels and then transferred to the testing room to prevent the mice observing the swim trials of other mice.

Probe Phase

The memory transfer efficiency was determined the day following the acquisition phase by removing the platform and calculating the percentage of time spent in each quadrant (day 6). These measures were determined both automatically (Water 2020 software) and manually by recording the position of the mouse each second using a multi-channel cell counter. Each mouse underwent three trials lasting 90 sec each, beginning at the northern cardinal point and rotating clock-wise.

Visual Testing

Mice underwent a final day of swimming (three trials starting from the east, south and west cardinal points) in clear water with the platform raised approximately 0.5 cm above the water surface to determine the visual acuity of the mice. The spatial cues (e.g. posters) were visible and in the same position as during the acquisition and probe phases. The platform was located in the south-east quadrant (i.e. opposite the target quadrant in the acquisition phase). Mice that failed to find the platform in all trials in the visual test would have been excluded from the study, however, all mice passed the visual test in this assessment.

6.2.3. Morris Water Maze Data and Statistical Analyses

The default settings for the Water 2020 software were utilised (percentage of time swimming slowly defined as swimming less than 5 m per second; thigmotaxic/circular swimming if the thigmotaxis diameter/pool diameter ratio was greater than 0.9).

Mr Craig Hirte of the Public Health Research Unit (CYWHS) performed all statistical analyses of Morris Water Maze data using SPSS (version 14.0) and Stata (version 9). The 4

trials in the acquisition phase were averaged each day to give one outcome per day and assessed using a linear-mixed model using a Greenhouse-Geisser correction with simple contrasts. For the probe phase, the percentage of time within each quadrant was assessed as unit level data (i.e. was not averaged) for the three trials using a linear mixed model with a Greenhouse-Geisser correction to allow a trial effect to be included as a predictor.

6.2.4. The Elevated Plus Maze

Unless otherwise stated all mice were male, naïve to the Elevated Plus Maze apparatus and from the congenic strain. Unaffected and MPS IIIA mice were 7-, 15-, 18- or 20-wks of age when tested and the group sizes for each of the Elevated Plus Maze trials are summarised in **Table 6.1**. These mice had not undergone testing in other behavioural tests with the exception of two groups: the 'repeat-tested' 18-wk-old group (which underwent testing in the Elevated Plus Maze at both 15- and 18-wks) and the automatically-assessed mice at 15-wks of age (tested in open field activity 8-days before assessment in the Elevated Plus Maze). The mice were group-housed where possible but some mice were single-housed due to aggressive tendencies (**Table 6.1**). The 7-wk-old mice were tested over 2 consecutive days, separated by gender.

Apparatus

The Elevated Plus Maze was constructed of wood so that four arms with dimensions of 50 cm x 7 cm intersected in a central square (7 cm x 7 cm) forming a "+" shape (**Fig. 6.2**). The maze floor was covered in white paper and clear plastic to prevent urine impregnation and to allow easy cleaning with ethanol between trials to eliminate odour transfer. Two opposing arms were enclosed by 40 cm high walls covered in black plastic ("closed arms") and the two arms without walls were designated "open arms". The open arms did not contain ledges. The maze floor was elevated 50 cm above ground level. The apparatus was placed on a green drape to provide contrast during the calibration of the Maze 2020 software.

Room Layout

The Elevated Plus Maze was conducted in the same room as the Morris Water Maze, described in **Section 6.2.2.** The glass pane of the door was covered with a black plastic sheet and a ceiling-mounted video camera was connected to a laptop with Maze 2020 software (**Fig. 6.3**). The operator was positioned at a 45° angle between an open and closed arm.

Table 6.1: Group sizes of Elevated Plus Maze trials. The number of mice tested in each Elevated Plus Maze trial is listed by genotype. The 7-wk-old male and female mice were tested over two consecutive days. Age-matched animals were tested on the same day. Mice indicated by an asterisk were tested on two separate occasions at 15- and 18-wks of age.

Age and Sex	Unaffected (n=)	MPS IIIA (n=)	Elevated Plus Maze Experience	Other Behavioural Test Experience (time prior to Elevated Plus Maze testing)	Housing Conditions
15 wk Males * *(Manual assessment)*	6	6	Naïve	None	Single/Group
7 wk Females	10	11	Naïve	None	Group
7 wk Males	10	8	Naïve	None	Group
15 wk Males	7	10	Naïve	Open Field (8 days)	Group
18 wk Males	11	9	Naïve	None	Group
18 wk Males *	9	9	Repeat-Tested	Elevated Plus Maze (3 wks)	Single
20 wk Males	10	9	Naïve	None	Single

Testing

Mice were brought into the behaviour room on the morning of assessment and were tested in a random order between 7 am and 2 pm. Each mouse was placed in the centre square facing the open arm furthest from the operator and allowed to freely explore the apparatus for 5 or 10 min. For the pilot study, where the data was manually-assessed, the path of the mouse recorded with a video camera for 10 min and the location of the mouse (open arm, closed arm or centre square) at every second was tallied using a multi-channel cell counter after the completion of all trials. For subsequent trials, the path of the mouse was recorded automatically by HVS Image Maze 2020 software (version 7/2003). At the completion of each trial, the quantity of faecal boli and number of urinary spots were counted. The floor and inner walls were cleaned with ethanol between each trial to prevent scent transfer.

Data Analysis

In the pilot study, the percentage of time spent in the open arms was calculated by summing the number of seconds in both of the open arms and dividing it by the total sum of the time in any arm. The number of open arm entries was defined as the two front paws crossing into an open arm.

Figure 6.2: The Elevated Plus Maze. The Elevated Plus Maze was constructed from wood with a white floor to improve the tracking of black mice by the automated tracker system. The dimensions of each arm were 7 cm x 50 cm meeting at a central square. Two opposing arms were enclosed by 40 cm high, black walls (“closed arms”) and the other two arms were designated “open arms”. The floor of the maze was elevated 50 cm above ground level and was situated on a green drape to improve contrast during the calibration of Maze 2020 software.

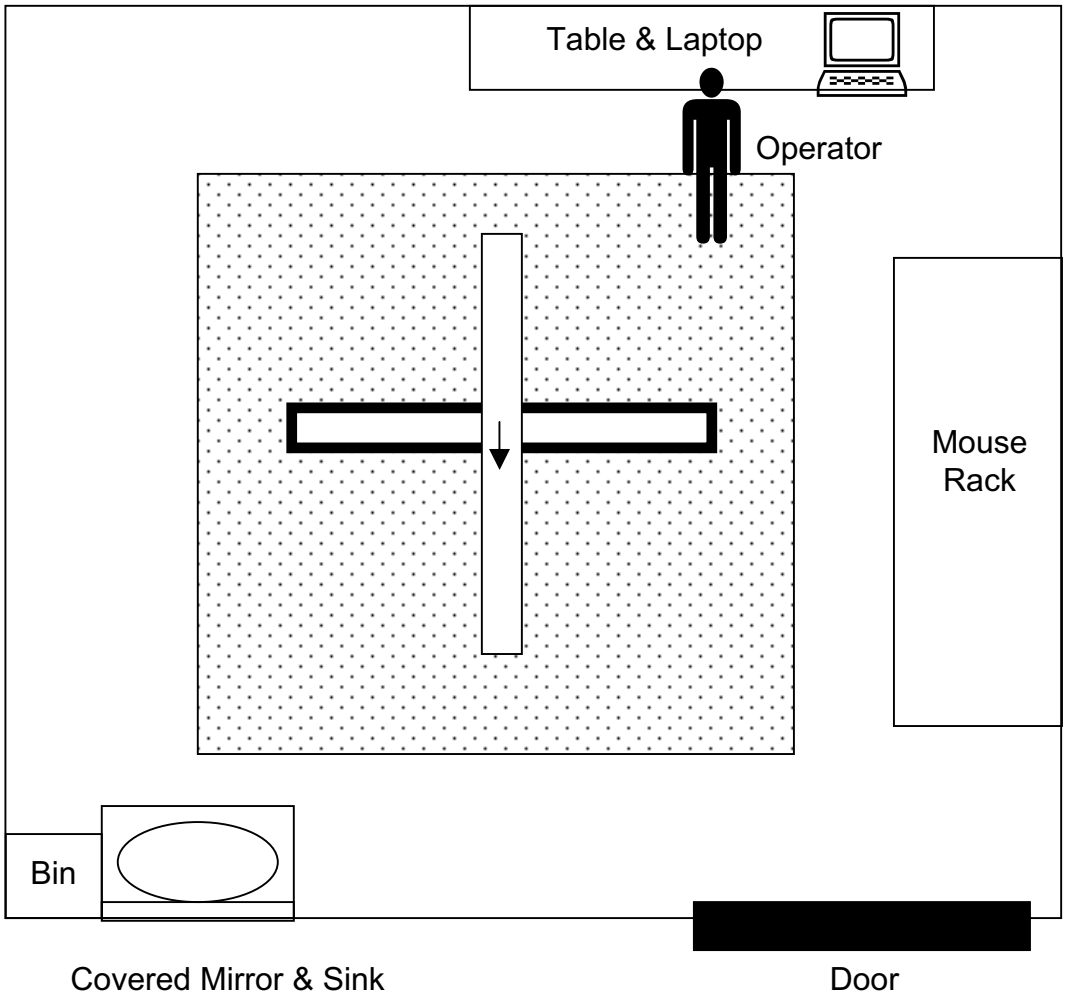


Figure 6.3: Schematic layout of the Elevated Plus Maze. Mice underwent a single trial in the Elevated Plus Maze in the CYWHS Animal House. The Elevated Plus Maze was constructed with two opposing "closed arms" (indicated in bold, horizontal in the schematic diagram) and the two unwalled arms referred to as "open arms". The mice were placed into the central square of the maze facing an open arm in the direction indicated by the arrow. The mice freely explored the apparatus for 5 or 10 min and the path of the animal was recorded using Maze 2020 software. At the completion of each trial, the apparatus was cleaned with ethanol to remove urine, faeces and scent trails.

For the subsequent trials, Maze 2020 software automatically determined the path length, percentage of time spent in all arms and the total number of arm entries. The following measures were calculated from the data provided by the Maze 2020 software:

$$\text{Percentage of entries into open arms} = \frac{\text{Entries in open arm A + Entries in open arm B x 100}}{\text{Total arm entries (open arm A + open arm B + closed arm C + closed arm D)}}$$

$$\text{Percentage of path length in open arms} = \text{\% Path in open arm A + \% Path in open arm B}$$

$$\text{Percentage of time spent in open arms} = \frac{\text{Time in open arm A + Time in open arm B x 100}}{\text{\% time in all arms}_{\text{tracker}}}$$

Statistical analyses were performed using a one-way ANOVA followed by a post-test calculation with GraphPad software (http://graphpad.com/quickcalcs/posttest2.cfm). This comparison uses a Bonferroni correction factor to adjust for multiple comparisons. The alpha value was set at 0.05 with a 95% confidence level.

6.3. RESULTS

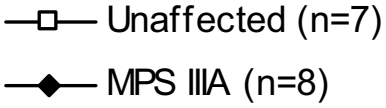
6.3.1. Morris Water Maze

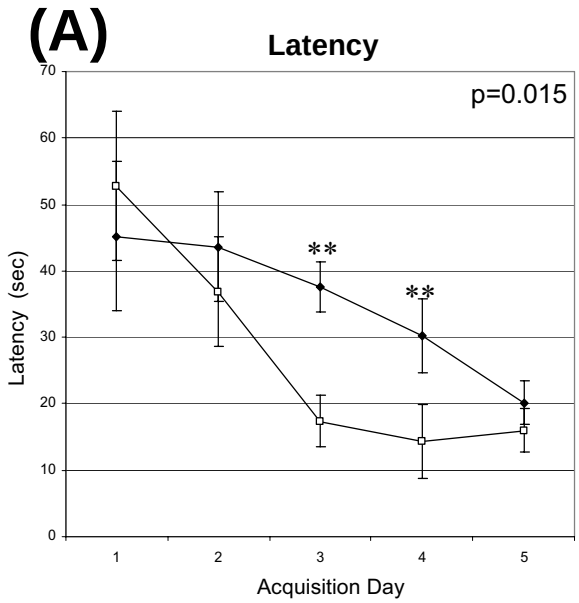
Male MPS IIIA and unaffected congenic mice were assessed in the Morris Water Maze as described in **Section 6.2.2** at approximately 16-wks of age. The dates of birth on the habituation day spanned from 15-wks and 5-days to 16-wks and 2-days. The mice had been assessed in the open field activity arena (13-days prior) and the Elevated Plus Maze (5-days prior) before initiating the Morris Water Maze. This was the first Morris Water Maze conducted by our group using the HVS Image tracker system and consequently many experimental parameters were investigated to determine whether there were significant differences between the disease and control groups.

The time taken by MPS IIIA and unaffected mice to find the hidden platform was similar on the first 2-days of the acquisition period (**Fig. 6.4A**) with average latencies of 53 and 45 sec for unaffected and MPS IIIA mice, respectively, on the first day of testing. However, the unaffected mice had significantly faster latencies on days 3 and 4, with unaffected mice improving their swim time to 17 and 14 sec, respectively, whilst the MPS

Figure 6.4: Acquisition phase of the Morris Water Maze in 16-wk-old congenic mice. Male, naïve unaffected (n=7) and MPS IIIA (n=8) mice of the C57Bl/6 congenic strain were assessed in the Morris Water Maze for their memory and learning abilities at 16-wks of age. Mice underwent 4 trials per day starting from each of the four cardinal points in succession, and the time taken to find a submerged platform located in the north-west quadrant was assessed up to a maximum of 90 sec. The path of the mouse was recorded by Water 2020 software.

The **(A)** latency time to find the platform in secs; **(B)** path length in metres; **(C)** average swim speed in metres/sec; **(D)** percentage of time that mice were swimming slowly (swimming <0.05 metres/sec); **(E)** percentage of path length spent in thigmotaxis; and **(F)** percentage of time spent in thigmotaxis were determined. The average of each measure is displayed over the 5 day acquisition period ± 1 SEM. The p-value displayed is the day x disease interaction as an indication of significant differences in the overall rate of learning between the unaffected and MPS IIIA mice. Significant differences between the unaffected and MPS IIIA mice on any individual day are indicated * p = 0.01-0.05, ** p = 0.01-0.001.




(A)
Latency
p=0.015
Latency (sec)
Acquisition Day
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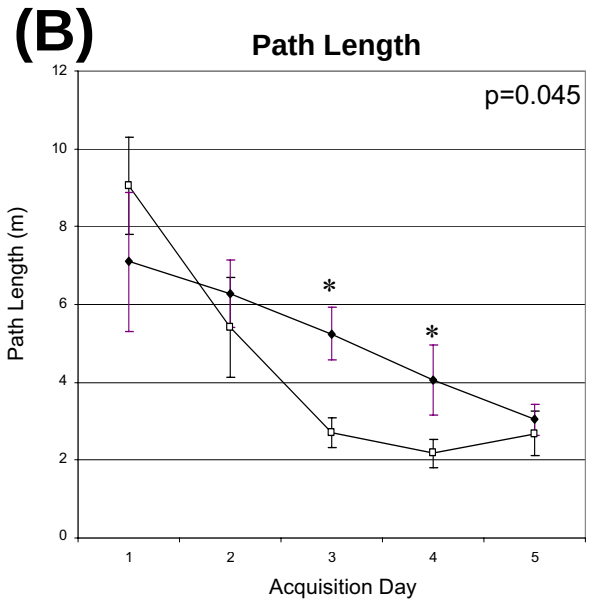
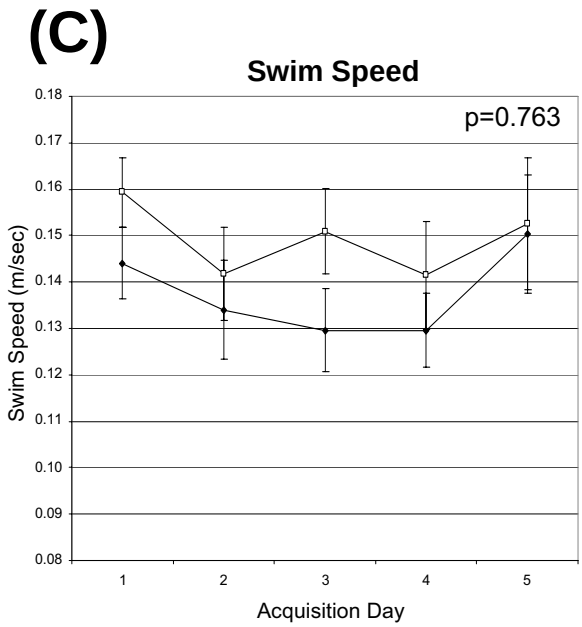
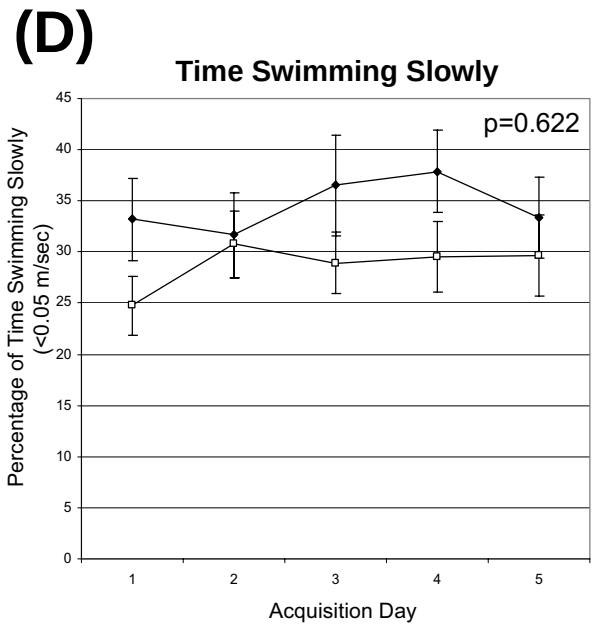
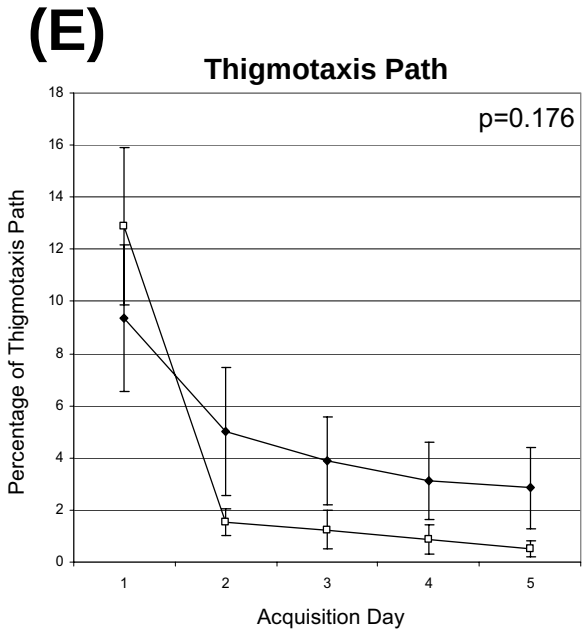
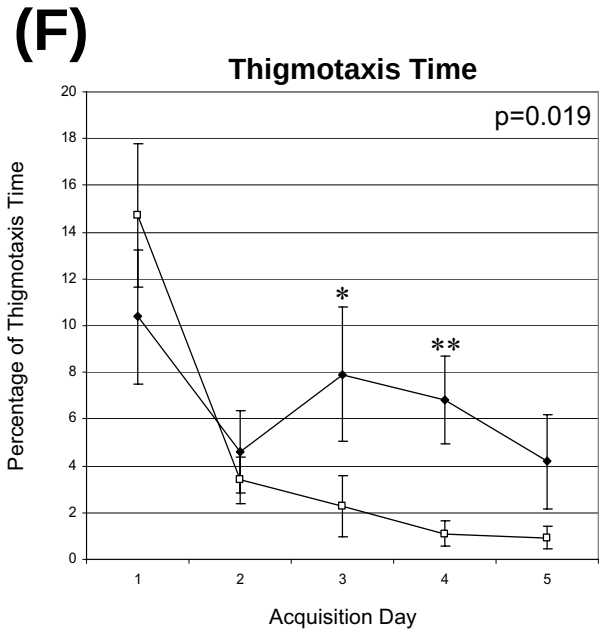

(B)
Path Length
p=0.045
Path Length (m)
Acquisition Day
*
*


(C)
Swim Speed
p=0.763
Swim Speed (m/sec)
Acquisition Day


(D)
Time Swimming Slowly
p=0.622
Percentage of Time Swimming Slowly (<0.05 m/sec)
Acquisition Day


(E)
Thigmotaxis Path
p=0.176
Percentage of Thigmotaxis Path
Acquisition Day


(F)
Thigmotaxis Time
p=0.019
Percentage of Thigmotaxis Time
Acquisition Day
*
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IIIA mice remained at 38 and 39 sec (p=0.001, p=0.005). This was reflected in a significant overall day x disease interaction (p=0.015), indicating that the unaffected mice learnt the position of the platform more quickly than the MPS IIIA mice. However, by acquisition day 5 both disease groups had similar search times (unaffected 15.4 sec; MPS IIIA 19.7 sec) suggesting that both groups of mice had learnt the task.

The path length displayed a similar trend with shorter path lengths observed in both groups on day 5 of the acquisition phase compared to day 1, indicating that the mice had improved their navigation to the hidden platform during training (**Fig. 6.4B**). The mean path length was significantly greater in the MPS IIIA mice compared to the unaffected mice on acquisition days 3 and 4 (p=0.011, p=0.040) but was similar on day 5 (p=0.508). As before, there was a significant overall day x disease interaction (p=0.045) indicating that the unaffected mice had a faster rate of learning compared to the MPS IIIA mice.

The shorter path lengths in the unaffected mice were not a result of faster swim speeds as there was no significant difference in the overall mean swim speed during the acquisition phase between the disease groups (day x disease interaction, p=0.763; **Fig. 6.4C**).

In some trials, mice floated for parts of the trial and were not actively searching for the hidden escape platform. To try and quantify this behaviour, the percentage of time mice spent swimming slowly, as defined as having swim speeds less than 0.05 metres per sec, was assessed (**Fig. 6.4D**). There were no significant differences between the unaffected and MPS IIIA mice (p=0.233) or in the percentage of time the mice swam slowly over the acquisition phase (day x disease interaction, p=0.622).

The percentage of path and time swum in a thigmotaxic pattern was determined (**Fig. 6.4E, F**). These parameters are indicators of anxiety and involve the mice searching for an exit by swimming in circles in close proximity to the wall. It is difficult to interpret the results in a statistically significant manner as the majority of trials recorded zero thigmotaxic measurements. The percentage of time and the percentage of path length spent swimming around the pool edge (thigmotaxis time, thigmotaxis path length) was reduced in both unaffected and MPS IIIA mice throughout the acquisition period (thigmotaxis time and path length, day x disease interaction p=0.019 and p=0.176, respectively). MPS IIIA mice were more likely to swim in a thigmotaxic pattern than unaffected mice on acquisition days 2 to 5, which was likely influenced by the unaffected mice learning the position of the hidden platform more quickly than the MPS IIIA animals.

For the probe phase, the percentage of time spent in the target quadrant was marginally higher in the unaffected mice compared to MPS IIIA mice, with an average of

44% and 35%, respectively (**Fig. 6.5A**). However, these changes were not statistically different between the disease and control groups (p=0.307). The mice showed a preference towards the target quadrant (NW) and the quadrant closest to the operator (NE) compared to the remaining quadrants (**Fig. 6.5B**). A significant trial effect was not observed in the target quadrant (p=0.214) suggesting that the mice were persisting in their search for the hidden platform over the three probe trials.

No obvious eye defects were detected in the tested mice. By the third trial in the visual acuity test on the final day of swimming, unaffected and MPS IIIA mice were able to find the visible platform in 10.9 ± 2.7 and 23.9 ± 3.9 sec, respectively (p=0.02). However, the swim speeds between the two diseases groups were indistinguishable (unaffected and MPS IIIA 0.156 ± 0.009 and 0.150 ± 0.006 metres/sec, respectively; p=0.567).

6.3.2. Elevated Plus Maze

6.3.2.1. Manual Assessment at 15-wks

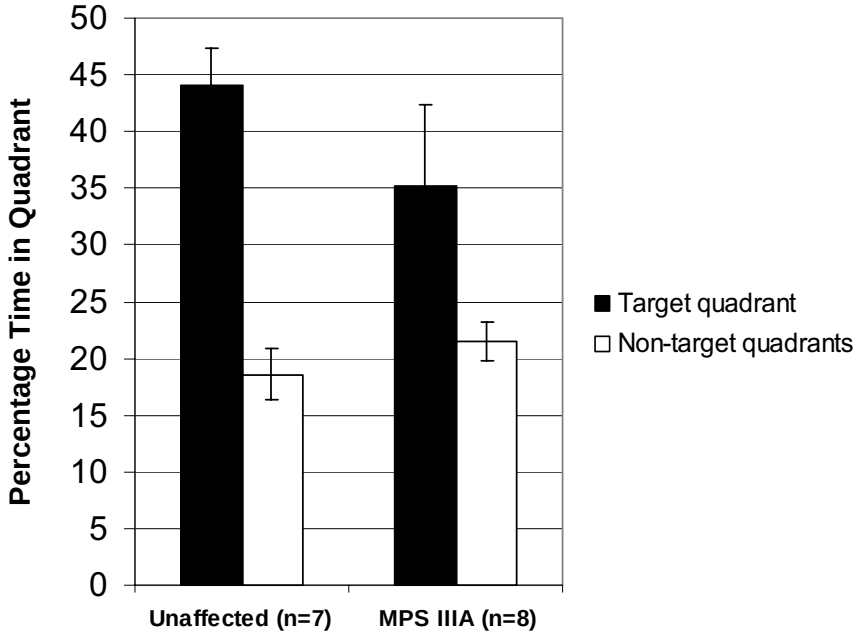
A pilot study was conducted in 15-wk-old male mice (n=6 for each genotype) and was analysed manually from a video-recording. No significant differences were detected in the number of open arm entries (p=0.69), with unaffected and MPS IIIA mice crossing into the open arms 15.8 ± 2.6 and 14.2 ± 3.1 times, respectively. Likewise, the number of faecal boli and urinary spots produced during the trial was similar (4.8 ± 1.0 and 4.8 ± 1.1) for unaffected and MPS IIIA animals.

In contrast, the percentage of time spent in the open arms was significantly elevated in MPS IIIA mice compared to unaffected mice over the 10 min trial (**Fig. 6.6**; p=0.029). MPS IIIA mice were more likely to explore the open arms compared to wild-type mice in the first 5 min of testing (p=0.02). However, in the second half of testing MPS IIIA mice tended to spend less time in the open arms compared to the first 5 min (p=0.37) whilst unaffected mice increased the percentage of time in the open arms over the same time period (p=0.66). For the 5-10 min time period there was a marginal difference between the disease groups (p=0.08). Consequently, successive trials in the Elevated Plus Maze were conducted for 5 min.

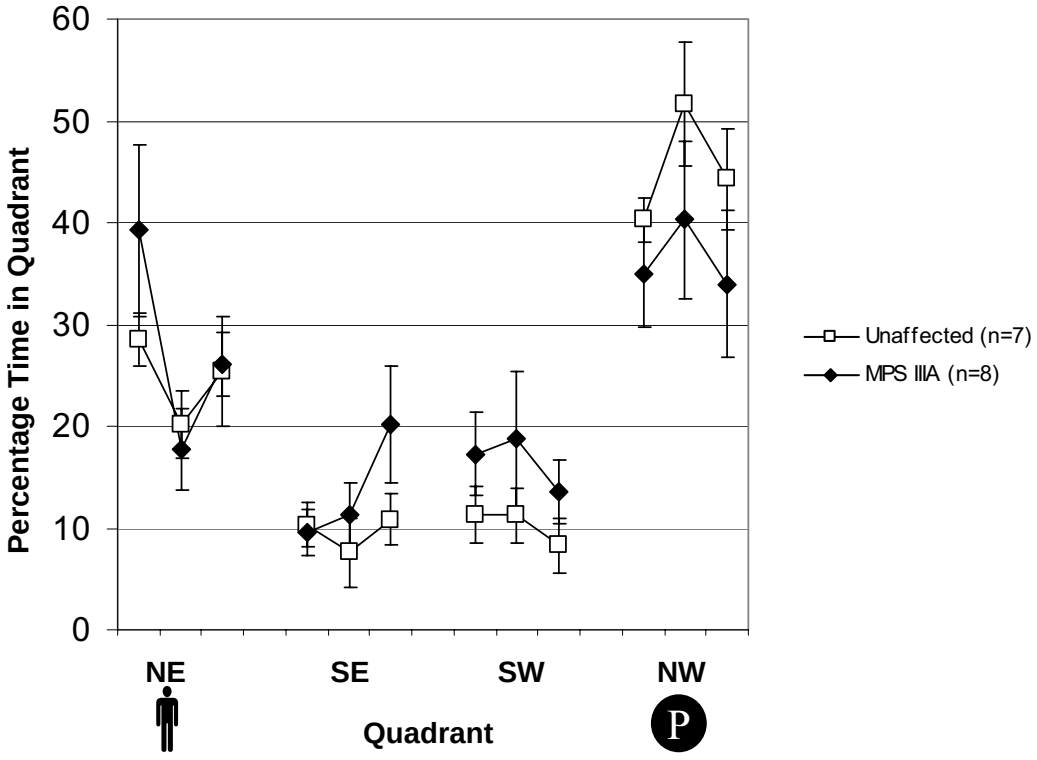
Figure 6.5: Quadrant times for probe phase of the Morris Water Maze in 16-wk-old congenic mice. Following the 5-day acquisition phase, the memory transfer (probe phase) was assessed in three trials lasting 90 sec each by measuring the percentage of time spent in each quadrant using an automated tracker system. The operator was located closest to the north-east quadrant (indicated by ) and the hidden platform was previously positioned in the north-west quadrant (indicated by 

) throughout the acquisition phase. The percentage of time spent in the target quadrant was marginally significantly greater for the unaffected mice compared to the MPS IIIA mice ($p=0.097$). **(A)** The data are presented as the mean percentage of time spent in the non-target quadrants for all trials (white bars) compared to the percentage of time in the target quadrant for all trials (black bars) ± 1 SEM or **(B)** the mean of the individual percentage of time spent in each quadrant per trial ± 1 SEM.

(A) **Mean Quadrant Times (Probe Phase, 16 wk)**



(B) **Individual Quadrant Times (Probe Phase, 16 wk)**



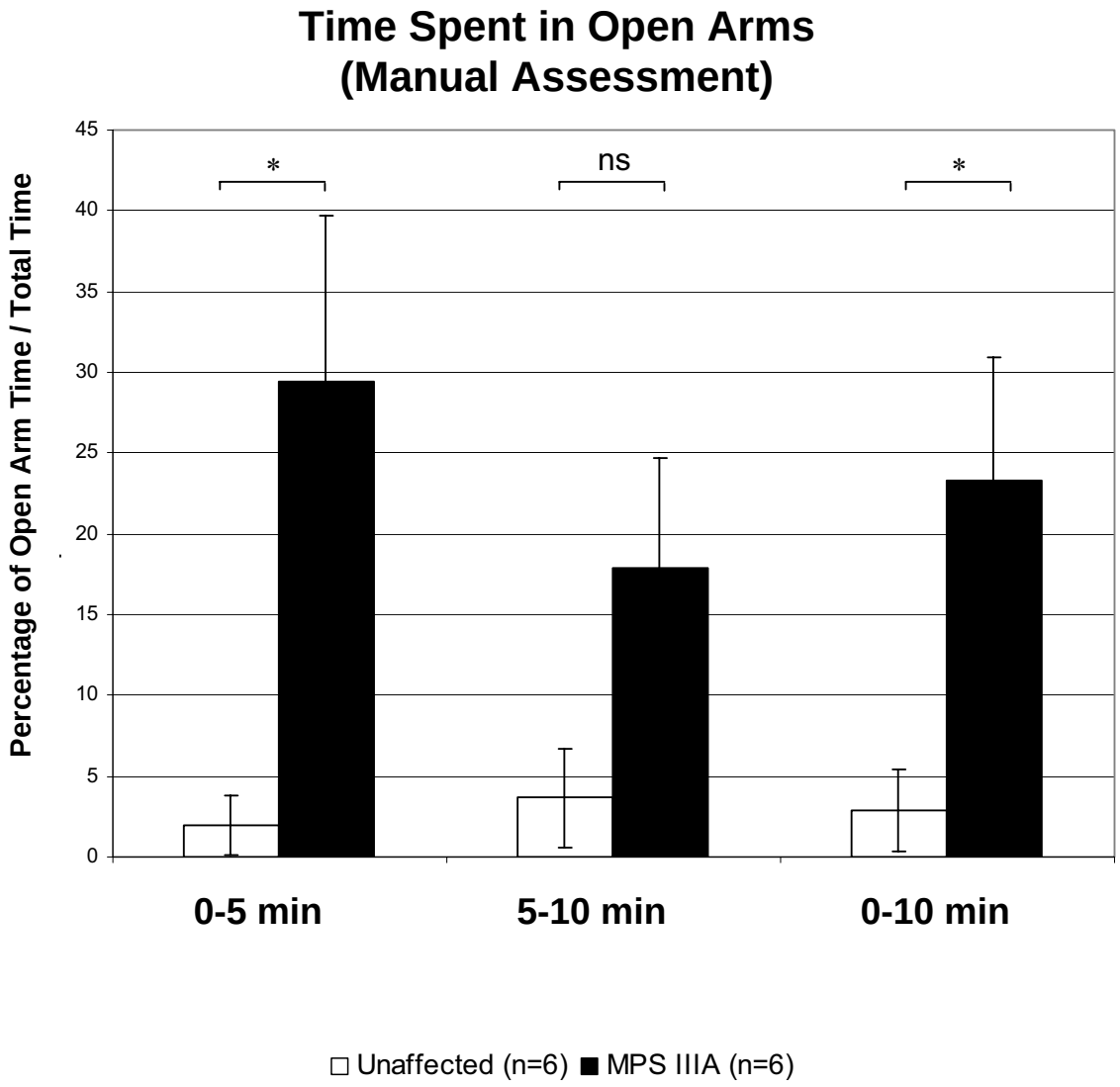


Figure 6.6: Percentage of time spent on the open arms in 15-wk-old mice. Male congenic mice were allowed to explore the Elevated Plus Maze apparatus for 10 min and the path was recorded with a video camera for manual determination of the position of the mouse at each second. The mean percentage of time spent in the open arms ± 1 SEM was calculated for unaffected and MPS IIIA mice in the first (0-5 min) and second (5-10 min) halves of testing or for the combined 10 min (0-10 min). * t-test $p<0.05$; ns, not significant.

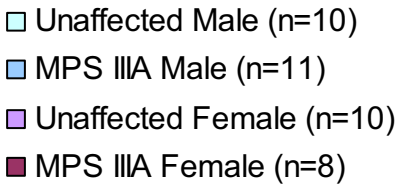
6.3.2.2. Automated Tracking in 7-wk-old Male and Female Mice

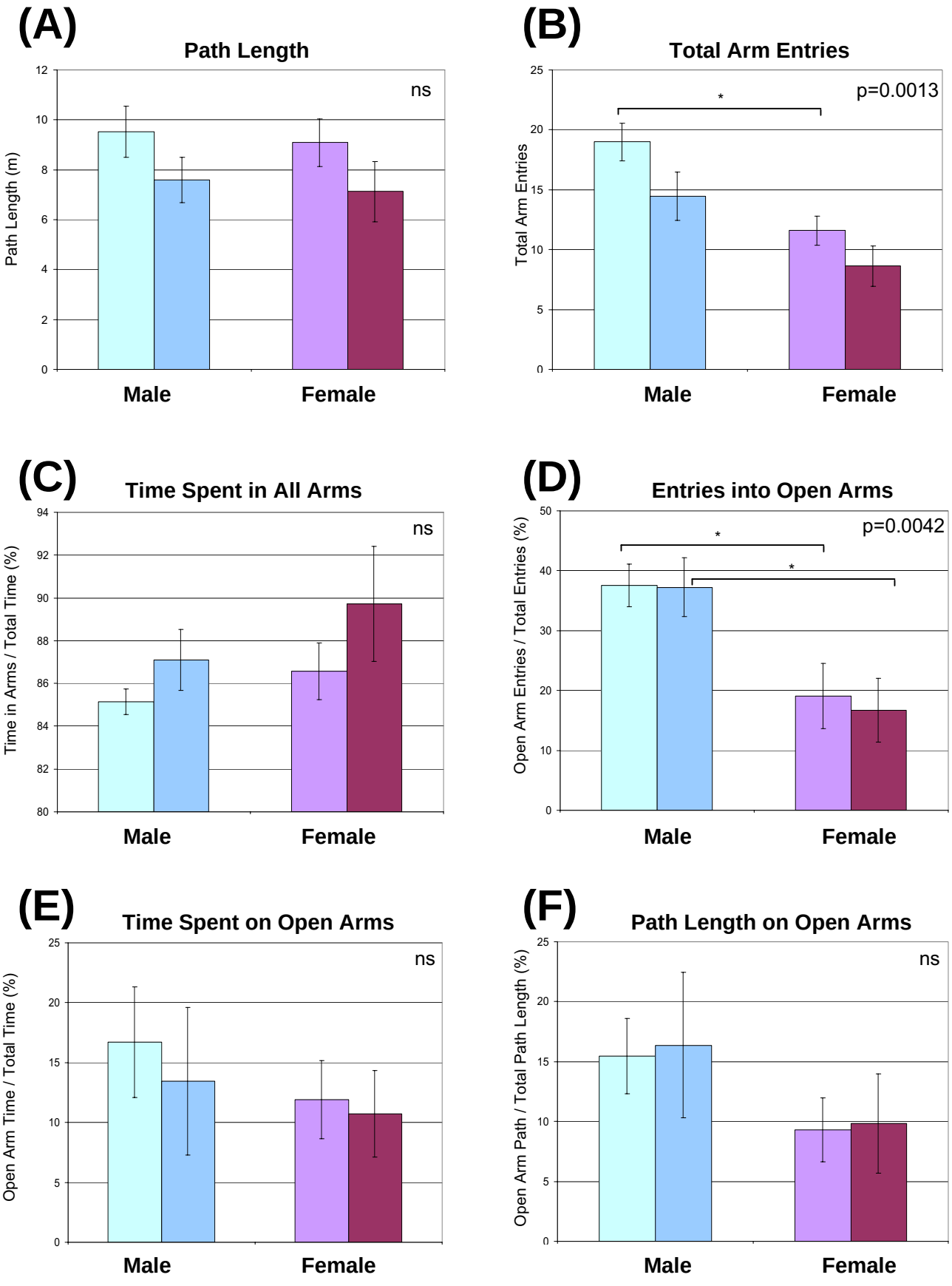
Other measures of interest, such as path length, were difficult to quantitate from the video recording in the pilot experiment. To address this issue, animals were tracked with an automated system equipped with Maze 2020 software. Seven-wk-old male and female mice were assessed to determine whether there were gender differences in the disease groups. In all parameters measured, there was no statistically significant difference between disease groups of mice with the same gender (i.e. MPS IIIA and unaffected male mice were similar; MPS IIIA and unaffected female mice were similar).

Unaffected mice had longer path lengths than gender-matched MPS IIIA mice (**Fig. 6.7A**) but there were no statistical differences between the groups. Similarly, the number of total entries (open and closed arms) was greater in unaffected animals compared to their MPS IIIA counter-parts (**Fig. 6.7B**). Males tended to enter the arms more frequently than females of the same genotype (**Fig. 6.7B**). Statistical differences in the total number of arm entries were only observed between unaffected males and females ($p<0.05$, $t=3.145$). Unaffected mice tended to spend less time in arms (or conversely spent more time in the centre square, as a less risky assessment of the open arms than direct exploration of the open arms) than MPS IIIA animals (**Fig. 6.7C**).

No differences in the number of entries into the open arms were observed between gender-matched mice (**Fig. 6.7D**). However, significant reductions in the number of entries into the open arms were detected between male and female mice of genotype-matched animals ($p<0.05$ for both; $t=2.708$ for unaffected male versus unaffected female; $t=2.908$ for MPS IIIA male versus MPS IIIA female). Males were more likely to spend time exploring the open arms than genotype-matched females (**Fig. 6.7E**) but not to a statistically significant degree. Male mice tended to have a greater percentage of path length on the open arms but no statistical differences between the genotypes or genders were found (**Fig. 6.7F**).

Figure 6.7: Elevated Plus Maze in 7-wk-old mice of both genders. Seven-wk-old congenic mice of both sexes were allowed to explore the Elevated Plus Maze for 5 min each. The path was recorded with an automated tracker and assessed using Maze 2020 software. The p-value of the one-way ANOVA analyses is presented for each measure. The parameters assessed were the: **(A)** total path length; **(B)** total arm entries (open and closed arms); **(C)** percentage of time spent in all arms; **(D)** percentage of entries into the open arms; **(E)** percentage of time spent in the open arms; and **(F)** percentage of path length on the open arms. Data are presented as the mean of each measure ± 1 SEM. The ANOVA p-value is displayed in the corner of each graph and significant post-test comparisons ($p<0.05$) are indicated with an asterisk. ns, not signifcant.




(A)
Path Length
ns
Path Length (m)
0
2
4
6
8
10
12
Male
Female
(B)
Total Arm Entries
p=0.0013
*
Total Arm Entries
0
5
10
15
20
25
Male
Female
(C)
Time Spent in All Arms
ns
Time in Arms / Total Time (%)
80
82
84
86
88
90
92
94
Male
Female
(D)
Entries into Open Arms
p=0.0042
*
*
Open Arm Entries / Total Entries (%)
0
10
20
30
40
50
Male
Female
(E)
Time Spent on Open Arms
ns
Open Arm Time / Total Time (%)
0
5
10
15
20
25
Male
Female
(F)
Path Length on Open Arms
ns
Open Arm Path / Total Path Length (%)
0
5
10
15
20
25
Male
Female

6.3.2.3. Elevated Plus Maze Assessment with Disease Progression

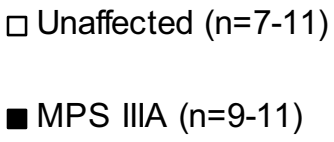
Age-matched, male unaffected and MPS IIIA congenic mice were assessed in the Elevated Plus Maze for 5 min each. The 7-, 15-, 18- and 20-wk naïve groups were tested on different days. The data presented for the 7-wk-old male mice are the same as that described in the previous section. At each time point, the unaffected mice tended to be more active than the age-matched MPS IIIA mice, as seen by increases in path length and total arm entries (**Fig. 6.8A, B**). However, neither of these measures were statistically significant although the total arm entries at 7-wks approached significance (p=0.097). The percentage of time spent in all arms gradually increased as the unaffected mice aged (**Fig. 6.8C**), indicating that the mice were spending less time in the centre square. In comparison, the percentage of time spent in all arms remained relatively constant for MPS IIIA males.

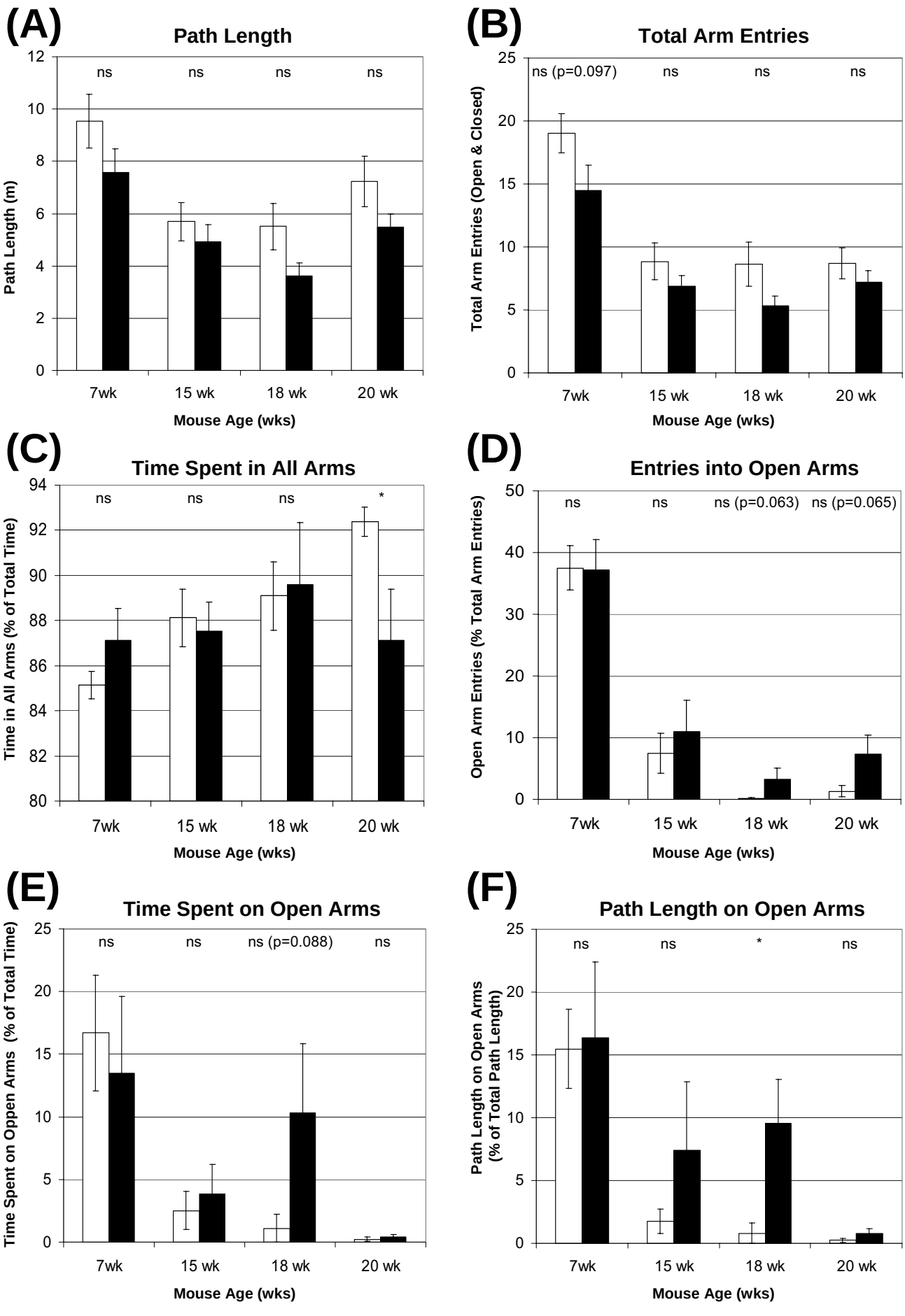
The most striking changes were observed in the measures involving open arm exploration. The percentage of entries into the open arms, time spent on the open arms and path length on the open arms were significantly elevated in the 7-wk-old male mice of both genotypes compared to the older age groups (**Fig. 6.8D-F**). MPS IIIA mice had more open arm entries than unaffected mice at 15-, 18- and 20-wks, with the latter two time-points marginally significant (p=0.063, 0.065; **Fig. 6.8D**). Likewise, the percentage of time spent on the open arms was elevated in MPS IIIA animals at 15-, 18- and 20-wks, and at 15-wks the differences were approaching significance (p=0.088; **Fig. 6.8E**). At all time points the MPS IIIA animals had a greater percentage of path length on the open arms compared to unaffected mice and this elevation was significant at 18-wks (p=0.0158, **Fig. 6.8F**).

6.3.2.4. Effect of Repeated Testing in the Elevated Plus Maze at 18-wks

To test the influence of prior maze experience, congenic male mice of both disease groups were tested at 15- and 18-wks (same group that was scored manually at 15-wks of age in **Section 6.3.2.1**) and the results obtained at 18-wks were compared with data collected from the naïve-tested animals at 18-wks of age (the same mice detailed in **Section 6.3.2.3**). The group size for the repeat-tested mice at 18-wks was larger (n=9 for each genotype) than when they were originally tested at 15-wks (n=6 for each genotype). This was because some of the data from the 15-wk trials were lost due to problems with the video camera during the manual scoring procedure (e.g. not capturing the entire 10 min trial, the video camera shifting mid-trial so that the position of the animal was not clear).

Figure 6.8: Elevated Plus Maze in male mice of various ages. Naïve groups of unaffected or MPS IIIA male mice were tested in the Elevated Plus Maze at 7-, 15-, 18- or 20-wks of age. The path of each mouse was automatically tracked for 5 min and the data analysed using independent t-tests. The measures assessed included the **(A)** total path length (metres); **(B)** total arm entries (i.e. entries into open and closed arms); **(C)** percentage of time spent in all arms; **(D)** open arm entries (as a percentage of total entries into all arms); **(E)** time spent on the open arms (as a percentage of total time spent in arms); and **(F)** path length on the open arms (as a percentage of total path length). Data are the mean of each measure ± 1 SEM. The t-test p-value for comparisons made between unaffected and MPS IIIA at each age is displayed and p-values approaching significance (p=0.05-0.1) are also indicated. * p=0.01-0.05.

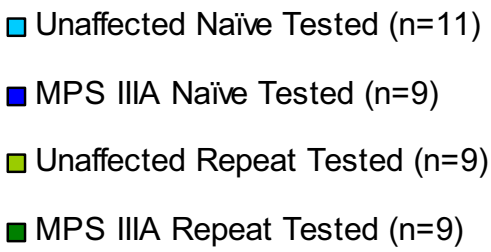


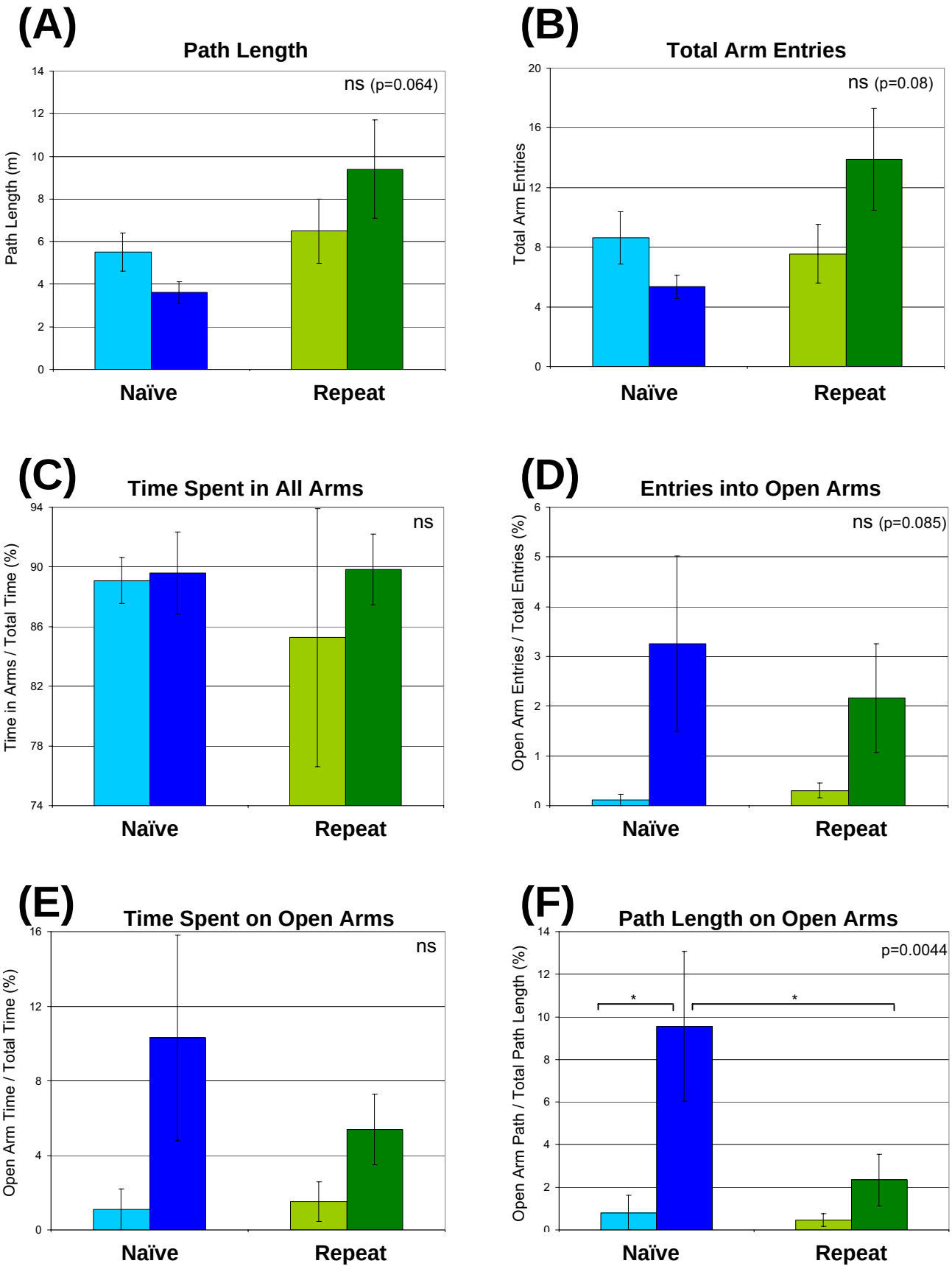

(A)
Path Length
Path Length (m)
ns
ns
ns
ns
7wk
15 wk
18 wk
20 wk
Mouse Age (wks)
(B)
Total Arm Entries
Total Arm Entries (Open & Closed)
ns (p=0.097)
ns
ns
ns
7wk
15 wk
18 wk
20 wk
Mouse Age (wks)
(C)
Time Spent in All Arms
Time in All Arms (% of Total Time)
ns
ns
ns
*
7wk
15 wk
18 wk
20 wk
Mouse Age (wks)
(D)
Entries into Open Arms
Open Arm Entries (% Total Arm Entries)
ns
ns
ns (p=0.063)
ns (p=0.065)
7wk
15 wk
18 wk
20 wk
Mouse Age (wks)
(E)
Time Spent on Open Arms
Time Spent on Oppen Arms (% of Total Time)
ns
ns
ns (p=0.088)
ns
7wk
15 wk
18 wk
20 wk
Mouse Age (wks)
(F)
Path Length on Open Arms
Path Length on Open Arms (% of Total Path Length)
ns
ns
*
ns
7wk
15 wk
18 wk
20 wk
Mouse Age (wks)

At 18-wks of age, mice which had been previously tested in the Elevated Plus Maze had different behavioural profiles to mice that were naïve to the apparatus. Naïve MPS IIIA mice were less active than naïve unaffected mice as indicated by reduced path lengths and total arm entries (**Fig. 6.9A**, **B**). Repeat testing had no effect on the activity of unaffected mice (**Fig. 6.9A**, **B**). In contrast, repeat-tested MPS IIIA mice were more active than naïve-tested MPS IIIA mice to a level that was approaching statistical significance (path length ANOVA $p=0.064$; total arm entries ANOVA $p=0.08$; **Fig. 6.9A, B**). The percentage of time spent in all arms was similar for both naïve- and repeat-tested mice (**Fig. 6.9C**).

Interestingly, the repeat-tested MPS IIIA mice were less likely to explore the open arms compared to naïve-tested MPS IIIA mice, with reductions observed in the number of entries into the open arms as well as the percentage of time and path length spent on the open arms (**Fig. 6.9D-F**). Repeat-tested, unaffected mice had slightly more open arm entries and percentage of time spent on the open arms than naïve unaffected mice, suggesting that prior maze experience marginally reduced the anxiety in unaffected animals (**Fig. 6.9D, E**). Whilst similar profiles were observed in all measures of open arm exploration (MPS IIIA mice were more active than unaffected mice, repeat-testing reduced the level of exploration on the open arms by MPS IIIA mice compared to naïve-tested MPS IIIA mice), significant differences were only detected in the percentage of path length on the open arms (ANOVA $p=0.0044$; **Fig. 6.9F**) with a significant reduction in exploration observed in repeat-tested MPS IIIA mice compared to naïve-tested mice of the same genotype (post test $p<0.05$, $t=2.724$).

Figure 6.9: Effect of repeat testing of 18-wk-old mice in the Elevated Plus Maze. Male congenic mice underwent a 5 min trial in the Elevated Plus Maze at 18-wks of age. Mice were either naïve to the Elevated Plus Maze apparatus or had previously been tested at 15-wks of age. An automated tracker and Maze 2020 software were used to record the path of the animal. The p-value of the one-way ANOVA analyses is presented for each measure. The parameters assessed were: **(A)** total path length; **(B)** total arm entries; **(C)** percentage of time spent in all arms; **(D)** percentage of entries into the open arms; **(E)** percentage of time spent in the open arms; and **(F)** percentage of path length on the open arms. Data are presented as the mean ± 1 SEM. The ANOVA p-value is displayed in the corner of each graph and significant ($p<0.05$) comparisons from the post test are also shown (*).




(A)
Path Length
ns (p=0.064)
Path Length (m)
0
2
4
6
8
10
12
14
Naïve
Repeat
(B)
Total Arm Entries
ns (p=0.08)
Total Arm Entries
0
4
8
12
16
20
Naïve
Repeat
(C)
Time Spent in All Arms
ns
Time in Arms / Total Time (%)
74
78
82
86
90
94
Naïve
Repeat
(D)
Entries into Open Arms
ns (p=0.085)
Open Arm Entries / Total Entries (%)
0
1
2
3
4
5
6
Naïve
Repeat
(E)
Time Spent on Open Arms
ns
Open Arm Time / Total Time (%)
0
4
8
12
16
Naïve
Repeat
(F)
Path Length on Open Arms
p=0.0044
*
*
Open Arm Path / Total Path Length (%)
0
2
4
6
8
10
12
14
Naïve
Repeat

6.4. DISCUSSION

Memory and Learning Deficits in MPS IIIA Mice

In this study, congenic male MPS IIIA mice at 16-wks of age displayed significant memory and learning impairments in the learning or acquisition phase compared to unaffected controls. The difference observed was not the result of variations in swim speeds between the disease and control groups. These data support previous findings outlining that C57Bl/6 mice are "good learners" in the Morris Water Maze. Wild-type C57Bl/6 mice outperform BALB/cByJ, BALB/c, 129/SvEvBrd, DBA/2J, 1291/SvlmJ, BTBR T + tf/tf, A/J, C3H/HeJ (retinal degeneration), FVB/NJ (retinal degeneration) strains in the Morris Water Maze (Upchurch and Wehner, 1988; Zaharia *et al*, 1996; Wahlsten *et al*, 2005; Van Dam *et al*, 2006).

Prior to undertaking the study detailed within this chapter, several Morris Water Maze tests had been conducted in 14- to 20-wk-old congenic mice by other researchers (Crawley *et al*, 2006a, unpublished results, LDRU). The earlier experiments established the necessity to increase the inter-trial interval from 2 min to approximately 10 min and to raise the water temperature from the 21-25°C used for the "New York" strain to 25°C ± 1°C to prevent hypothermia in the congenic mice (Crawley *et al*, 2006a). Learning is inhibited by hypothermia caused by low water temperatures and/or short inter-trial intervals (Iivonen *et al*, 2003). In studies conducted by other LDRU researchers in the congenic MPS IIIA mice, the latencies were manually recorded and the mice were held in the corridor adjacent to the testing room until immediately prior to testing. However, in the present study, alterations to the classification levels of holding rooms within the Animal House resulted in the plan of the Animal House being modified such that the access point to the PC2 holding room was now situated across the corridor from the behavioural testing room, greatly increasing the amount of foot traffic and noise in the corridor during testing. It was decided that whilst it was not ideal to keep mice in the testing room throughout the behavioural assessments, this option would provide greater reproducibility and control of experimental conditions.

Spatial Cognition and Neuropathology in MPS IIIA

Several brain regions have been implicated in spatial learning, as assessed by the Morris Water Maze, including the hippocampus, striatum, basal forebrain, cerebellum and neocortex (reviewed extensively in D'Hooge and De Deyn, 2001). The importance of the hippocampus in spatial memory has been demonstrated using a virtual computer simulation of

the Morris Water Maze task in 10 human patients with unilateral hippocampal resections (Astur *et al*, 2002). Similar results have been obtained in C57Bl/6 mice receiving unilateral or bilateral chemical lesions in the hippocampus (Logue *et al*, 1997; Gerlai *et al*, 2002). Mild lysosomal storage within the dentate gyrus of the hippocampus has been documented in a 24-wk-old, vehicle-treated MPS IIIA mouse (n=1; Savas *et al*, 2004). In addition, 2- to 10-month-old MPS IIIA mice (n=6) display moderate to high amounts of HS storage as well as secondary storage of G_{M2} and G_{M3} gangliosides and cholesterol in the hippocampus (McGlynn *et al*, 2004). These pathological changes may have contributed to the spatial learning impairments observed in the MPS IIIA mice during the acquisition phase of the Morris Water Maze.

However, no statistically significant differences were observed between the two disease groups at 16-wks of age when the efficacy of memory transfer was tested in the probe phase. It is possible that prior behavioural training (in the open field activity and Elevated Plus Maze) in the fortnight prior to Morris Water Maze testing may have contributed to the lack of overall significant differences in the probe phase. The behavioural profile of mice is greatly influenced by the training history (i.e. naïve-tested versus battery-tested) of the mice (discussed further in **Chapter 7**; McIlwain *et al*, 2001; Voikar *et al*, 2004). In combination with the comparable escape latency and path length measures on the final day of the acquisition phase, these data suggest that both unaffected and MPS IIIA mice ultimately learnt the position of the platform, although the rate of learning was slower in MPS IIIA mice. Consequently, subsequent Morris Water Maze assessments undertaken in congenic mice were performed at a later stage of disease progression, at approximately 20-wks of age (**Chapter 7**).

Manual and Automated Scoring of 15-wk-old Male Mice

The design of the Elevated Plus Maze was based on that described in Crawley (2000). A comprehensive appraisal by questionnaire of approximately 30 research groups revealed a great deal of variability in both the design (presence/absence of open arm ledges, colour of floor and walls, dimensions of the maze) and the testing procedures (bright or dim illumination, the use of red lights, length of trial, method of scoring) for the Elevated Plus Maze (Hogg, 1996). A grey maze floor (which influences the maze brightness) was considered as the percentage of time that male C57Bl/10J mice spent exploring the open arms was greater when using a grey floor compared to a white floor (Lamberty and Gower, 1996). However, we ultimately selected a white floor to improve the contrast, and thus accuracy, of the automated HVS tracker system.

In the pilot study, 15-wk-old mice explored the maze apparatus for 10 min. The percentage of time spent in open arm exploration was significantly higher in MPS IIIA mice compared to unaffected controls in both the first and second halves of testing (5 min blocks). However, the MPS IIIA mice appeared to habituate over the course of the trial with less time spent in the open arms during the second block of testing. Similar observations were made over the course of 5 min trials when the path of the mice was assessed in 1 min increments (Rodgers *et al*, 1996; Zhu *et al*, 2006). To avoid habituation in the MPS IIIA mice, the subsequent maze trials were conducted for 5 min.

At 15-wks of age, MPS IIIA mice were significantly more likely to spend a greater percentage of time on the open arms (scored manually) over the 5 min trial ($p=0.02$) compared to analysis with Maze 2020 software. Considerable variation in this parameter was observed with manual scores of 2.0% and 29.5% and automated scores of 2.5% and 3.8% for unaffected and MPS IIIA mice, respectively. Part of this variation may be attributed to the way in which an open arm entry is defined: for manual scoring, an open arm entry is defined as "the two front paws crossing into the open arm", whilst for the automated scoring the software follows the "nominal centre" of the animal (usually the rear haunches as this provides the greatest colour contrast; Mr Richard Baker, HVS Image, personal communication). Consequently, the majority of an animal's torso is required to enter into the open arm for the automated tracker system to classify the movement as an 'open arm entry', and is likely to reduce the number of open arm scores when compared to the 'two front paws' definition of an open arm entry. Better comparisons may have been drawn between the manually-assessed and Maze 2020-calculated data if the definition of an open arm entry was revised so that the whole body was required to cross into an arm to be counted as an entry in the manual assessments.

In addition, the manually-assessed mice were housed randomly (i.e. single- or group-housed) whilst all 15-wk-old mice scored with the automated system were group-housed. Long-term individual housing (7-wks prior to testing) significantly reduced anxiety-like behaviours in male C57Bl/6JolaHsd mice, with faster open arm latencies and higher percentages of open arm entries and time spent on the open arms when tested in the Elevated Plus Maze (Voikar *et al*, 2005). Altered emotional behaviours were also detected after social isolation in alternative anxiety tests (novel cage, dark-light test, hyponeophagia) (Voikar *et al*, 2005). Furthermore, male and female C57Bl/6 mice housed in isolation for 4-months before testing were less fearful than their environmentally-enriched/group-housed counter-parts in the Elevated Plus Maze (Zhu *et al*, 2006). As a result of the behavioural changes described in

these published studies, great care was taken in this study to standardise the housing conditions. Data from single- and group-housed animals has not been combined in the following, automatically-assessed studies.

Female Mice are More Anxious than Male Mice

It has been widely reported that human females have a greater tendency for depressive and/or anxiety-related disorders, and by age six, females are twice as likely as males to have experienced an anxiety disorder (Breslau *et al*, 1995; Lewinsohn *et al*, 1998). In an attempt to determine whether behavioural distinctions related to the gender of the mice were evident, 7-wk-old unaffected and MPS IIIA animals of each gender were assessed. Male mice were found to be significantly less fearful/anxious than females as measured by the percentage of entries into the open arms. This finding was supported by the trends observed in the time and path length spent on the open arms. Similar observations were made when C57Bl/6NIA mice were tested in an Elevated Plus Maze (Frick *et al*, 2000), with males tending to be less anxious than females (time spent in the open arms, entries into the open arms) and significant gender differences were detected at 17-months of age for parameters influenced by locomotion (time in closed arms, closed arm entries).

These findings contrast with the Elevated Plus Maze results presented by Teixeira *et al* (2004) where no gender effect was observed between Swiss male and female mice in the diestus phase of the estrous cycle. Furthermore, when 5 common mouse genetic strains of each gender were tested, there were statistically insignificant gender-associated changes in the Elevated Plus Maze, although male mice tended to be less fearful than the females of the corresponding strain in 4 of the 5 strains (Voikar *et al*, 2001). Due to the gender-related differences observed in this study, subsequent behavioural experiments were only conducted with male mice.

Male Mice are Less Active and Display More Anxiety-Related Behaviours with Age

A longitudinal study was conducted using unaffected and MPS IIIA mice ranging from 7- to 20-wks of age. Both disease groups were less active with age, as seen by reduced path lengths and total arm entries. Similar to the hypoactivity displayed by MPS IIIA mice at 6- to 15-wks in the open-field activity testing (Hemsley and Hopwood, 2005), MPS IIIA mice were less active in the Elevated Plus Maze compared to unaffected controls at every time point assessed.

Younger mice (7-wks of age) were more prone to reckless behaviour than the older mice (15- to 20-wks) with young/adolescent mice spending more time in the central platform and exhibiting a greater level of open arm exploration (entries into the open arms, percentage of time spent on the open arms and percentage of path length on the open arms). These observations mirror those described by Macri *et al* (2002), where adolescent mice (~7-wks) were approximately 80% more likely to enter an open arm and spend a significantly higher proportion of time in the open arms compared to adult mice (~9-wks). This is further supported by studies where younger (5-month-old) female C57Bl/6NIA mice had significantly more open arm entries and spent a greater percentage of time on the open arms than 17-month-old females (Frick *et al*, 2000). Comparable trends have also been observed using rats of various ages (Imhof *et al*, 1993; Boguszewski and Zagrodzka, 2002).

Effect of Repeated Testing

In this study, unaffected mice displayed nearly identical values when tested naïvely or when retested for all of the measures assessed. This conflicts with the results reported in vehicle-treated mice (Rodgers *et al*, 1996; Espejo, 1997) or rats (Fernandes and File, 1996; Bertoglio and Carobrez, 2000) in the Elevated Plus Maze where a marked decrease was observed in the percentage of open arm entries and time spent on the open arms when retested 1- to 7-days after the initial exposure. When unaffected congenic male mice are tested in the open field, the mice with previous exposure to the test eventually habituate to the testing arena and are hypoactive and less exploratory than naïve mice (Allison Crawley, LDRU, unpublished results; Crawley *et al*, 2006a). In comparison, MPS IIIA mice tend to have similar or higher levels of open field activity after re-testing which may indicate that the affected mice do not effectively remember the testing apparatus and continue to actively explore the open field (LDRU unpublished results; Crawley *et al*, 2006a).

Naïve and repeat-tested MPS IIIA mice display reduced anxiety/fearfulness at 18-wks of age compared to wild-type controls in all parameters assessed (entries into the open arms, percentage of time spent on the open arms, percentage of path length on the open arms). In contrast to the data collected from unaffected mice, 18-wk-old MPS IIIA mice with previous exposure to the maze appeared to habituate during re-testing, with reductions for all anxiety-related measures (entries into the open arms, percentage of path length and time spent on the open arms). Based on these observations, it was decided that repeated testing altered the behavioural profile of MPS IIIA mice in the Elevated Plus Maze and consequently mice in subsequent studies were only tested once at a single time-point (**Chapter 7**).

Reduced Anxiety in MPS IIIA Mice

The amygdala of the brain has been implicated in influencing anxiety or fearfulness in humans (Richardson *et al*, 2000, as reviewed in Davis, 1992; Rauch *et al*, 2003). The accumulation and distribution of storage material has been studied in four models of MPS disease (MPS I, IIIA, IIIB and VII) in mice ranging in age from 2- to 10-months (McGlynn *et al*, 2004). The amygdala is a major site of neuropathology in these diseases, with high to intense HS storage reported in all of the models assessed (McGlynn *et al*, 2004). In addition, appreciable accumulation of glycosphingolipids (G_{M2} and G_{M3} gangliosides), free cholesterol and autofluorescent storage material was also observed in the amygdala of MPS mice (McGlynn *et al*, 2004).

The occurrence of behavioural problems, including hyperactivity, disruptive tendencies and poor impulse control has been documented in Sanfilippo children (Nidiffer and Kelly, 1983). Qualitative increases in anxiety were also reported with 64% of affected children displaying fear/anxiety when parents (n=30) were assessed via questionnaire (Nidiffer and Kelly, 1983). Our quantitative findings using the Elevated Plus Maze support those in MPS IIIB mice (Cressant *et al*, 2004), suggesting that MPS IIIA and MPS IIIB mice are significantly less anxious than their wild-type counterparts.

6.5. SUMMARY AND CONCLUSIONS

The Morris Water Maze revealed spatial learning differences between unaffected and MPS IIIA congenic male mice at 16-wks. However, MPS IIIA mice eventually learnt the position of the hidden escape platform, displaying comparable escape latencies to those of unaffected animals. This indicates that the disease impairs the speed at which learning occurs in MPS IIIA mice.

Furthermore, the Elevated Plus Maze was developed to measure anxiety-related and emotional behaviour. Significant gender-related differences were detected at 7-wks of age, with males more inclined to explore the open arms than the female mice. The best discrimination between MPS IIIA and control groups of males was obtained at 18-wks of age. The analyses conducted in this chapter provided the baseline evaluations of different aspects of the MPS IIIA behavioural phenotype. This characterisation was essential to enable the evaluation of the functional effect of CAV-NS-mediated therapy.

CHAPTER 7:
Therapeutic Assessment of Gene Transfer in Newborn MPS IIIA Mice

7.1. INTRODUCTION

In the experiments detailed in the previous chapters, the administration of CAV-NS vector in newborn mice resulted in widespread GFP expression in the brains of injected mice (**Chapter 5**). Dose-dependent transgene expression was observed for both the rhNS and GFP transgenes, with the optimal titre determined to be 10^9 particles/hemisphere (**Chapter 5**). Neither antibodies towards rhNS nor neutralising antibodies to the CAV-2 capsid were detected in PBS- or CAV-NS-injected-mice (**Chapter 5**). In addition, the behavioural phenotype of MPS IIIA mice was thoroughly characterised, both in **Chapter 6** and by other members of the LDRU in studies independent of this thesis, to provide an insight into the functional changes resulting from NS deficiency.

The major aim of the experiments described in this chapter was to determine whether early intervention with the CAV-NS vector could improve the clinical outcome in MPS IIIA mice in long-term studies using the methods developed and validated in the preceding chapters.

7.2. SPECIFIC METHODS

7.2.1. Evan's Blue Toxicity Test

To determine whether Evan's Blue dye (used to illuminate the cerebral ventricles during injection) was toxic to CAV-2 vectors, $1x10^5$ DKCre cells/well were plated in 24-well trays and incubated overnight at 37°C. A series of dilutions were prepared from a 0.2% (w/v) solution of filter-sterilised Evan's Blue Dye diluted in PBS, combined with an equal volume of 20 particles/cell CAV-GFP, and the mixture was then applied to DKCre cells to give a final concentration of 0.1% (w/v) Evan's Blue dye in the most concentrated samples. Twenty-four hrs post-inoculation, quadruplicate samples were analysed by flow cytometry to quantitate the number of GFP-positive cells (**Section 2.2.9**).

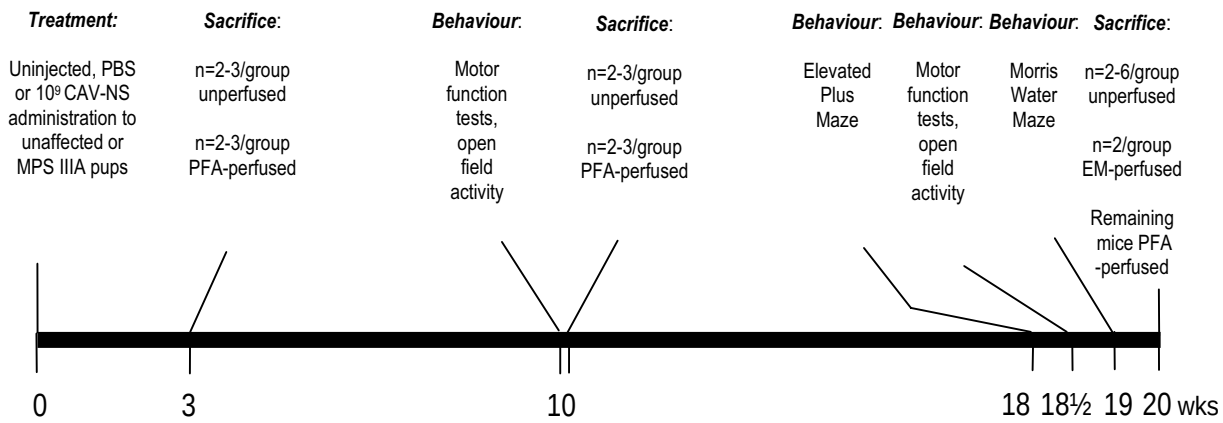
7.2.2. Study Design and Bilateral Injections

Dr Kim Hemsley performed some of the PBS injections. Ms Amanda Luck assisted with the collection of the peripheral organs and sera at 20-wks post-injection and also performed the homogenisation and sonication of fresh brain segments of the mice sacrificed at 20-wks.

Newborn mice were bilaterally-injected with $1x10^9$ particles CAV-NS in 0.025% (w/v) Evan's Blue dye per hemisphere or 0.025% (w/v) Evan's Blue dye in sterile saline as a vehicle control on the day of birth (as described in **Section 2.2.5**). Additional unaffected and MPS IIIA pups that did not receive the cryo-anaesthesia/bilateral injections (i.e. remained uninjected) were also included in the study design as controls for the behavioural analyses. For brevity, these groups will be referred to as uninjected, PBS-injected or CAV-NS-injected, although the buffer for the injected groups also contained Evan's Blue dye.

Mice were sacrificed at each of 3-, 10- or 20-wks to provide an on-going assessment of CAV-NS-mediated transgene expression and the effect on pathology. Female mice were preferentially selected for euthanasia at the earlier time points to maximise the number of males available for the behavioural analyses conducted at 10- to 20-wks of age. At 3- and 10-wks post-injection, the brains of 2-3 mice per genotype treated with PBS or CAV-NS were sacrificed without fixation (**Section 2.2.5.3**) or perfused with saline and then 4% (w/v) PFA in PBS (**Section 2.2.5.2**). At 20-wks of age, unaffected or MPS IIIA mice (uninjected, PBS-injected or CAV-NS-injected; n=2-6/group) were also sacrificed without fixation. In addition, 2 mice from each of the six groups (2 genotypes, 3 treatments) were euthanased and perfused with PBS followed by EM fixative. The remaining mice underwent perfusion with PBS before switching to 4% (w/v) PFA.

A time-line for the injection, behavioural analyses and sacrifice times post-injection is shown:



In several of the figures and tables presented in this chapter, data from the preceding chapters are also presented to better allow comparisons between CAV-NS doses (ranging from $5x10^6$ to $1x10^9$ particles/hemisphere in **Chapter 5** versus $1x10^9$ particles/hemisphere in this chapter; **Fig. 7.7** and **Table 7.4**), at various times post-treatment (1- or 6-wks post-injection in **Chapter 5** versus 3-, 10- or 20-wks post-injection in this chapter; **Figs. 7.9, 7.10**) or between groups of mice in the behavioural analyses (non-battery-tested mice described in **Chapter 6** versus battery-tested mice in this chapter; **Fig. 7.5**). In addition, based on the results in this chapter, data obtained from further analysis of samples collected from mice described in **Chapter 5** (CAV-NS dose of $1x10^9$ particles/hemisphere, sacrificed 1-wk post-injection) are presented in **Table 7.2** and **Fig 7.18**.

7.2.3. Behavioural Test Battery

Behavioural testing and data processing were performed without knowledge of the treatment and genotype of the animals (i.e. blinded). Statistical analyses were performed using a one-way ANOVA for the data collected at each individual time-point followed by a post-test calculation with GraphPad software (http://graphpad.com/quickcalcs/posttest2.cfm). This comparison uses a Bonferroni correction factor to adjust for multiple comparisons. The alpha value was set at 0.05 with a 95% confidence level.

Timing of Behavioural Tests

Gait, negative geotaxis, neuromuscular grip strength and open field locomotor activity were originally validated in the "New York" mixed mouse strain (Hemsley and Hopwood, 2005) and then compared to the congenic strain (LDRU unpublished observations and Crawley *et al*, 2006a). For this study, male mice were battery-tested in a series of tests examining locomotion, cognition and emotional behaviours (similar to components of the behavioural phenotyping battery reviewed in Crawley, 1999). Animals were born over a 9-day period. The distribution of the date of births of the male mice was as follows, with the number of mice born each day shown in parentheses: day 1 (n=3); day 2 (n=2); day 3 (n=1); day 4 (n=8); day 5 (n=8); day 6 (n=12); day 7 (n=11); day 8 (n=11); and day 9 (n=17). The reported age at which each behavioural test was performed in this chapter is based on the age of mice born on the sixth day. Mice were housed in same-sex groups of 8-10 animals per cage. All males were separated into single cages at approximately 15-wks of age due to aggression in some mice.

Motor function (gait, negative geotaxis, neuromuscular grip strength) was investigated at 10-wks. The mice were naïve-tested in open field activity 2-days later (10-wks). At 18-wks of age, mice were randomly divided into two groups for the Elevated Plus Maze and underwent one trial on either of two consecutive days after overnight acclimatisation to the behaviour room. Three- or 4-days later, mice were again examined in the motor function tests (18.5-wks) and then open field activity the following day. Mice were then divided on the basis of age into two groups and assessed in the Morris Water Maze with the habituation day performed at approximately 19-wks of age for each group. The latter test was performed over two consecutive weeks. Mice were sacrificed at 20-wks of age.

7.2.3.1. Gait

The walking pattern of mice was assessed in a walled corridor lined with paper with a width of approximately 12 cm and a length of 50 cm. A darkened goal box was positioned at the end of the corridor. The hind paws of each mouse were dipped into non-toxic food colouring and the mouse allowed to freely walk towards the goal box. At least two sets of tracks were assessed for each mouse with a minimum of three pairs of footprints included in the analysis. The mean length of the right and left strides (length) and the perpendicular distance between each stride (width) were assessed. The first and last two sets of footprints were not included in the analysis due to changes in speed associated with initiating movement and slowing down as the mouse approached the goal box.

7.2.3.2. Negative Geotaxis

Immediately following the gait analysis, mice were positioned on a wire grid (15 x 30 cm) and slowly rotated vertically so that the mouse was facing downwards. The time taken for the mouse to re-position itself so that it was facing towards the ceiling was determined up to a maximum of 30 sec.

7.2.3.3. Neuromuscular Grip Strength

Mice were placed onto a horizontal wire grid suspended 50 cm above a container filled with soft bedding material. Grid squares were 1 cm^2. The time taken for mice to fall from the grid from a single attempt up to a maximum of 60 secs was recorded.

7.2.3.4. Open Field Locomotor Activity

After overnight acclimatisation to the behaviour room, mice were tested between 7 am and 1 pm in a plastic enclosure divided into 15 zones in a 5 x 3 grid formation. Each zone had dimensions of 9 x 10 cm. Mice were placed facing the corner closest to the operator (designated south-east) and the number of line crossings, rears (both fore-legs not in contact with the floor) and faecal boli were manually recorded over 3 min. The HVS tracker system equipped with Field 2020 software (version 10/20/04) determined path length and the total number of cell entries. The open field was swabbed with 70% (v/v) ethanol between trials.

7.2.3.5. Elevated Plus Maze

Male mice were tested at 18-wks of age as described in **Section 6.2.4**. The mice were acclimatised in the testing room overnight and randomly allocated to one of two consecutive days of testing. The group tested on the second day was kept in the testing room during the first day of testing and for an additional night. Several trials were excluded due to inaccurate tracking by the automated Maze 2020 software prior to unblinding the identity of each mouse.

7.2.3.6. Amendments to Morris Water Maze Protocol

The mice born on days 1-7 and 7-9 (n=34, n=33, respectively) were tested in a 7-day protocol (day 0 habituation phase, days 1-5 acquisition phase, day 6 probe phase) over 2 consecutive weeks. Between the performing of experiments described in **Chapter 6** and the present study, changes were made to the Morris Water Maze protocol based on observations made within the LDRU. These included the use of a curtain to surround the pool to obscure all visual cues on the habituation day (e.g. stationary cues such as the experimenter, sink, door etc) and a more visible marker on the platform (a brightly-coloured paperweight approximately 10 cm in height). To habituate each mouse, they were placed into the pool at each of the cardinal points in succession (4 trials in total with an inter-trial interval of approximately 5-10 min) whilst the platform remained located in the north-eastern quadrant.

Mice were swum in a different, random order each day of the acquisition and probe phases (based on a random number table) and were warmed on heat pads between trials. For the acquisition phase, the platform was located in the north-western quadrant. The operator was blinded to the identity and the treatment group of the mice. Due to hardware failure on the first day of the acquisition phase during the first week of testing, the data from this group was only recorded manually. Mice underwent four trials in the probe phase beginning at each of the four cardinal points.

7.2.4. Measurement of Anti-GFP Antibody Titres

The ELISA detection method used to assay for the presence of anti-rhNS antibodies in sera (**Section 2.2.10**) was adapted to test whether a humoral response was being generated against the GFP protein in CAV-treated mice. The coating antigen was recombinant GFP protein (5 μg/well). A mouse monoclonal anti-GFP antibody (1/125 diluted in blocking solution) was used as a positive control. All other assay procedures were conducted as previously described (**Section 2.2.10**).

7.2.5. NS Activity Time-course in Mixed, Primary Neural Cells

Newborn, primary cell cultures derived from unaffected mice were harvested and plated into four 6-well trays (**Section 2.2.2.1**). Seven-days post-plating, the cells were mixed with 1000 particles/cell of CAV-NS in complete media (or media only as a control) and cultured under standard conditions. Cells were viewed using an inverted fluorescent microscope to confirm GFP expression. The conditioned media was collected at harvest (days 3, 6 or 9 post-transduction) and stored at 4°C. For the wells yet to be harvested, the cells were fed with an additional 2 mL/well of fresh complete media. The cell layer was harvested as described in **Section 2.2.2.3,** resuspended in 200 μL of 20 mM Tris/0.5 M NaCl (pH 7.0) and the cell contents liberated by six alternate cycles of freezing/thawing. NS activity in conditioned media and cell extract samples was determined as detailed in **Section 2.2.12,** after dialysis overnight in 0.2 M sodium acetate (pH 5.0), and was normalised to total cellular protein (**Section 2.2.13**).

7.2.6. CytoTox-One™ Membrane Integrity Assay

Unaffected primary murine neural cells derived from newborn or adult mice were plated into 6-well trays as described in **Section 2.2.2.1**. Seven-days post-plating, duplicate samples were mixed with 100 or 1000 particles/cell of CAV-GFP or CAV-NS vector in complete primary cell culture media excluding the sodium pyruvate supplement in a volume of 3 mL/well. Untransduced cells (treatment negative control) and media without cells (assay negative control) were also included. The media was not replaced after inoculation.

After 2- or 7-days post-treatment, the conditioned media was removed and an assay positive control well corresponding to 100% lysis/cytotoxicity was also prepared by adding 12 μL of lysis buffer/well. The lactate dehydrogenase activity was measured as detailed in the

manufacturer's instructions as an indicator of membrane integrity and cytotoxicity in the CAV-NS-treated cells. In brief, triplicate wells containing 100 μL of sample per well were aliquoted into 96-well tissue culture trays, mixed with an equal volume of cytotoxic substrate and mixed for 30 sec on the Milenia plate shaker (Micromix 4, Biomediq; setting 5). After incubation at 22-24°C in the dark for 10 min, 50 μL stop buffer per well was added, mixed for 10 sec and the fluorescence read on a Victor^2 (1420 Multilabel counter, Wallac, Finland) with an excitation of 544 nm and an emission of 590 nm. The percentage of cytotoxicity was determined using the following equation:

$$\% \text{ Cytotoxicity} = \frac{(\text{Experimental} - \text{Culture Medium Background})}{(\text{Maximum LDH Release} - \text{Culture Medium Background})} \times 100\%$$

7.3. RESULTS

7.3.1. Evan's Blue Toxicity to CAV-NS Vector Particles

To improve the consistency and reproducibility of targeting the lateral ventricles of newborn mice, the use of Evan's Blue dye in the dilution buffer was considered. However, it was first necessary to determine if the dye was toxic to CAV-2 vectors (**Section 7.2.1**). No significant differences in transduction efficiencies were observed in the samples containing Evan's Blue dye, even at the highest concentration (0.1% (w/v) final concentration), when compared to viral particles in the absence of dye (unpaired t-test, p=0.4475; **Table 7.1**).

It was noted that the recommended concentration of 0.1% (w/v) Evan's Blue dye for newborn injections (Dr Allison Crawley, personal communication) produced very strong and intense colour in the cell culture media. Consequently a lower dose of 0.025% (w/v) Evan's Blue dye was examined *in vivo* in the first instance in an attempt to minimise the exposure of the pups and CAV-NS particles to high concentrations of dye. Visualisation of the lateral ventricles was sufficient using 0.025% (w/v) Evan's Blue dye and consequently all PBS vehicle control and CAV-NS-treated mice were injected with Evan's Blue dye at this concentration.

Table 7.1: **Transduction efficiency of CAV-2 in the presence of Evan's Blue dye**. CAV-GFP (20 particles/cell) was mixed with two-fold dilutions of Evan's Blue dye ranging from a final concentration of 0.00625% to 0.1% (w/v) Evan's Blue dye. The mixture was applied to DKCre cells and the transduction efficiency (with GFP-positive cells as the readout) was counted by flow cytometry 24-hrs later (**Section 2.2.9**). The mean of quadruplicate wells is presented ± 1 SEM. The GFP expression observed with viral vector mixed with 0.1% Evan's Blue dye was not significantly different from the control CAV-GFP particles without Evan's Blue dye present in the buffer composition (unpaired t-test p=0.4475).

Evan's Blue Dye (% w/v)	% GFP-positive cells ± 1 SEM
0 %	14.4 ± 0.66
0.00625 %	13.9 ± 0.35
0.0125 %	15.2 ± 0.51
0.025 %	13.6 ± 0.68
0.05 %	12.6 ± 0.19
0.1 %	13.0 ± 0.56

7.3.2. Body and Organ Weights, Survival Rates and Adverse Events

In this experiment, all pups receiving saline or 1 x 10^9 particles/hemisphere of CAV-NS vector were born to experienced dams (second to fourth litters) and recovered well from the cryo-anaesthesia/bilateral injections. Of the 27 litters injected in this study, 22 of the pairings were heterozygote females x MPS IIIA males, 2 pairs were heterozygote females x heterozygote males and 3 pairs were MPS IIIA females x MPS IIIA males.

After all of the pups had been injected with CAV-NS vector, the transduction efficiency of the vector stock was re-titrated as described in **Section 2.2.3.4** and the particle-to-transduction ratio after freeze/thawing was found to be 5.0 to 1, slightly poorer than the original ratio of 4.5 to 1 (as determined in **Chapter 3**).

The survival rates of the pups until weaning were 92.6% (63 pups surviving of 68 born), 94.4% (51/54 pups) and 86.7% (46/53 pups) for uninjected, saline-injected or CAV-NS-injected mice, respectively. Of the 7 pups receiving CAV-NS who died before weaning, 3 were littermates that were lost the day after a leaky water bottle flooded the cage. Hydrocephalus developed in 4 of the 107 pups (3.7%) injected as newborns: two at 6-wks of age (a MPS IIIA mouse treated with PBS and a heterozygote mouse treated with CAV-NS)

and one at each of 8- and 16-wks post-injection (both heterozygotes treated with CAV-NS). Mice exhibiting hydrocephalus were euthanased and perfusion-fixed with 4% (w/v) PFA in PBS.

Congenic uninjected MPS IIIA male mice were significantly heavier than unaffected uninjected male mice at 20-wks of age (post-test $p<0.05$, $t=3.913$; **Fig. 7.1**). Cryo-anaesthetised/injected pups were similar in weight to genotype-matched uninjected pups at most time-points with no statistical differences detected.

The liver and spleen weights were determined in unperfused, 20-wk-old, male mice to determine if CAV-NS treatment had normalised the hepatosplenomegaly associated with the MPS IIIA disease phenotype (**Fig. 7.2**; Crawley *et al*, 2006a). Statistical analyses could not be performed due to the small sample sizes, however, no obvious differences were observed in liver and spleen weights between unaffected and MPS IIIA mice.

7.3.3. Behaviour

7.3.3.1. Motor Function Tests

Gait

Male mice were assessed at 10-wks to determine whether CAV-NS administration had an effect on gait, as had been observed from Ad-induced demyelination (Fathallah-Shaykh *et al*, 2000). Mice were also assessed at 19-wks to determine if CAV-NS administration altered the clinical disease progression in MPS IIIA mice. Statistically significant differences were not found in the gait length or width at either time point (ANOVA $p=0.26$-0.57). Subtle changes were observed between the trials conducted between 10- and 19-wks of age. The stride length of the 19-wk-old mice was longer, and the gait width narrower than genotype- and treatment-matched mice at 10-wks of age (**Fig. 7.3A-D**). Nineteen-wk-old unaffected mice tended to have wider gait tracks than their MPS IIIA counterparts (**Fig. 7.3D**).

Negative Geotaxis

No differences were observed in the time taken for male mice to re-orientate themselves at 10- or 19-wks of age (ANOVA $p=0.11$-0.19; **Fig. 7.3E, F**). There did not appear to be an age- or treatment-related trend between the groups at either time-point.

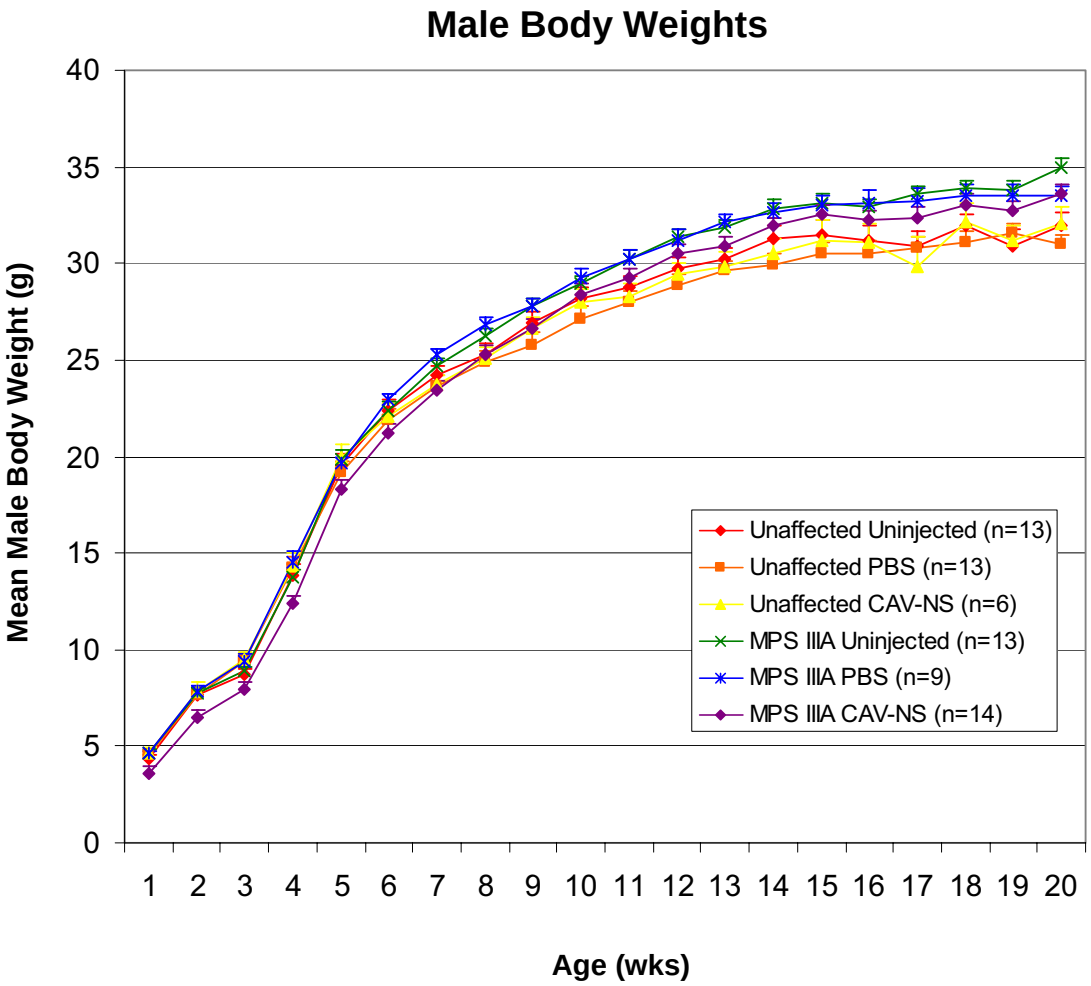


Figure 7.1: Body weights. Unaffected and MPS IIIA mice were cryo-anaesthetised and bilaterally-injected into the lateral ventricles with PBS or 1 x 10^9 CAV-NS particles/hemisphere on the day of birth. These mice were weighed weekly up until 20-wks of age and compared to uninjected mice. The group sizes at 20-wks of age are listed in the legend. The mean body weight of male mice per group + 1 SEM is displayed.

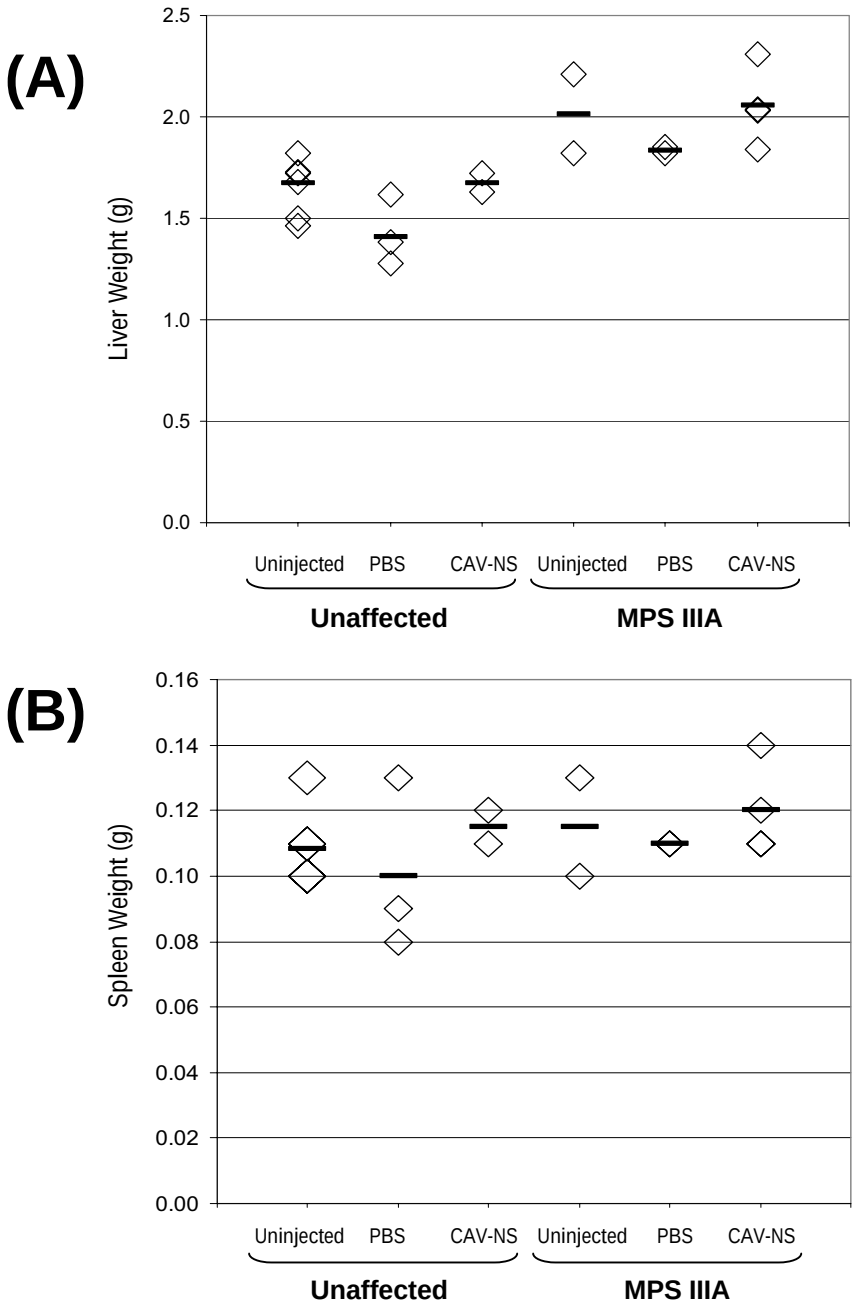
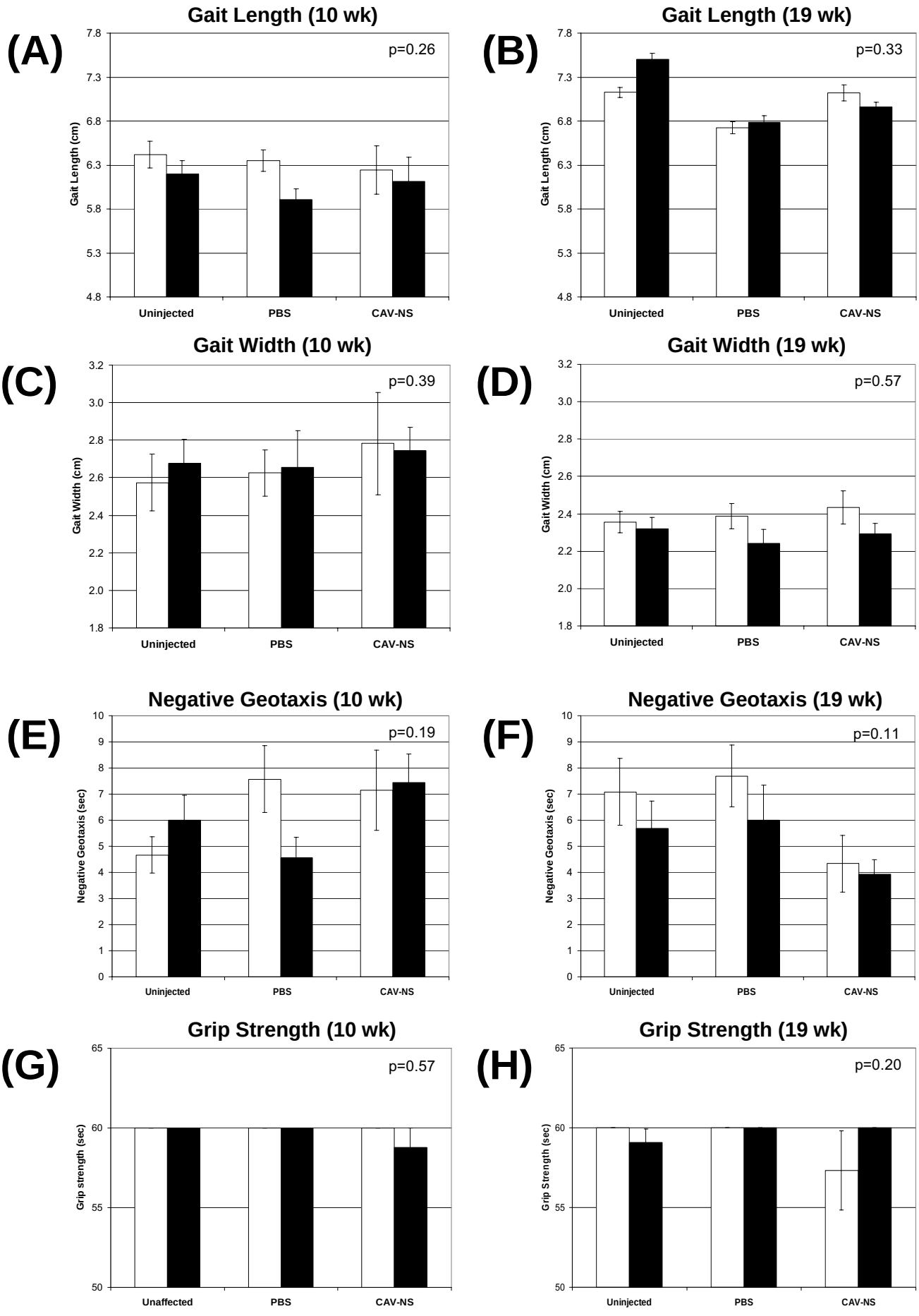


Figure 7.2: Liver and spleen weights. At 20-wks post-injection, male mice were asphyxiated with carbon dioxide and the liver and spleen weights determined. Each individual marker represents one mouse (group sizes n=2-6). Livers from unaffected mice tended to be lighter than livers obtained from MPS IIIA mice, regardless of the treatment administered. The spleen weight of unaffected mice appears similar to the weight of spleens from MPS IIIA mice.

Figure 7.3: Motor Function. Male mice were subjected to a battery of non-invasive behavioural tests. The motor function of the mice was assessed at 10-wks (left column; **A, C, E, G**) or 19-wks (right column; **B, D, F, H**) of age. The changes in the gait of the animals were investigated by dipping the hind paws of the mice in non-toxic food colouring and measuring the length **(A, B)** and width **(C, D)** of at least three strides in duplicate runs. Immediately following the gait assessment, male mice were placed on a wire grid situated over soft bedding material and orientated to face downwards. The time taken for the mouse to re-position itself to face towards the ceiling (negative geotaxis, **E, F**) was measured up to a maximum of 30 sec. Finally, the neuromuscular grip strength (**G, H**) of the animals was evaluated by allowing the mice to hang from a wire grid and measuring the latency of the mice to fall into soft bedding material up to a maximum of 60 sec. The mean of each measure ± 1 SEM is displayed. The ANOVA p-value is shown for each graph. Note that the y-axis is the same scale between each row.

	Group Size (n=)	
Treatment	**10 wks**	**19 wks**
Unaffected Uninjected	13	13
Unaffected PBS	13	13
Unaffected CAV-NS	7	6
MPS IIIA Uninjected	13	13
MPS IIIA PBS	9	9
MPS IIIA CAV-NS	14	14


(A)
Gait Length (10 wk)
p=0.26
Gait Length (cm)
7.8
7.3
6.8
6.3
5.8
5.3
4.8
Uninjected
PBS
CAV-NS
(B)
Gait Length (19 wk)
p=0.33
Gait Length (cm)
7.8
7.3
6.8
6.3
5.8
5.3
4.8
Uninjected
PBS
CAV-NS
(C)
Gait Width (10 wk)
p=0.39
Gait Width (cm)
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
Uninjected
PBS
CAV-NS
(D)
Gait Width (19 wk)
p=0.57
Gait Width (cm)
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
Uninjected
PBS
CAV-NS
(E)
Negative Geotaxis (10 wk)
p=0.19
Negative Geotaxis (sec)
10
9
8
7
6
5
4
3
2
1
0
Uninjected
PBS
CAV-NS
(F)
Negative Geotaxis (19 wk)
p=0.11
Negative Geotaxis (sec)
10
9
8
7
6
5
4
3
2
1
0
Uninjected
PBS
CAV-NS
(G)
Grip Strength (10 wk)
p=0.57
Grip strength (sec)
65
60
55
50
Unaffected
PBS
CAV-NS
(H)
Grip Strength (19 wk)
p=0.20
Grip Strength (sec)
65
60
55
50
Uninjected
PBS
CAV-NS

Neuromuscular Grip Strength

All mice performed well in the neuromuscular grip strength test (**Fig. 7.3G,H**) with only 1 (at 10-wks of age) or 2 mice (at 19-wks of age) recording fails. Rather than falling off the grid due to lack of neuromuscular strength, these mice appeared to fall while exploring the wire matrix. No significant differences were measured between the treatment groups at either time-point (ANOVA p=0.20-0.57).

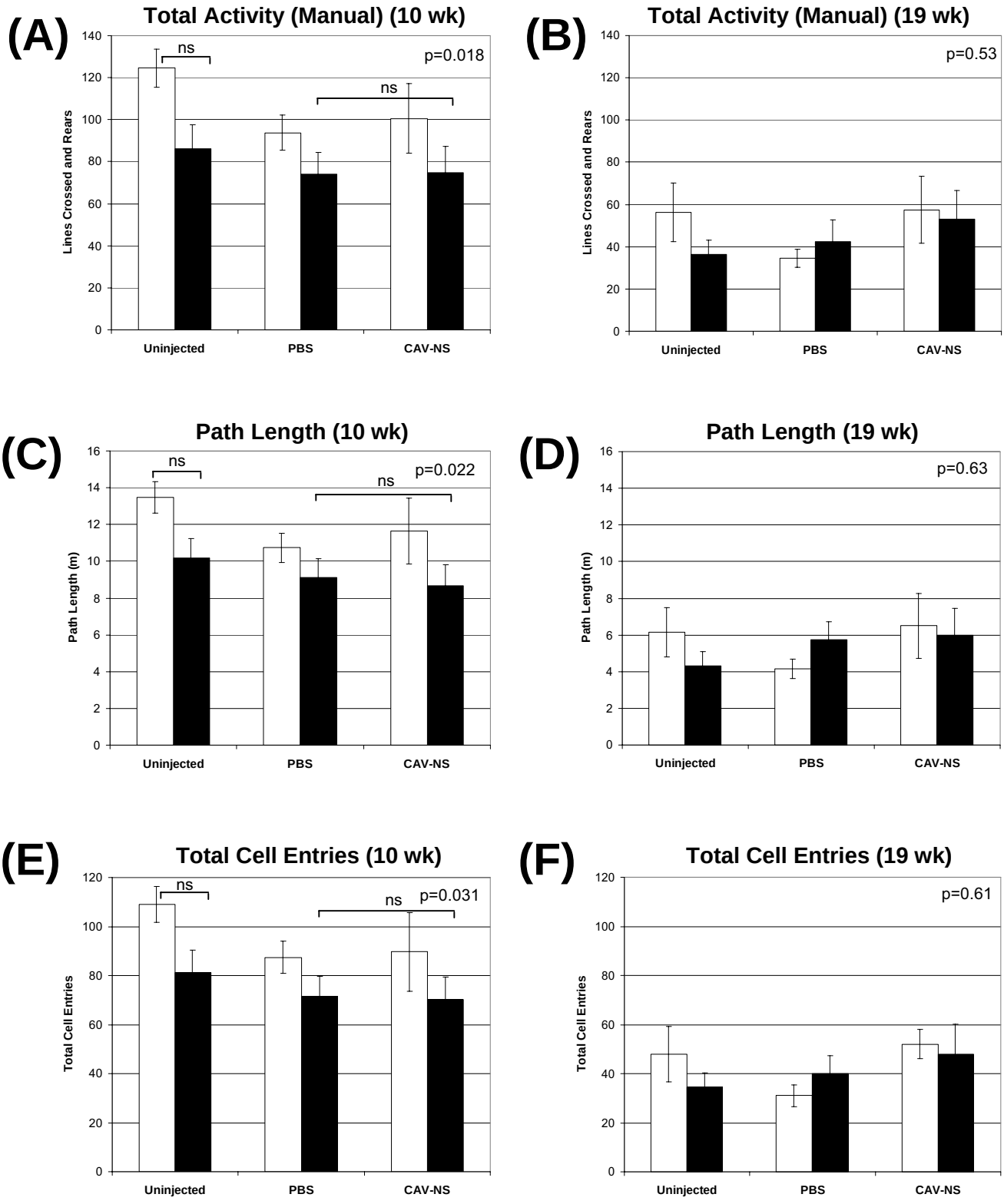
7.3.3.2. Open Field Locomotor Activity

One male MPS IIIA mouse treated with PBS was excluded from all open field activity tests due to anophthalmia in both eyes (i.e. had no eyes, a defect known to occur in the C57Bl/6 strain of mice). At 10-wks of age, male MPS IIIA mice were less active than their unaffected counterparts when assessed manually (**Fig. 7.4A**) and via an automated tracker system (**Fig. 7.4C, E;** ANOVA p=0.018-0.031). Hypoactivity in MPS IIIA animals was observed in all of the measures assessed (total activity encompassing both horizontal line-crossing and vertical rearing exploration, path length and cell entries). Post-hoc test comparisons revealed that no statistically significant differences were present for each of the measures at 10-wks of age for the following pair-wise tests: unaffected uninjected versus MPS IIIA uninjected; unaffected PBS versus MPS IIIA PBS; unaffected uninjected versus unaffected PBS; MPS IIIA uninjected versus MPS IIIA PBS; unaffected PBS versus unaffected CAV-NS; MPS IIIA PBS versus MPS IIIA CAV-NS.

When tested a second time at 19-wks of age, mice appeared less active than the corresponding group at 10-wks. Similar to observations at 10-wks, 19-wk-old uninjected, unaffected mice were more active than uninjected MPS IIIA mice (**Fig. 7.4B, D, F**), however, no significant differences were measured (ANOVA p=0.53-0.63). This contrasts with the trend observed in PBS-treated mice, with the MPS IIIA mice treated with PBS being more active than the unaffected mice treated with PBS. CAV-NS treatment of MPS IIIA mice did not appear to influence locomotor activity and exploration when compared to control uninjected and PBS-treated MPS IIIA mice.

Figure 7.4: Open Field Activity. One- to 2-days after assessment in the motor function tests, male mice underwent testing in the open field arena at 10-wks **(A, C, E)** or 19-wks **(B, D, F)** of age. The testing enclosure consisted of a plastic, rectangular box arbitrarily divided into 15 cells, each with dimensions of 9 x 10 cm. Male mice were placed in the corner closest to the operator and allowed to freely explore the enclosure for 3 min. The arena was cleaned between trials with ethanol to remove scent trails. The total number of rears and lines crossed in each trial were scored manually **(A, B)** to give an indication of total activity (vertical and horizontal activity). An automated tracker system equipped with Field 2020 software was also used to monitor the path of the animal, and the total path length in metres **(C, D)** and number of cells entered **(E, F)** were calculated. The mean of each measure ± 1 SEM and the ANOVA p-value is displayed for each parameter. Note that the y-axis is the same scale between each row. ns, not significant.

	Group Size (n=)	
Treatment	**10 wks**	**19 wks**
Unaffected Uninjected	13	13
Unaffected PBS	13	13
Unaffected CAV-NS	7	6
MPS IIIA Uninjected	13	13
MPS IIIA PBS	8	8
MPS IIIA CAV-NS	14	14


(A)
Total Activity (Manual) (10 wk)
Lines Crossed and Rears
ns
ns
p=0.018
Uninjected
PBS
CAV-NS
(B)
Total Activity (Manual) (19 wk)
Lines Crossed and Rears
p=0.53
Uninjected
PBS
CAV-NS
(C)
Path Length (10 wk)
Path Length (m)
ns
ns
p=0.022
Uninjected
PBS
CAV-NS
(D)
Path Length (19 wk)
Path Length (m)
p=0.63
Uninjected
PBS
CAV-NS
(E)
Total Cell Entries (10 wk)
Total Cell Entries
ns
ns
p=0.031
Uninjected
PBS
CAV-NS
(F)
Total Cell Entries (19 wk)
Total Cell Entries
p=0.61
Uninjected
PBS
CAV-NS

7.3.3.3. Elevated Plus Maze

Behavioural battery-tested mice were assessed for changes in anxiety-related behaviours in the Elevated Plus Maze at 18-wks (an age where unaffected mice can be distinguished from MPS IIIA mice in non-battery-tested animals, as determined in **Chapter 6**). Whilst generally displaying the same trends (**Fig. 7.5**), the absolute values between the uninjected *battery-tested* and uninjected *non-battery*-tested animals were different, resulting in less separation between the battery-tested unaffected and MPS IIIA groups. Consequently, statistical differences were not detected in any of the parameters (p=0.41-0.97), with the exception of the percentage of time spent in the open and closed arms (p=0.011; **Fig. 7.5C**).

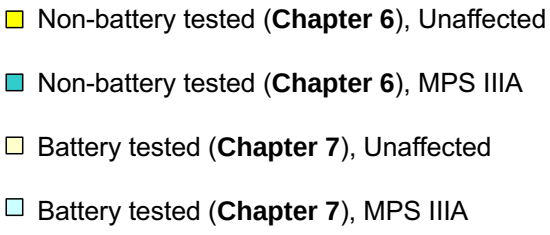
The path length and total arm entries of uninjected mice (**Fig. 7.5A,B**) displayed similar patterns to non-battery-tested mice, with unaffected animals more active than MPS IIIA mice in both measures. Likewise, unaffected PBS-treated and CAV-NS-treated mice were slightly more active than their MPS IIIA counterparts.

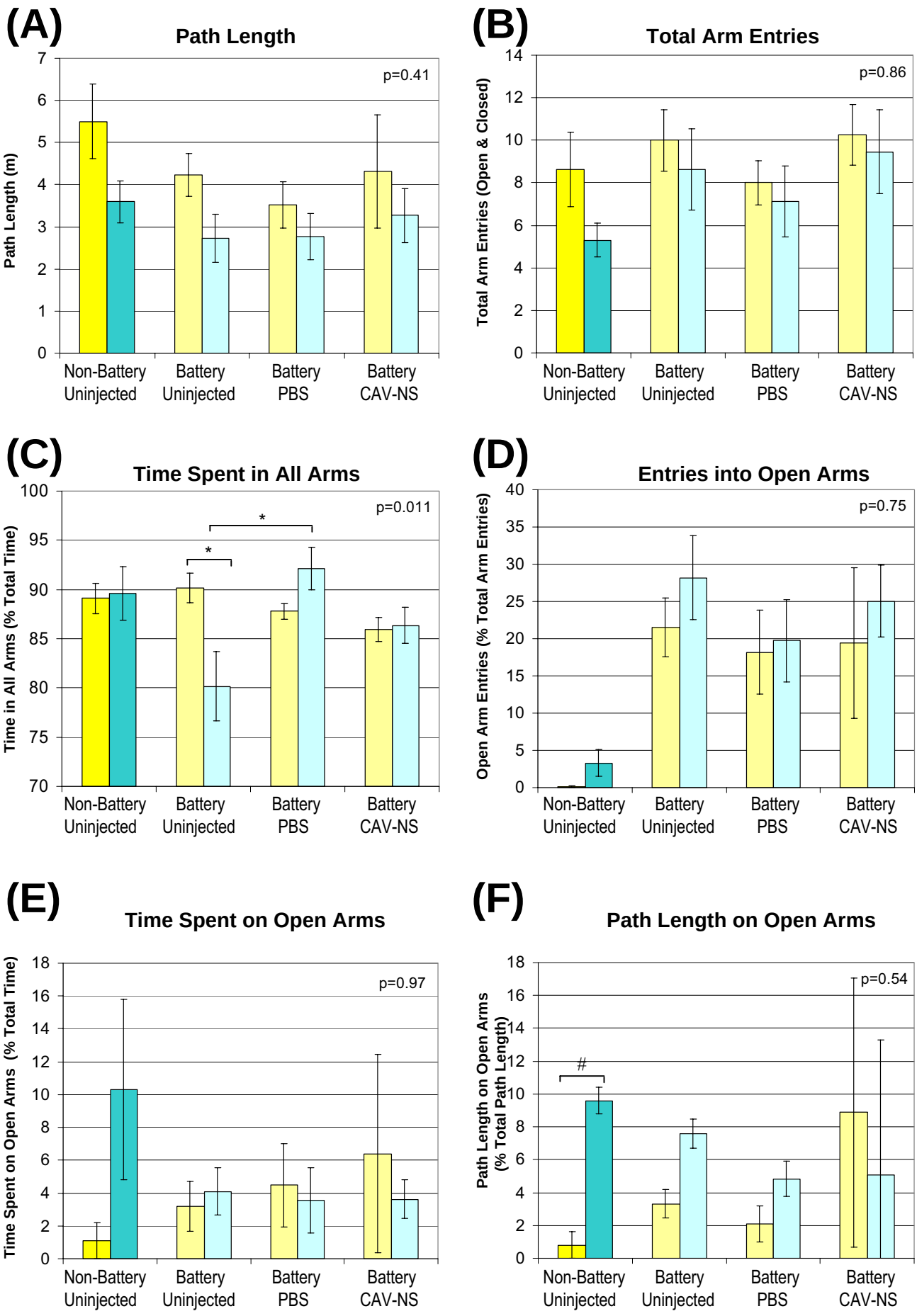
In the only measure to reach statistical significance (time spent in all arms), battery-tested, uninjected unaffected mice spent more time on arms than uninjected MPS IIIA mice (ANOVA p=0.11, post test t=3.237; **Fig. 7.5C**). Battery-tested, uninjected MPS IIIA mice were also different to PBS-treated MPS IIIA animals (post-test t=3.532). There were no differences between the two groups of PBS-treated animals, the two groups of CAV-NS-treated mice or between MPS IIIA saline-treated and MPS IIIA CAV-NS-treated mice.

All groups of mice entered the open arms more frequently than the non-battery-tested mice from **Chapter 6** (**Fig. 7.5D**). Of the mice tested in the behavioural battery, MPS IIIA mice were slightly more likely to enter the open arms compared to the treatment-matched, unaffected animals, but statistical significance was not achieved. Although the MPS IIIA mice were marginally more likely to enter the open arms, the percentage of time spent on the open arms was not different between the groups of battery-tested mice (**Fig. 7.5E**). Uninjected and PBS-treated MPS IIIA mice were more likely to spend a greater proportion of the path length on the open arms compared to the corresponding treatment group of unaffected mice (**Fig. 7.5F**). This trend was not maintained in CAV-NS-treated mice, with unaffected mice receiving CAV-NS spending a greater proportion of path length on the open arms compared to MPS IIIA mice receiving CAV-NS. However, the variation in these groups was high.

Figure 7.5: Elevated Plus Maze. Male unaffected or MPS IIIA mice underwent testing in a battery of behavioural tests to evaluate motor function and open field activity (at 10-wks of age for both) prior to assessment in the Elevated Plus Maze. At 18-wks, male mice were tested in a random order over 2 consecutive days. The mice were placed in the central square facing an open arm and freely explored the apparatus for 5 min. After each trial, the inner maze walls and floor were cleaned with 70% (v/v) ethanol to remove scent trails. The path of the animal was automatically recorded using Maze 2020 software and the following parameters measured: **(A)** path length in metres; **(B)** total arm entries (open and closed arms); **(C)** percentage of time spent in all arms; **(D)** percentage of entries into the open arms; **(E)** percentage of time spent in the open arms; **(F)** percentage of path length on the open arms. Data from the 6 groups of battery-tested mice (naïve to the Elevated Plus Maze, pale-coloured bars) are displayed as the mean ± 1 SEM and were assessed via a one-way ANOVA (p-value shown for each measure). Statistically significant ($p<0.05$) post-test comparisons are indicated (*). The data presented in **Chapter 6** from 18-wk-old, non-battery-tested, uninjected mice (dark-coloured bars) have been included for comparison and were not included in the statistical analyses. # $p<0.05$ in the non-battery tested mice.

Treatment	n=
Unaffected Uninjected	11
Unaffected PBS	11
Unaffected CAV-NS	4
MPS IIIA Uninjected	13
MPS IIIA PBS	8
MPS IIIA CAV-NS	13




(A)
Path Length
p=0.41
Path Length (m)
0
1
2
3
4
5
6
7
Non-Battery Uninjected
Battery Uninjected
Battery PBS
Battery CAV-NS
(B)
Total Arm Entries
p=0.86
Total Arm Entries (Open & Closed)
0
2
4
6
8
10
12
14
Non-Battery Uninjected
Battery Uninjected
Battery PBS
Battery CAV-NS
(C)
Time Spent in All Arms
p=0.011
*
*
Time in All Arms (% Total Time)
70
75
80
85
90
95
100
Non-Battery Uninjected
Battery Uninjected
Battery PBS
Battery CAV-NS
(D)
Entries into Open Arms
p=0.75
Open Arm Entries (% Total Arm Entries)
0
5
10
15
20
25
30
35
40
Non-Battery Uninjected
Battery Uninjected
Battery PBS
Battery CAV-NS
(E)
Time Spent on Open Arms
p=0.97
Time Spent on Open Arms (% Total Time)
0
2
4
6
8
10
12
14
16
18
Non-Battery Uninjected
Battery Uninjected
Battery PBS
Battery CAV-NS
(F)
Path Length on Open Arms
p=0.54
#
Path Length on Open Arms (% Total Path Length)
0
2
4
6
8
10
12
14
16
18
Non-Battery Uninjected
Battery Uninjected
Battery PBS
Battery CAV-NS

7.3.3.4. Morris Water Maze

Male mice were tested at 19-wks of age for their memory and spatial learning ability in the Morris Water Maze. Due to the large number of mice to be tested (n=67), the mice were divided into 2 groups on the basis of their date of birth so that older mice were tested in the first week (n=34) and the younger mice (n=33) in the second week. This strategy enabled mice to be tested closer to exactly 19-wks of age by the same operator and at the same stage of the day (i.e. morning to early afternoon).

The habituation phase was the only day where the automated tracker was functional in both weeks of testing due to hardware break-down during the Morris Water Maze testing conducted in the first week. Consequently, to determine whether testing over two consecutive weeks had a significant effect, comparisons of each measure between week 1 and week 2 of testing and between the 6 treatment groups (unaffected or MPS IIIA mice either uninjected, or treated with PBS or CAV-NS) were made using a two-way ANOVA on the habituation day.

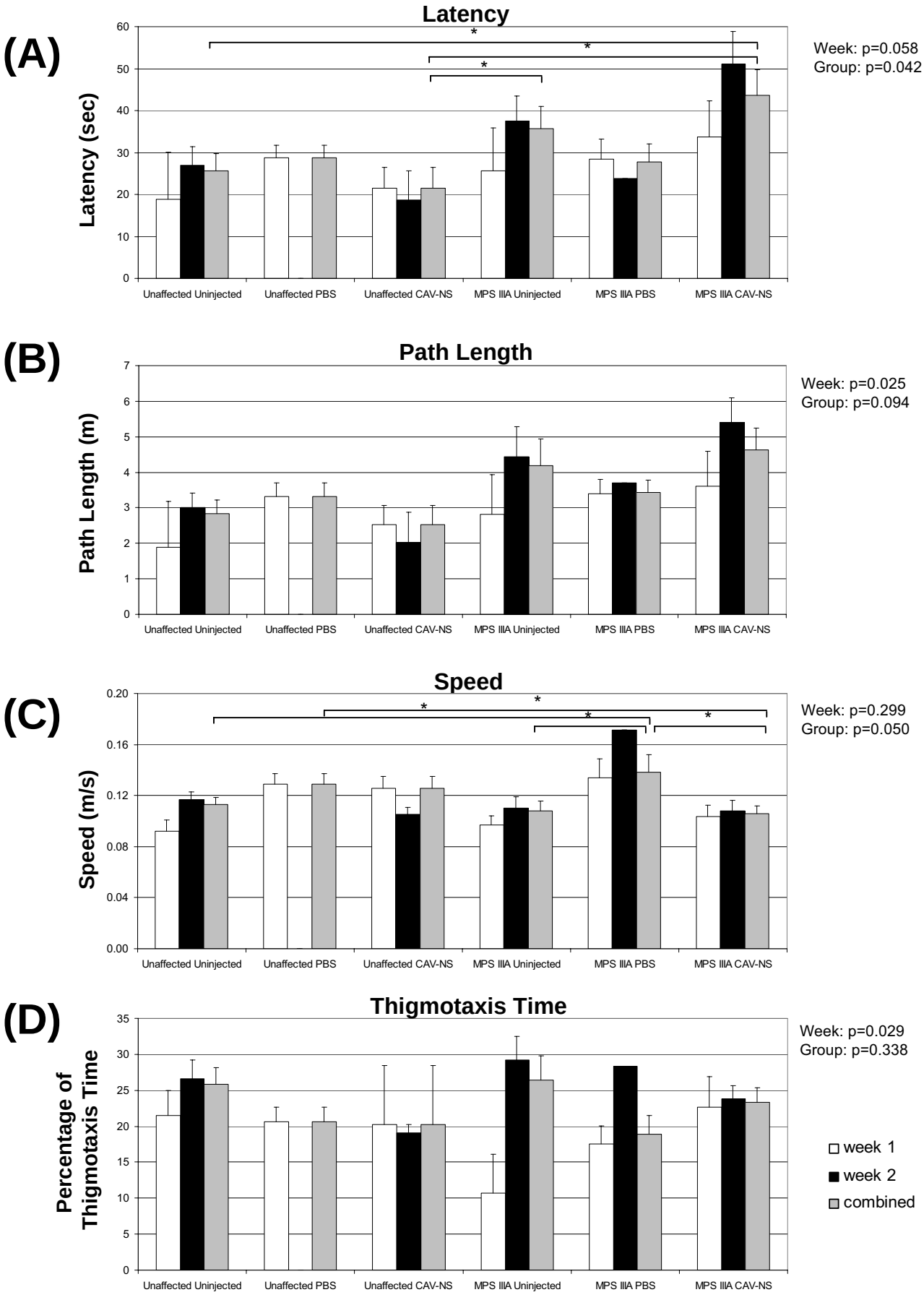
Mice tested in week 1 swam to the visible platform quicker than mice of the same genotype/treatment in the following week on the habituation day (**Fig. 7.6A**). The faster latency times of the mice tested in week 1 were marginally different ($p=0.058$) to the mice swum in week 2 when controlling for a possible treatment group effect and was significantly affected by the treatment group ($p=0.042$). Statistically significant ($p\leq0.05$) post-hoc pair-wise comparisons were observed between the following pairs: unaffected uninjected and MPS IIIA CAV-NS; unaffected CAV-NS and MPS IIIA uninjected; and unaffected CAV-NS and MPS IIIA CAV-NS. For the path lengths on the habituation day (**Fig. 7.6B**), significant differences were detected between the 2-wks of testing ($p=0.025$) but not between the 6 treatment groups ($p=0.094$), with mice tested in the first week swimming to the platform in a shorter path length. The week in which the mice were tested did not significantly affect the mean swim speeds ($p=0.229$, **Fig. 7.6C**), however, the treatment group of the mouse influenced the swim speed ($p=0.050$). The least significant difference post-hoc tests showed differences between the following groups: unaffected uninjected and MPS IIIA PBS; unaffected PBS and MPS IIIA CAV-NS; MPS IIIA uninjected and MPS IIIA PBS; and MPS IIIA PBS and MPS IIIA CAV-NS. In contrast, the percentage of time that mice swam in a thigmotaxic pattern (**Fig. 7.6D**) was significantly reduced in the mice assessed in week 1 compared to those tested in week 2 ($p=0.029$), but was not affected by the treatment group of the mouse ($p=0.338$).

Figure 7.6: Morris Water Maze. Male mice were subjected to a behavioural test battery from 10-wks of age prior to assessment of their memory and spatial learning ability in the Morris Water Maze at 19-wks of age. Mice were divided into two assessment groups (wk 1 or wk 2, see table below) on the basis of their date of birth. The mice were assessed blinded in a random order each day. Mice were habituated to the testing arena over four trials in one day with clear water, a platform marked with a colourful paperweight and with all external room cues obscured by a curtain surrounding the pool (day 0). During the habituation phase, the **(A)** latency to find the visible platform in sec, **(B)** path length in metres, **(C)** average speed in metres/sec and **(D)** percentage of time swimming in a thigmotaxic pattern for week 1 (white bars), week 2 (black bars) or both weeks combined (grey bars) was determined. The ANOVA p-value for the comparisons between weeks and between groups and all statistically significant post-hoc test comparisons from the treatment groups are indicated (*) for the habituation phase.

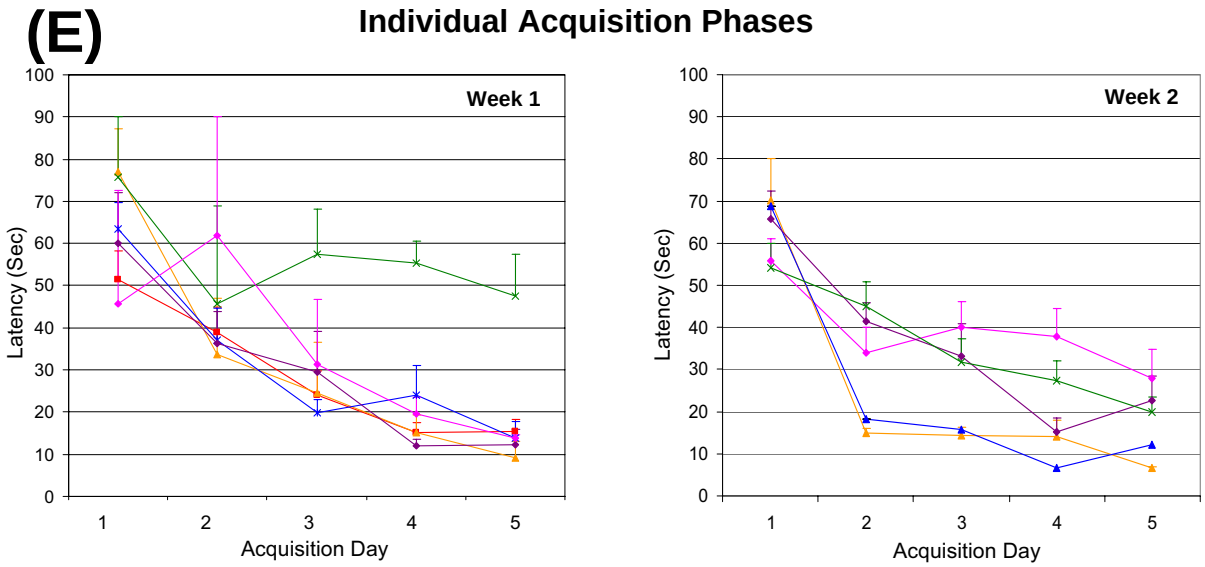
Following this, mice were tested in the acquisition phase (days 1-5) with the water made opaque with white, non-toxic paint and the platform moved to a new position (north-western quadrant). Each mouse underwent four trials of up to 90 sec each (with an inter-trial interval of 5-10 min) and the latency to find the hidden platform was recorded. The **(E)** mean latency in the first and second week of testing, or **(F)** combined over both weeks, on each day of the acquisition phase, is displayed.

On day 6 (probe phase), the platform was removed from the pool and mice were tested for their memory of the platform location over four trials lasting 90 sec each. **(G)** The mean percentage of time spent in target (the quadrant where the platform was previously located) and non-target quadrants is displayed. All data are presented as the mean + 1 SEM.

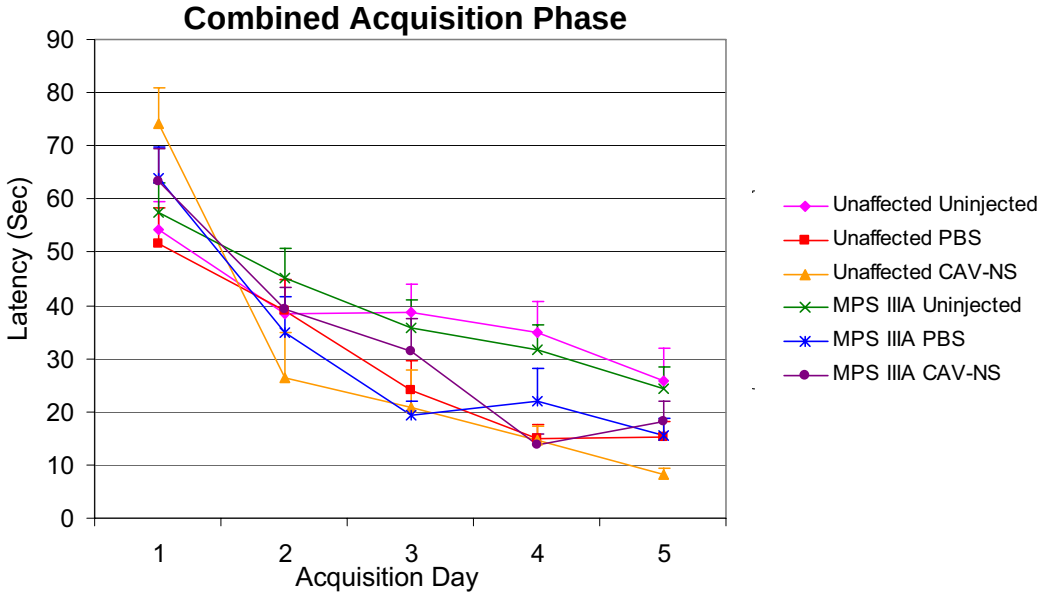
	Group Size (n=)		
Treatment	**Wk 1**	**Wk 2**	**Total**
Unaffected Uninjected	2	11	13
Unaffected PBS	13	0	13
Unaffected CAV-NS	4	2	6
MPS IIIA Uninjected	2	11	13
MPS IIIA PBS	7	1	8
MPS IIIA CAV-NS	6	8	14


(A)
Latency
Latency (sec)
60
50
40
30
20
10
0
*
*
*
Unaffected Uninjected
Unaffected PBS
Unaffected CAV-NS
MPS IIIA Uninjected
MPS IIIA PBS
MPS IIIA CAV-NS
Week: p=0.058
Group: p=0.042
(B)
Path Length
Path Length (m)
7
6
5
4
3
2
1
0
Unaffected Uninjected
Unaffected PBS
Unaffected CAV-NS
MPS IIIA Uninjected
MPS IIIA PBS
MPS IIIA CAV-NS
Week: p=0.025
Group: p=0.094
(C)
Speed
Speed (m/s)
0.20
0.16
0.12
0.08
0.04
0.00
*
*
*
*
Unaffected Uninjected
Unaffected PBS
Unaffected CAV-NS
MPS IIIA Uninjected
MPS IIIA PBS
MPS IIIA CAV-NS
Week: p=0.299
Group: p=0.050
(D)
Thigmotaxis Time
Percentage of Thigmotaxis Time
35
30
25
20
15
10
5
0
Unaffected Uninjected
Unaffected PBS
Unaffected CAV-NS
MPS IIIA Uninjected
MPS IIIA PBS
MPS IIIA CAV-NS
Week: p=0.029
Group: p=0.338
week 1
week 2
combined

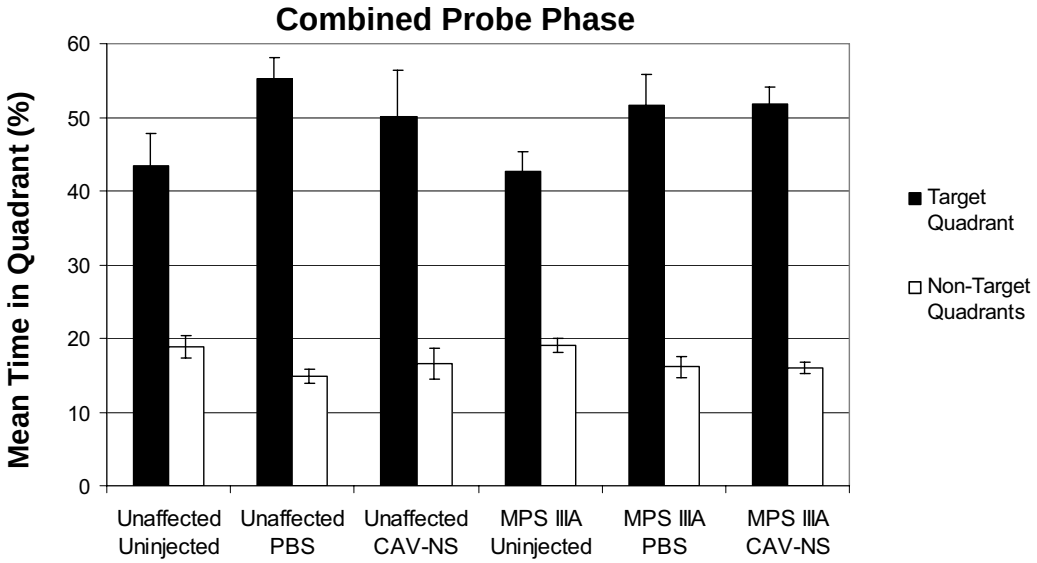
(E)


Individual Acquisition Phases
Week 1
Week 2
Latency (Sec)
Acquisition Day
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5

(F)


Combined Acquisition Phase
Latency (Sec)
Acquisition Day
0
10
20
30
40
50
60
70
80
90
1
2
3
4
5
Unaffected Uninjected
Unaffected PBS
Unaffected CAV-NS
MPS IIIA Uninjected
MPS IIIA PBS
MPS IIIA CAV-NS

(G)


Combined Probe Phase
Mean Time in Quadrant (%)
0
10
20
30
40
50
60
Unaffected Uninjected
Unaffected PBS
Unaffected CAV-NS
MPS IIIA Uninjected
MPS IIIA PBS
MPS IIIA CAV-NS
Target Quadrant
Non-Target Quadrants

The latency data from the acquisition phase was recorded manually in both weeks of testing (**Fig. 7.6E, F**) and was statistically analysed as described in **Chapter 6**. Over the course of the acquisition phase there were no significant differences between unaffected and MPS IIIA mice when adjusted for the treatment group (p=0.870) or across all treatments and days (p=0.332). However, the treatment group had a significant impact on the latency of the mice when adjusted for any possible disease effects (p=0.001). Mice who were not injected had greater latencies to find the hidden platform on the final days of the acquisition phase (days 3 to 5) compared to both cryo-anaesthetised/bilaterally-injected groups, regardless of the genotype of the mice. This was reflected in the significant values in latency between the treatment groups on acquisition days 3 to 5 (p=0.002, <0.001, 0.03, respectively), although there were no significant differences detected on acquisition days 1 and 2 (p=0.436, 0.727).

For the data collected in the probe phase, a two-way ANOVA was performed with the two variables as disease state (i.e. unaffected or MPS IIIA) and injection type (uninjected, PBS-injected, CAV-NS-injected). As seen in **Fig. 7.6G**, there were no statistically significant differences between the unaffected and MPS IIIA mice in the percentage of time spent in the target quadrant (p=0.743) or the mean of the non-target quadrants (p=0.749). Mice that underwent surgical procedures (PBS- and CAV-NS-treated mice) were more likely to spend a greater proportion of time in the target quadrant than uninjected mice (p=0.005), regardless of the genotype of the mice, suggesting that uninjected mice did not learn as effectively as the injected mice. Correspondingly, uninjected mice were more likely to spend time in the non-target quadrants compared to the injected mice (PBS- or CAV-NS-treated; p=0.006). The differences between the treatment groups was not evident in the quadrant closest to the operator (p=0.170) or the quadrant furthest from the operator (p=0.354) but could be detected in the quadrant opposing the target quadrant (p=0.010).

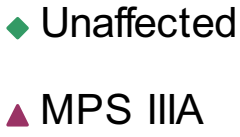
7.3.4. Biochemistry

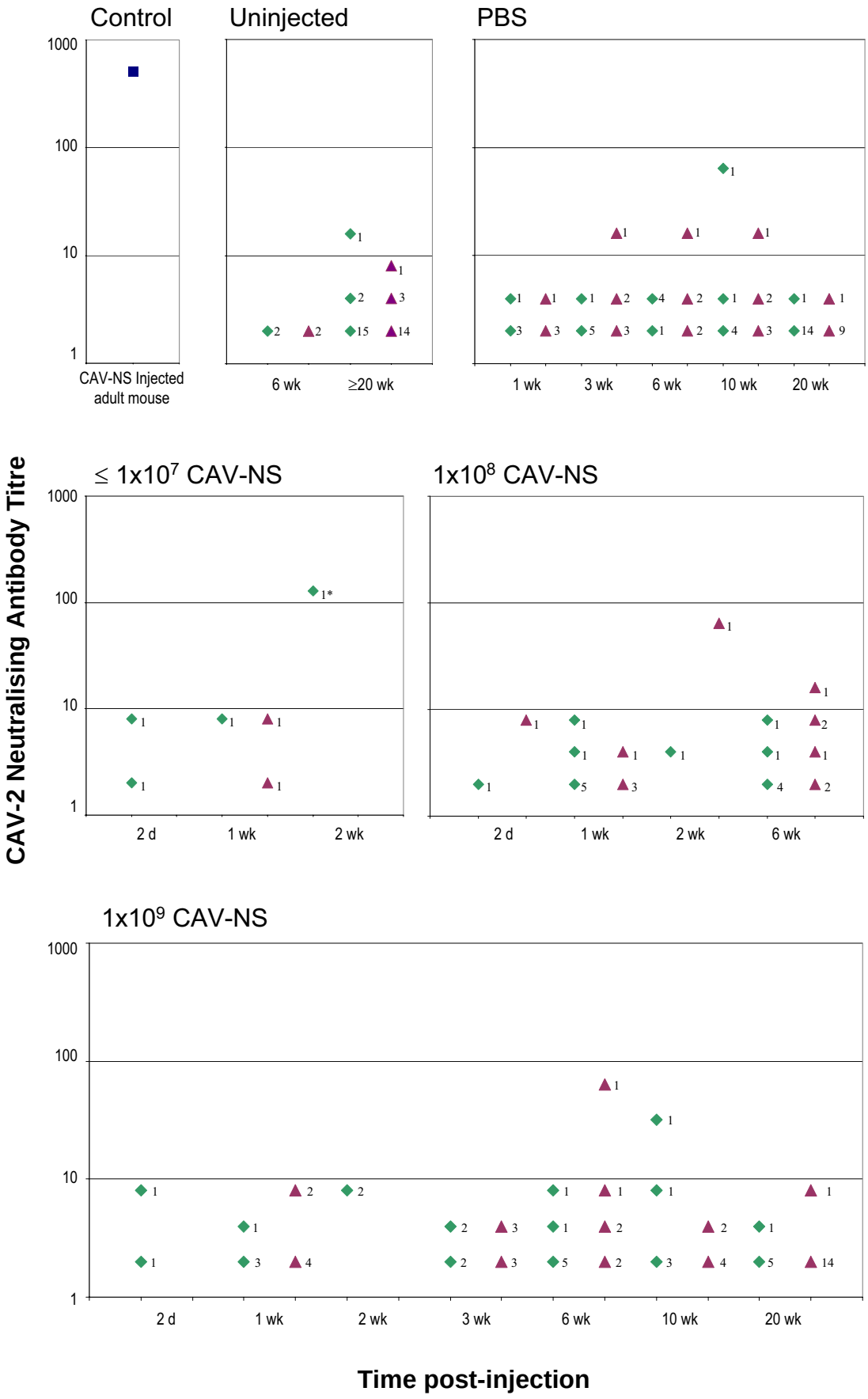
7.3.4.1. Humoral Immune Response

Neutralising Antibodies Against the CAV-2 Capsid

Sera were collected at euthanasia and assessed for the presence of neutralising antibodies against the CAV-2 capsid proteins (**Section 2.2.9**). Significant titres were classified as ≥16 according to previous studies (Perreau *et al*, submitted). The CAV-2 neutralising antibody titres for all mice injected at birth and euthanased between 2-days and 20-wks post-injection is displayed in **Fig. 7.7**.

Figure 7.7: Neutralising antibody titres. Newborn mice were bilaterally-injected with one of four CAV-NS doses (in particles/hemisphere), PBS control or remained uninjected. Sera was collected at euthanasia at various times post-injection (2-days, 1-wk, 2-wks and 6-wks post-injection, data from **Chapter 5**; 3-wks, 10-wks and 20-wks post-injection, data from **Chapter 7**), complement-inactivated and assessed for the presence of CAV-2 neutralising antibodies via a transduction inhibition assay. The percentage of transduced cells was determined via a flow cytometer with GFP expression as the readout. The CAV-2 neutralising antibody titre is the inverse of the highest dilution that gave a 50% reduction in the number of GFP-positive cells and is presented on a logarithmic scale. A significant CAV-2 neutralising titre is ≥16 (Perreau *et al*, submitted). The group size is listed to the right of each symbol. One mouse (indicated with an asterisk) had insufficient sera volume for the neutralising antibody detection assay and had a titre of ≤ 160. The highest titre detected after intracerebral injection of CAV-NS into adult mice is presented as a comparison ("Control"; **Chapter 4**).




Control
Uninjected
PBS
1000
100
10
1
CAV-NS Injected adult mouse
6 wk
≥20 wk
1 wk
3 wk
6 wk
10 wk
20 wk
≤ 1x10⁷ CAV-NS
1x10⁸ CAV-NS
1x10⁹ CAV-NS
CAV-2 Neutralising Antibody Titre
2 d
1 wk
2 wk
3 wk
6 wk
10 wk
20 wk
Time post-injection

In comparison to the adult mice receiving intracerebral injections of CAV-NS, very few mice receiving intraventricular injections during the neonatal period developed neutralising antibodies against CAV-2. When assessing the effect of CAV-NS dose on the percentage of mice with significant CAV-2 neutralising antibody titres (data from **Chapter 5** and this chapter), the percentage of responding mice were similar for all treatment groups: uninjected controls, 2.5% (1/40 mice); PBS-treated controls, 6.0% (4/67); and CAV-NS-treated, 0% (0/5), 7.4% (2/27) or 2.9% (2/69) for mice injected with $\leq 1x10^7$, $1x10^8$ or $1x10^9$ CAV-NS particles/hemisphere.

Detection of Transgene Specific Antibodies by ELISA

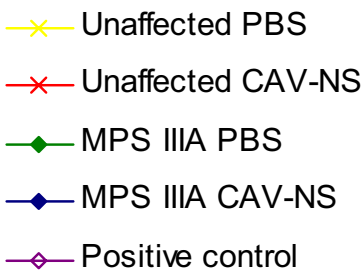
To determine whether transgene-specific antibodies had developed in CAV-NS-treated mice, sera samples were collected from saline- or CAV-NS-injected mice euthanased at 3-, 10- or 20-wks post-injection (**Fig. 7.8**). With the exception of one mouse, there were no differences in the rhNS- or GFP-specific antibodies between the PBS- and CAV-NS-treated mice at any of these time-points. One unaffected mouse receiving 10^9 particles/hemisphere of CAV-NS, and euthanased at 10-wks post-injection, displayed a slightly elevated rhNS antibody titre (1/800) compared to the other samples analysed in this subset, but was much lower than the positive control plasma collected from a mouse receiving 20 μg of purified rhNS into the cerebrospinal fluid (>1/1600, previously determined to be 1/51,200) (Hemsley *et al*, 2007).

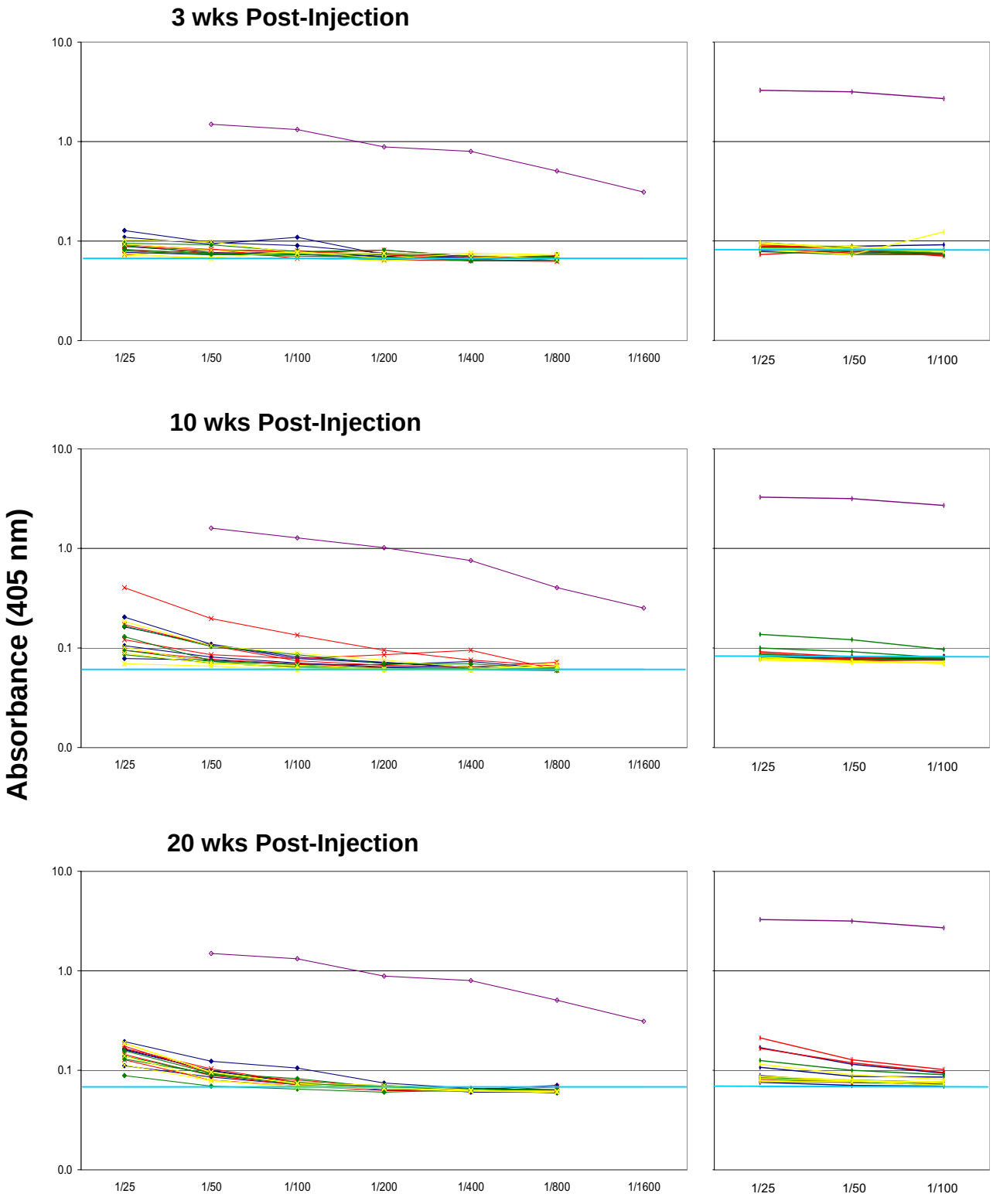
7.3.4.2. Detection of rhNS Protein and Activity

Concurrently, brain tissues were sliced as demonstrated in **Fig. 2.2**, sonicated and the tissue homogenates from an olfactory bulb or the hemi-coronal slice containing the lateral ventricle injection site were assessed for rhNS protein and activity. Similar to the results obtained in mice treated with 1×10^9 particles/hemisphere of CAV-NS at 1-wk post-injection (**Chapter 5** and **Fig. 7.9**), unaffected mice receiving 1×10^9 CAV-NS particles/hemisphere had 6.4 and 1.9 ng rhNS/mg total protein after 3-wks of treatment (data from individual mice; **Fig. 7.9**). MPS IIIA mice treated with CAV-NS had slightly lower measurements for rhNS protein at 3-wks (1.5, 0.6, 0.1 ng rhNS/mg total protein). The rhNS protein concentration was 0.2-0.4 ng rhNS/mg total protein or undetectable for PBS-treated unaffected and PBS-treated MPS IIIA mice, respectively.

As the maximum rhNS and GFP expression was determined to be at 1-wk post-injection (as evaluated by rhNS protein concentrations and the GFP immunostaining), NS

Figure 7.8: Trangene specific antibody response. Newborn mice were bilaterally injected with PBS or $1x10^9$ particles of CAV-NS per hemsiphere and monitored up to 20-wks of age. Sera samples were collected from mice euthanased at 3-, 10- or 20-wks post-injection. The complement was inactivated and assessed for transgene-specific antibodies using an ELISA-based assay. Serial dilutions of 4 randomly selected mice per group are displayed on a logarithmic scale. The positive control samples used for the rhNS-specific and GFP-specific antibody detection ELISA assays were serum collected from a MPS IIIA mouse receiving purified rhNS into the cerebrospinal fluid or a mouse anti-GFP monoclonal antibody, respectively. The assay background absorbance value is indicated with a pale blue line.




3 wks Post-Injection
10 wks Post-Injection
20 wks Post-Injection
Absorbance (405 nm)
10.0
1.0
0.1
0.0
1/25
1/50
1/100
1/200
1/400
1/800
1/1600
rhNS Dilution Factors
GFP Dilution Factors

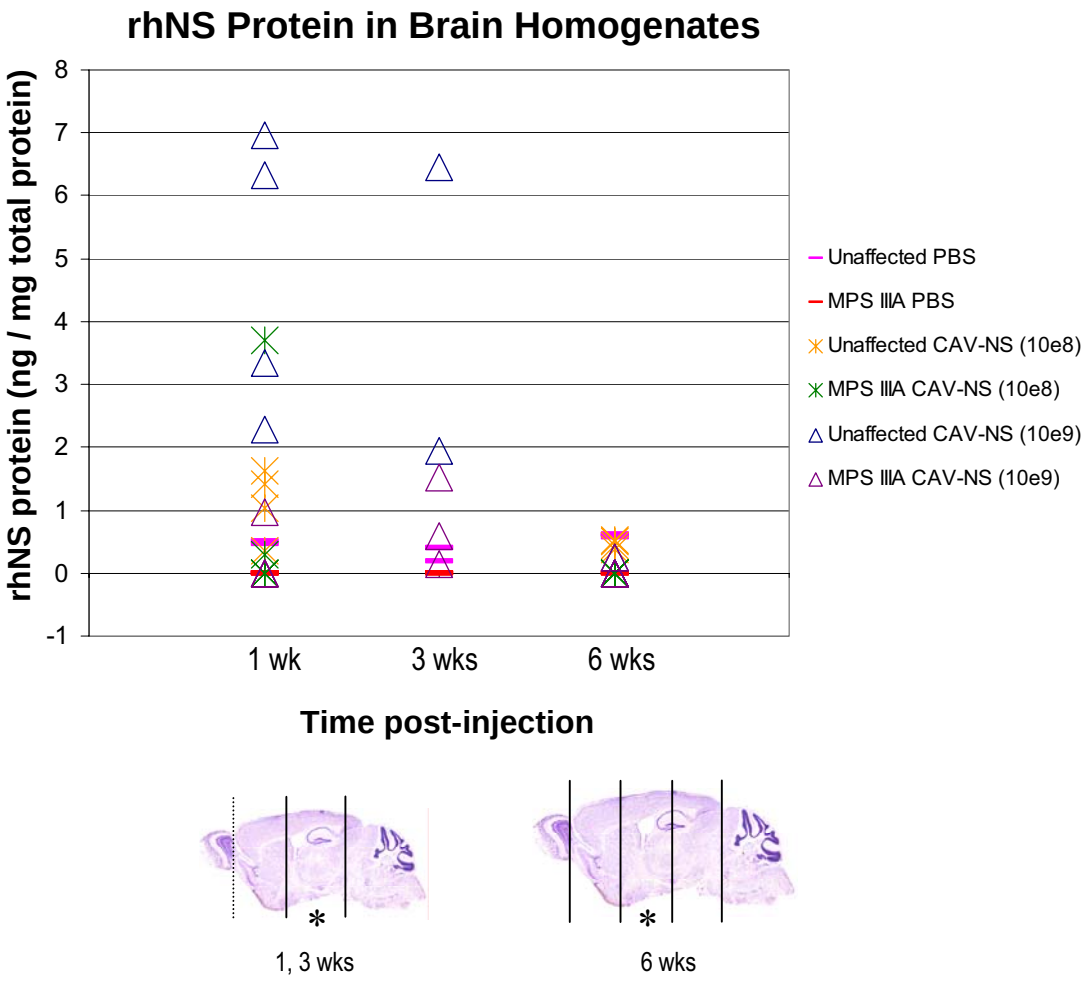


Figure 7.9: Quantitation of rhNS in brain homogenates. Brain tissues from mice injected with PBS or CAV-NS (10^8 or 10^9 particles/hemisphere) were divided along the mid-sagittal fissure and into coronal segments. The hemi-coronal slice containing the lateral ventricle injection (indicated with an asterisk) was homogenised, sonicated and the amount of rhNS protein (normalised to total protein content) was quantitated in a sandwich ELISA (DELFIA) assay. The group sizes were n=4, n=2-3, and n=4 for 1-, 3- and 6-wks post-injection, respectively. The 1- and 6-wk post-treatment data are the same as that presented in tabular format in previous sections (**Chapter 5**, **Table 5.3**).

activity was measured in brain homogenates containing the injection site at this time point (**Table 7.2**). NS activity was not detected in any brain sample taken from PBS-treated MPS IIIA mice. In the majority of unaffected mice receiving PBS, NS activity was not measured above the assay background, with only one mouse displaying recordable NS activity (for each of the brain regions). Thus, a correlation between NS activity (**Table 7.2**) and the amount of rhNS protein (**Fig. 7.9**) in the CAV-NS-treated brain homogenates was not detected.

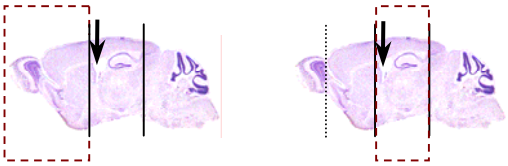
7.3.4.3. HNS-UA Oligosaccharides in Brain Homogenates

HNS-UA was measured using tandem mass spectrometry in brain homogenates from unaffected and MPS IIIA mice treated at birth with CAV-NS or saline as a control. Due to constraints in the number of samples that could be processed in each batch, the samples were analysed in three separate groups (1-wk and 6-wks; 3-wks and 20-wks; and 10-wks), which had the potential to lead to batch differences, thus complicating the comparisons between each batch group. The intra-batch coefficient of variation was 7.9% (n=10; 1-wk and 6-wks), 9.5% (n=10; 3-wks and 20-wks) and 9.2% (n=8; 10-wks) and the inter-batch coefficient of variation was 26.2% (n=10 experiments) for an untreated MPS IIIA brain quality control sample at an equivalent protein concentration. HNS-UA was not measured above the assay background in unaffected animals at any time-point, regardless of the treatment administered.

In the portion of the brain containing the lateral ventricle injection site, PBS-treated MPS IIIA mice showed a steady accumulation of HNS-UA with disease progression up to 6-wks of age, after which the HNS-UA concentrations reached a plateau (**Fig. 7.10**). A similar distribution over the 20-wks was observed in the olfactory bulb samples of PBS-treated MPS IIIA mice, with the exception of the 10-wk time-point where a slight depression in HNS-UA was noted in comparison to the adjacent 6- and 20-wk time points.

CAV-NS treatment at birth had variable effects on HNS-UA storage in MPS IIIA mice depending on the brain region. Some, but not all, MPS IIIA mice treated with CAV-NS exhibited a moderate reduction in HNS-UA storage in the olfactory bulb with up to 30%, 44% and 65% reductions in HNS-UA concentration in individual mice at 6-, 10- and 20-wks, respectively, compared to the average concentration found in age-matched, PBS-treated MPS IIIA mice (**Fig. 7.10**). On average, HNS-UA disaccharide storage in the olfactory bulb samples of treated MPS IIIA mice was reduced by CAV-NS administration, with 12%, 18%, 18% and 8% reductions at 1-, 3-, 10- and 20-wks post-injection, respectively. A 7% increase was measured in CAV-NS-treated MPS IIIA mice at 6-wks post-injection, compared to PBS-treated MPS IIIA animals.

Table 7.2: NS activity in brain homogenates in 1-wk-old mice. Newborn mice were bilaterally-injected with PBS or with 10^8 or 10^9 particles/hemisphere CAV-NS into the lateral ventricles. After 1-wk, mice were sacrificed and the brain sectioned along the midline and then into three coronal slices, as depicted in the schematic diagram below. Homogenates from the hemi-coronal slice containing the olfactory bulb (slice 1) and the injection site (slice 2) were assessed for NS activity at 60°C for 16 hrs. The injection site is indicated with an arrow. The NS activity in brain samples from individual mice per group is displayed in the same order as presented in **Table 5.3** (i.e. in descending rhNS protein concentration). nd, not detected; NA, not available.

Genotype	Treatment	NS activity (pmol/min/mg total protein) 	
		Slice 1	**Slice 2**
Unaffected	PBS	NA, nd, 0.54, nd (0.37 ± 0.23 ng)	0.59, nd, nd, nd (0.15 ± 0.15 ng)
Unaffected	CAV-NS (10^8 particles)	nd, nd, 0.22, nd (0.06 ± 0.06 ng)	1.2, 0.08, NA, 0.41 (0.61 ± 0.25 ng)
Unaffected	CAV-NS (10^9 particles)	0.25, 0.15, 0.98, nd (0.35 ± 0.22 ng)	0.81, 1.07, nd, 0.58 (0.62 ± 0.23 ng)
MPS IIIA	PBS	nd, nd, nd, nd (0 ± 0 ng)	nd, nd, nd, NA (0 ± 0 ng)
MPS IIIA	CAV-NS (10^8 particles)	nd, nd, nd, nd (0 ± 0 ng)	0.03, 0.07, nd, nd (0.01 ± 0.01 ng)
MPS IIIA	CAV-NS (10^9 particles)	0.18, NA, nd, nd (0.06 ± 0.04 ng)	nd, nd, nd, nd (0 ± 0 ng)

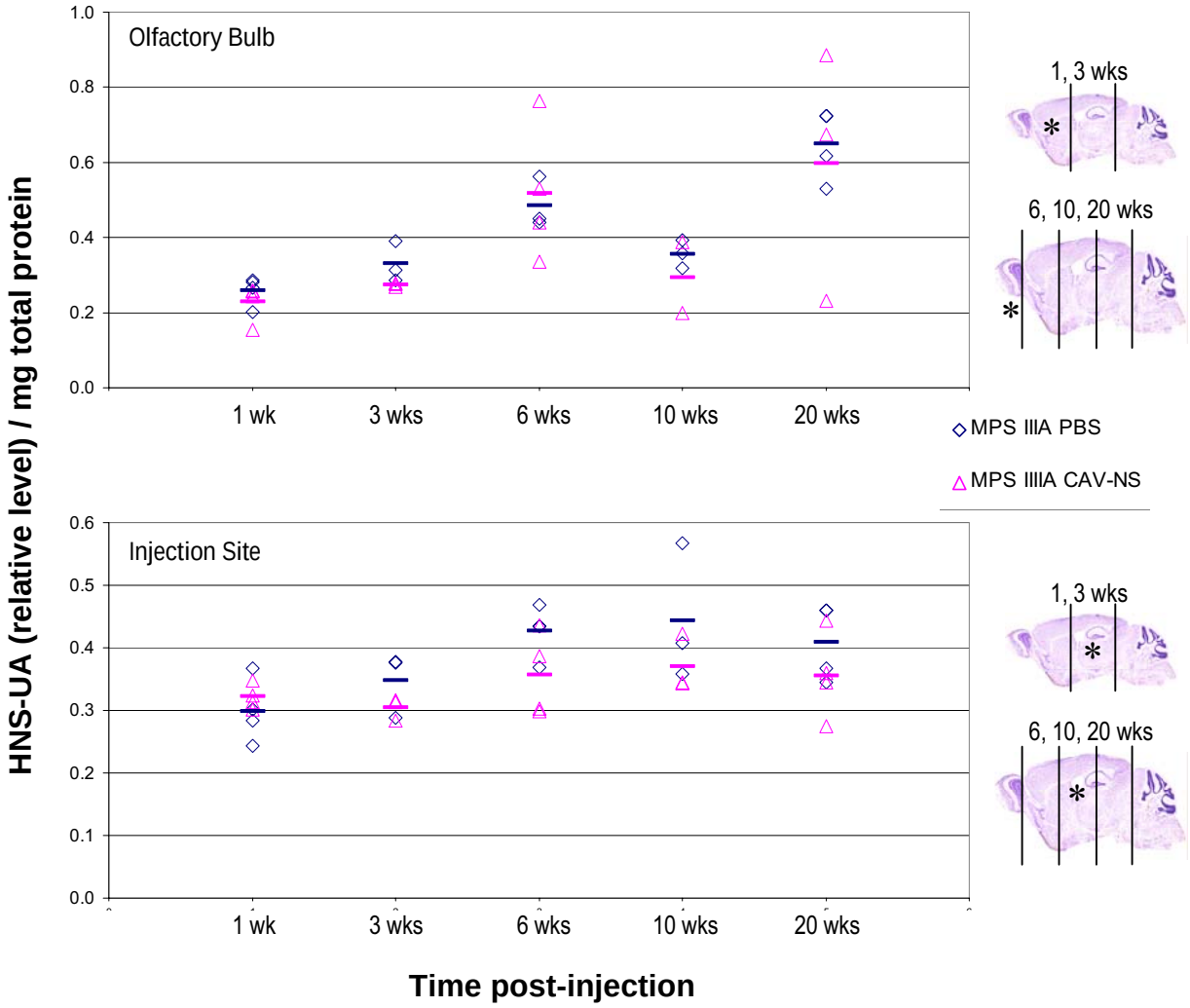


Figure 7.10: HNS-UA in brain homogenates. Brain tissues from mice injected with PBS or 10^9 particles/hemisphere of CAV-NS were divided into hemi-coronal portions and the olfactory bulb and the region containing the lateral ventricle injection site (marked with an asterisk) were homogenised, sonicated and derivatised with PMP. The relative level of a HS-derived disaccharide marker (HNS-UA) were determined by electrospray-ionisation tandem mass spectrometry and normalised to total protein content. The results were processed in three separate batches (1-wk and 6-wks; 3-wks and 20-wks; and 10-wks). HNS-UA in unaffected mice injected with PBS or CAV-NS could not be detected above the assay background (not displayed). Each data point represents an individual mouse and the bars are the mean of each group.

A CAV-NS treatment effect was less evident in the brain portion containing the injection site. With the exception of the mice assessed at 1-wk post-injection, CAV-NS-treated MPS IIIA mice had 12-17% less HNS-UA compared to PBS-injected MPS IIIA mice. However, no statistical differences were measured between CAV-NS- and PBS-treated MPS IIIA animals at any time-point in either brain region (unpaired t-test p=0.12-0.78).

7.3.5. Histology

7.3.5.1. GFP Transgene

Brain tissues (n≥4 sagittal sections/mouse) from random mice from each treatment and genotype group were assessed histologically for GFP expression via immunostaining at 3-, 10- or 20-wks post-injection (**Fig. 7.11** and **Table 7.3**). At 3-wks after delivery of $1x10^9$ particles/μL of CAV-NS, GFP was detected in all brain tissue sections that were assessed (1 unaffected, 2 MPS IIIA mice). GFP-positive cells were observed in the ependymal layer of the lateral ventricle (both endogenous and immunostained GFP expression) and the adjacent choroid plexus in all mice treated with CAV-NS. GFP expression was also detected in the choroid plexus of the fourth ventricle, hippocampus, corpus callosum, olfactory bulb and the pontine nucleus.

At 10-wks post-injection, no endogenous GFP expression was detected in CAV-NS-treated mice. However, GFP was immunodetected in the lateral ventricle wall in all mice injected with CAV-NS that were assessed (1 unaffected, 2 MPS IIIA mice) and was occasionally observed in the choroid plexus adjacent to the lateral ventricle injection site.

Twenty-wks post-injection was the longest time-point assessed. Immunopositive GFP-expressing cells could still be detected in the ependymal layer of the lateral ventricle wall of all unaffected and MPS IIIA mice (n=2 per genotype) 20-wks after the delivery of 10^9 particles/hemisphere of CAV-NS.

As expected, GFP expression was not observed in any of the uninjected or PBS-treated unaffected or MPS IIIA mice at 3-wks (n=4 in total), 10-wks (n=4 in total) or 20-wks post-injection (n=8 PBS-treated; n=8 uninjected).

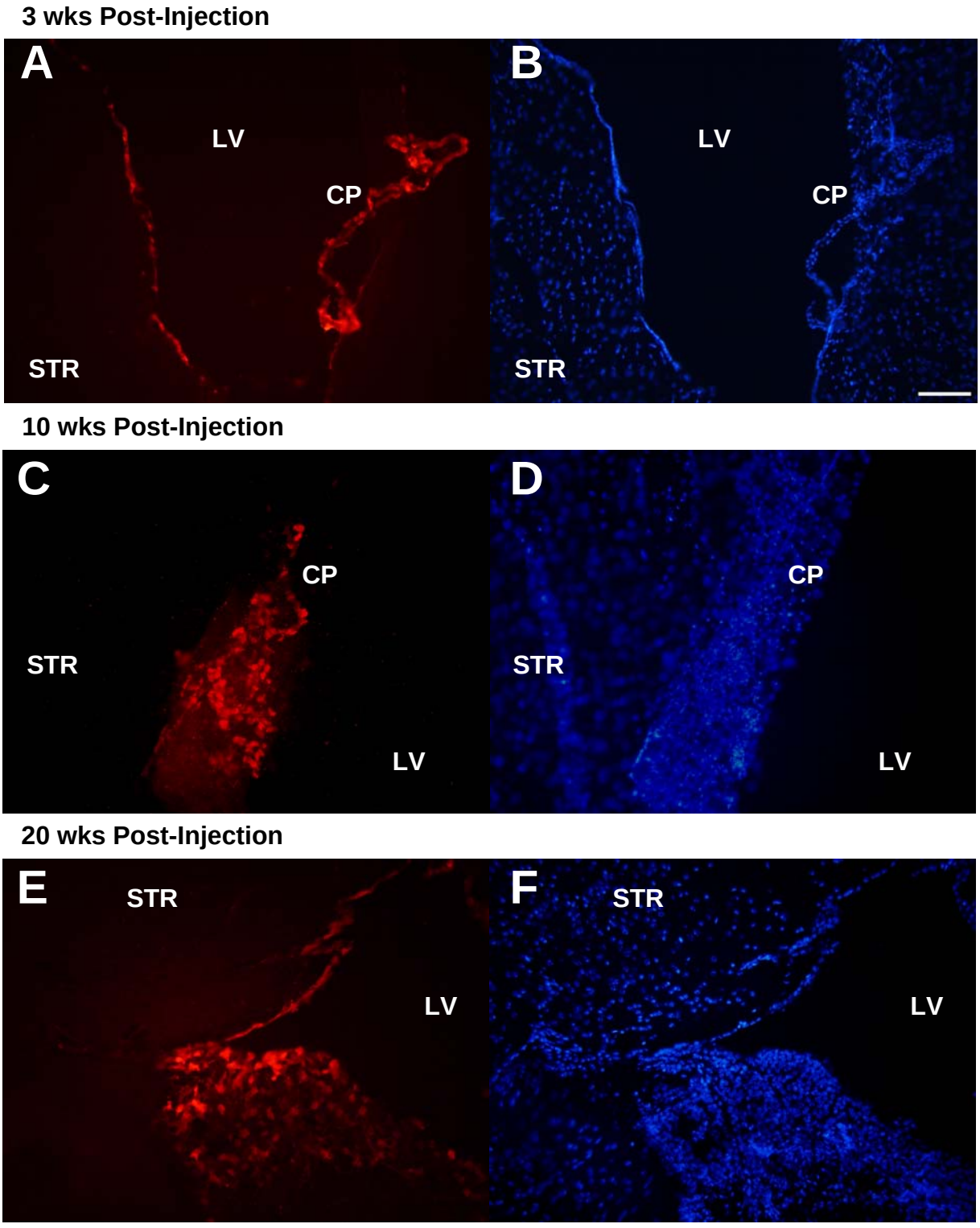
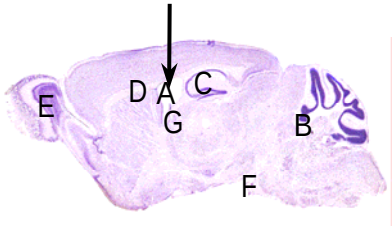


Figure 7.11: GFP Expression in the Lateral Ventricle. Sagittal, frozen sections were immunostained with a polyclonal anti-GFP antibody and GFP-positive cells (red; **A, C, E**) and the same field of view of DAPI-stained cell nuclei (blue; **B, D, F**) are shown from representative CAV-NS-treated mice at 3 wks **(A, B)**, 10 wks **(C, D)** and 20 wks **(E, F)** post-injection. Scale bar is 100 μm. LV, lateral ventricle; CP, choroid plexus; STR, striatum.

Table 7.3: GFP biodistribution in newborn mice injected with CAV-NS. Mice were bilaterally-injected on the day of birth with 1 x 10^9 CAV-NS particles/hemisphere into the lateral ventricles (indicated with an arrow in schematic diagram). At 3-, 10- or 20-wks post-injection, the mice were euthanased before the brain tissues were fixed with 4% (w/v) PFA, frozen in OCT compound and GFP-positive cells detected via immunofluorescent staining. The brain regions displaying GFP immunoreactivity from CAV-NS-injected mice of both genotypes at each time-point (≥4 sections; n=3-4 mice) are listed below. The regions marked in bold indicate that GFP could also be visualised without immunostaining.

Time post-injection		
3-wks (n=3 mice)	**10-wks** (n=3 mice)	**20-wks** (n=4 mice)
lateral ventricle (n=3; A)	lateral ventricle (n=3; A)	lateral ventricle (n=4; A)
choroid plexus (n=3; A, B)	choroid plexus (n=2; A)	
hippocampus (n=1; C)	3rd ventricle (n=1; G)	
corpus callosum (n=1; D)		
olfactory bulb (n=1; E)		
pontine nucleus (n=1; F)		

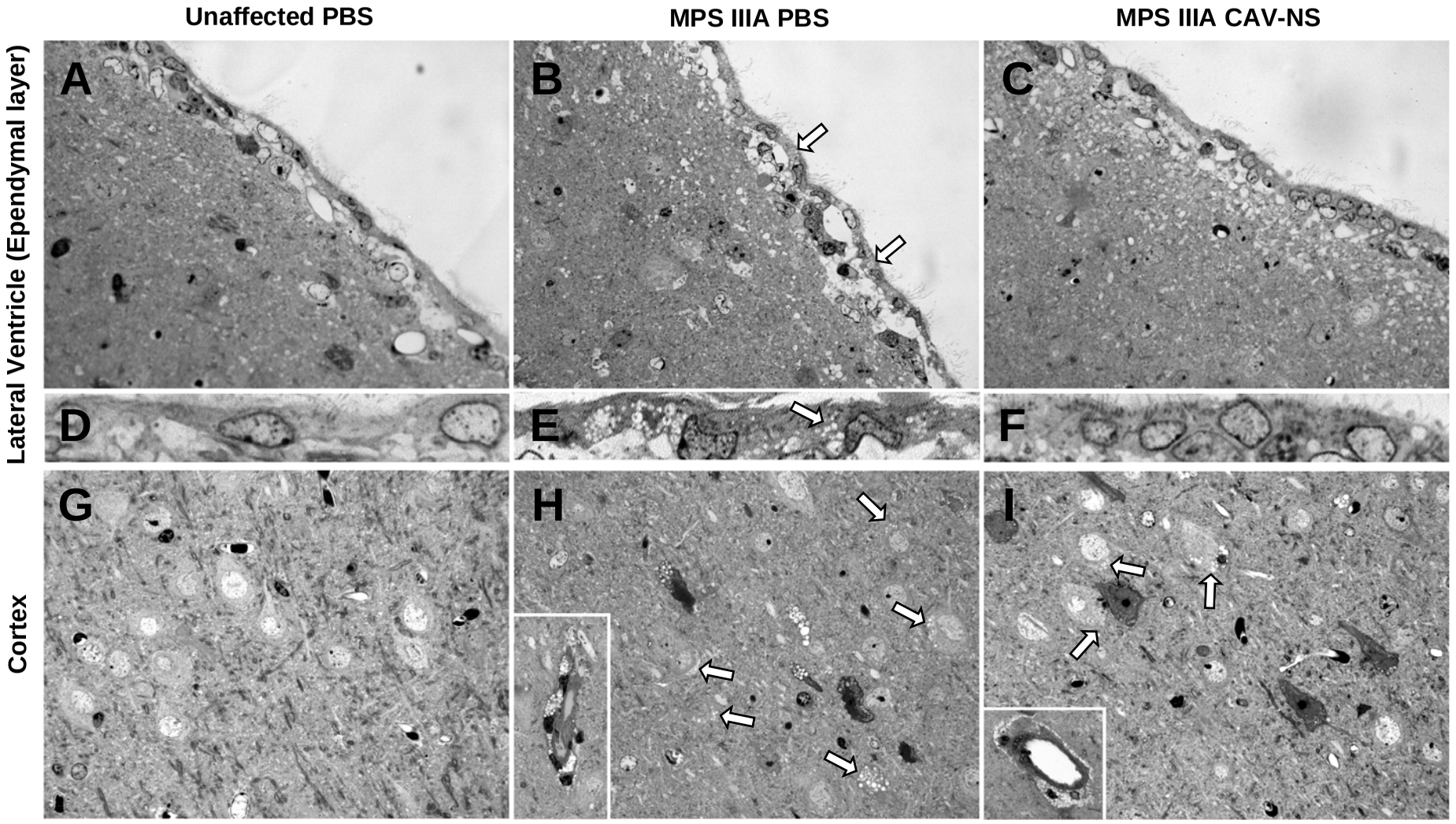


7.3.5.2. Toluidine Blue-Stained Epoxy Resin-Embedded Sections

To examine whether neonatal CAV-NS administration mediated visible reductions in the amount of lysosomal storage present in 20-wk-old MPS IIIA mice, 1 μm semi-thin brain sections were stained with toluidine blue and assessed (blinded) by light microscopy under oil immersion (**Section 2.2.6.3**). Although it was expected that most of the brain would display lysosomal inclusions, only the olfactory bulb and the ependymal and choroidal cells of the lateral ventricle were examined due to the high expression of transgene in these brain regions.

Both ependymal and choroidal cells of the lateral ventricle were filled with numerous small clear inclusions in uninjected and PBS-treated MPS IIIA mice (**Fig. 7.12B, E**) and could be easily distinguished from unaffected mice (**Fig. 7.12A, D**). Neonatal CAV-NS treatment dramatically reduced the frequency of storage vacuoles in ependymal and choroidal cells in MPS IIIA mice (**Fig. 7.12C, F**), with one CAV-NS-treated MPS IIIA animal displaying no discernable inclusions (similar to the appearance of ependymal/choroidal cells in wild-type animals), and a second animal exhibiting only occasional inclusions in these cell types at the light microscopy level.

Figure 7.12: Toluidine blue-stained semi-thin brain sections. EM-fixed brains from 20-wk-old mice were embedded in epoxy resin and 1 μm thick sections were stained with toluidine blue. Representative images from **(A, D, G)** PBS-treated unaffected, **(B, E, H)** PBS-treated MPS IIIA and **(C, F, I)** CAV-NS-treated MPS IIIA mice are shown. Lysosomal storage inclusions (see arrows) were observed in both the **(A-F)** ependymal layer of the lateral ventricle and the **(G-I)** cerebral cortex. Perivascular cells in the cortex were frequently observed with numerous inclusions (inset, **H**, **I**). Scale bar is 300 μm **(A-C, G-I)** and 100 μm **(D-F)**.


Unaffected PBS
MPS IIIA PBS
MPS IIIA CAV-NS
Lateral Ventricle (Ependymal layer)
Cortex
A
B
C
D
E
F
G
H
I

Similar observations were made in the cortex situated directly above the lateral ventricle. Microglia and oligodendrocytes could not be distinguished at 100x magnification and are subsequently referred to collectively as "glia". Uninjected and PBS-treated MPS IIIA mice displayed numerous inclusions in the neurons of all cortical layers (**Fig. 7.12H**), in some instances exceeding more than 25 inclusions per cell. Perivascular and free glia were also laden with storage, filling the entire cytoplasm in the majority of inclusion-containing cells assessed (**Fig. 7.12H inset**). Compared to cortical neurons, glial cells contained fewer vacuoles per cell but the inclusions were generally larger than those observed in neurons. Lysosomal inclusions were not observed in unaffected mice, regardless of the treatment administered (**Fig. 7.12G**).

In CAV-NS-treated MPS IIIA mice, it appeared that fewer cortical neurons contained inclusions and, of those with storage, the inclusions were less frequent and smaller in size than in similar cell populations in MPS IIIA mice either injected with PBS or not injected (**Fig. 7.12I**). As the treatment effect in cortical cells was not as definitive as that observed in the ependymal and choroidal cells of the lateral ventricle, the percentage of neuronal and glial cells with lysosomal storage inclusions was determined in cells near the cortical surface (layers II-III, further from the injection site) or in deeper layers (layers V-VI, closer to the injection site) to establish whether CAV-NS treatment had ameliorated lysosomal storage (as described in **Section 2.2.6.3**).

In total, between 71-156 cortical neurons and 19-34 glial cells were assessed per cortical region from each mouse. As shown in **Fig. 7.13**, CAV-NS treatment reduced the percentage of neurons and glia containing inclusions at both the cortical surface and in deeper layers of MPS IIIA mice compared to PBS-injected MPS IIIA animals. At the cortical surface, inclusions were seen in 62.9 ± 6.4% and 73.7 ± 2.3% of neurons and glia, respectively, in PBS-treated MPS IIIA animals. Delivery of CAV-NS at birth to MPS IIIA mice reduced the percentage of cells with storage vacuoles for both cell populations (neurons 41.5 ± 7.8%, $p=0.17$; glia 60.1 ± 1.8%, $p=0.04$, compared to PBS-treated MPS IIIA mice).

Likewise, the deeper cortical layers of PBS-treated MPS IIIA mice exhibited neurons with inclusions (49.7 ± 9.5%) and, on treatment with CAV-NS, the percentage of vacuolated neurons was reduced to 21.9 ± 13.3% ($p=0.23$; **Fig. 7.13**). A similar trend was observed for glial cells from deeper layers of the cortex with 73.3 ± 8.6% and 56.5 ± 14.4% of glial cells containing inclusions for PBS- and CAV-NS-treated cells, respectively ($p=0.42$).

There were no differences in the percentage of dark neurons in the PBS- and CAV-NS-treated MPS IIIA mice at the cortical surface (12.4 ± 4.6% versus 10.6 ±7.8% for PBS-

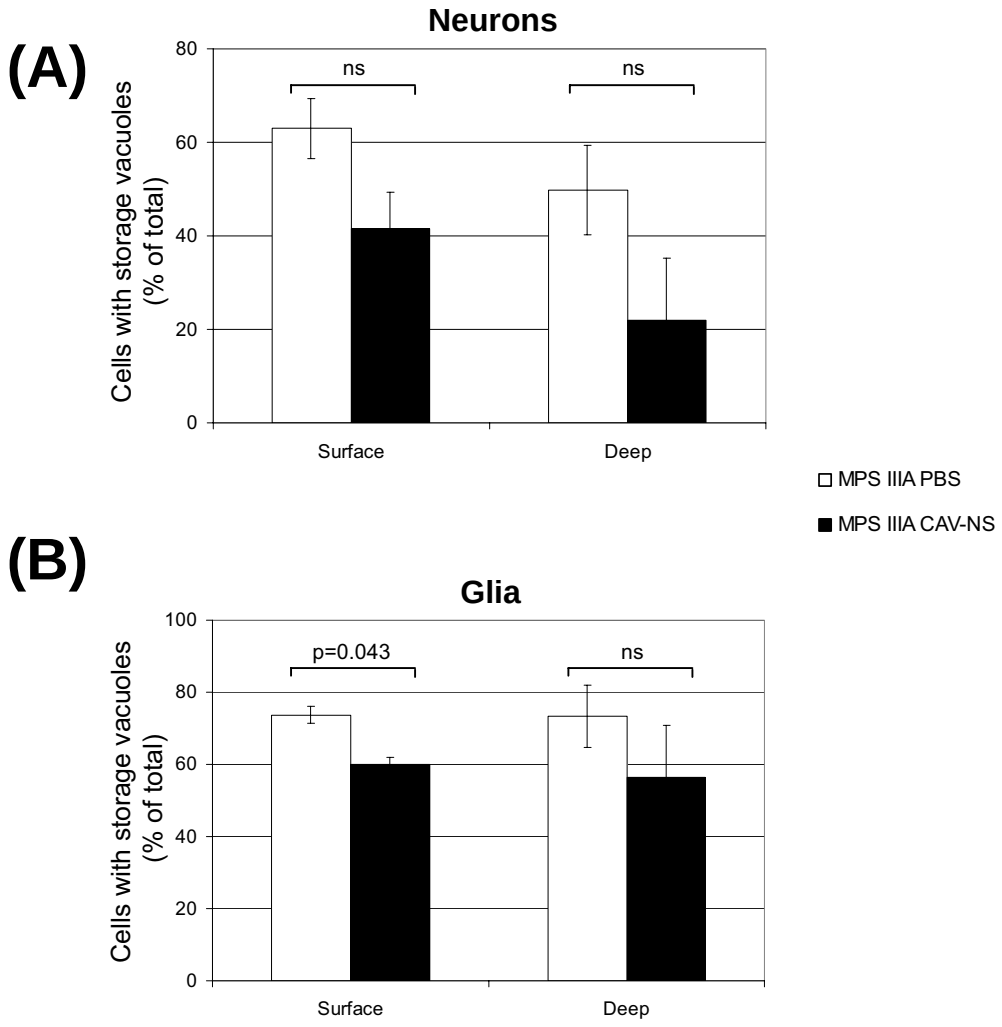


Figure 7.13: Lysosomal storage in the cerebral cortex. Toluidine blue-stained brain sections were assessed for the presence or absence of lysosomal inclusions in **(A)** neuronal or **(B)** glial cell populations at layers II-III (surface) or layers V-VI (deep) of the cerebral cortex in PBS- or CAV-NS-treated MPS IIIA mice. Error bars are ± 1 SEM. Statistically significant unpaired t-test p-values are indicated. ns, not significant.

and CAV-NS-injected mice, respectively; p=0.88) or in deeper cortical layers (15.9 ± 11.8% versus 5.5 ± 2.3 for PBS- and CAV-NS-injected mice, respectively; p=0.47).

7.3.5.3. GFAP

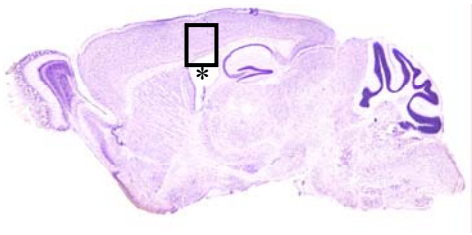
To determine the effect of CAV-NS treatment on the astrogliosis associated with MPS IIIA disease pathology, paraffin-embedded brain sections from uninjected, PBS-treated and CAV-NS-treated mice were immunostained with a polyclonal GFAP antibody to detect glia. The olfactory bulb and the cells lining the lateral ventricle injection site were the only regions examined. GFAP-reactive cells were observed in the glomerular layer (outermost layer) of the olfactory bulb, with GFAP-positive cells displaying a diffuse distribution in this region. Minimal immunoreactivity was observed in the (innermost) granular cell layer of the olfactory bulb of unaffected and MPS IIIA mice. No obvious difference in GFAP immunoreactivity in the olfactory bulb was observed between the genotype and treatment groups.

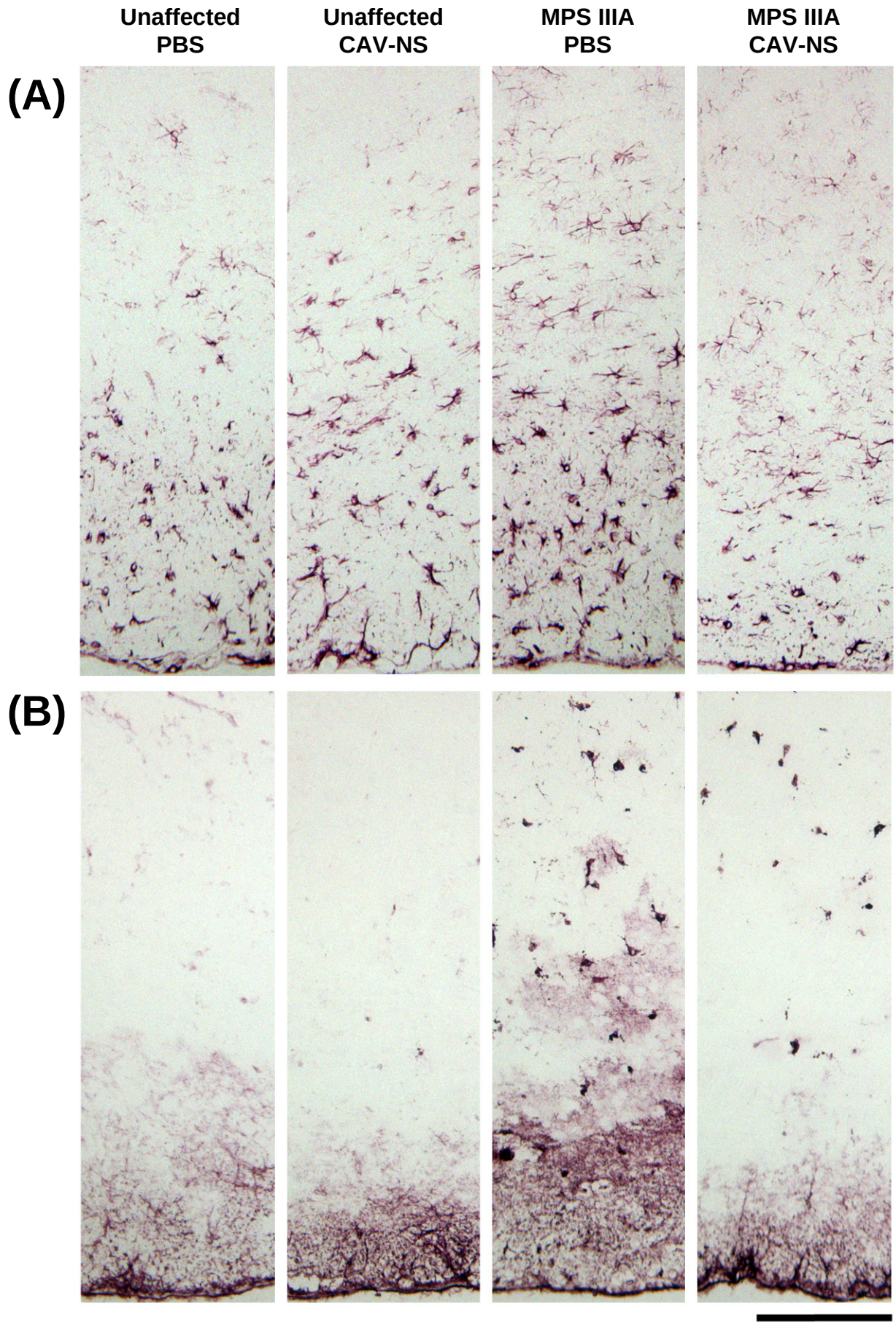
In contrast, in the cerebral cortex of uninjected MPS IIIA mice, GFAP-positive cells displayed a diffuse distribution with greater GFAP expression compared to their unaffected counterparts (**Fig. 7.14A**). This was particularly evident in layers II-IV of the cortex. Within the deeper layers of the cortex (layers V-VI), the immunopositive glia from MPS IIIA mice appeared darker than those from unaffected mice. There were no visible differences in staining or GFAP-positive cell distribution between uninjected and PBS-treated mice of the same genotype.

CAV-NS treatment at birth appeared to reduce the intensity of staining in GFAP-expressing glia in the cortex adjacent to the lateral ventricle of MPS IIIA mice compared to the expression in PBS-injected and uninjected MPS IIIA mice (**Fig. 7.14A**). In an attempt to verify these observations in a quantitative manner, unbiased comparisons of the percentage of GFAP-immunostained areas were made for each of the treatment groups using Metamorph imaging software whilst blinded to the genotype and treatment of each tissue during image capture and data processing.

Similar to the qualitative findings, GFAP expression was elevated in the cerebral cortex of uninjected MPS IIIA mice in comparison to their wild-type counterparts (MPS IIIA 4.27 ± 0.23%; unaffected 1.99 ± 0.48%; **Fig. 7.15A**). A similar trend was observed in the cortex of PBS-treated animals (MPS IIIA 4.69 ± 0.49%; unaffected 2.63 ± 0.48%). Interestingly, an increase in the percentage of GFAP-expressing area (4.11 ± 0.86%) was measured in the cortex of 20-wk-old unaffected mice treated with CAV-NS at birth, compared

Figure 7.14: Immunohistochemical detection of inflammatory markers. Unaffected or MPS IIIA mice treated at birth with CAV-NS or PBS vehicle control were sacrificed at 20-wks post-treatment. Immunohistochemical detection of astrocytes (GFAP, **A**) and activated microglia (Isolectin B_4, **B**) was conducted on paraffin-embedded brain tissues (see panel in schematic diagram; injection site indicated with an asterisk) and sections were imaged on a light microscope. The cortex (top of each panel) and the ependymal wall of the lateral ventricle (bottom of each panel) are depicted. Scale bar is 100 μm.




Unaffected PBS
Unaffected CAV-NS
MPS IIIA PBS
MPS IIIA CAV-NS
(A)
(B)

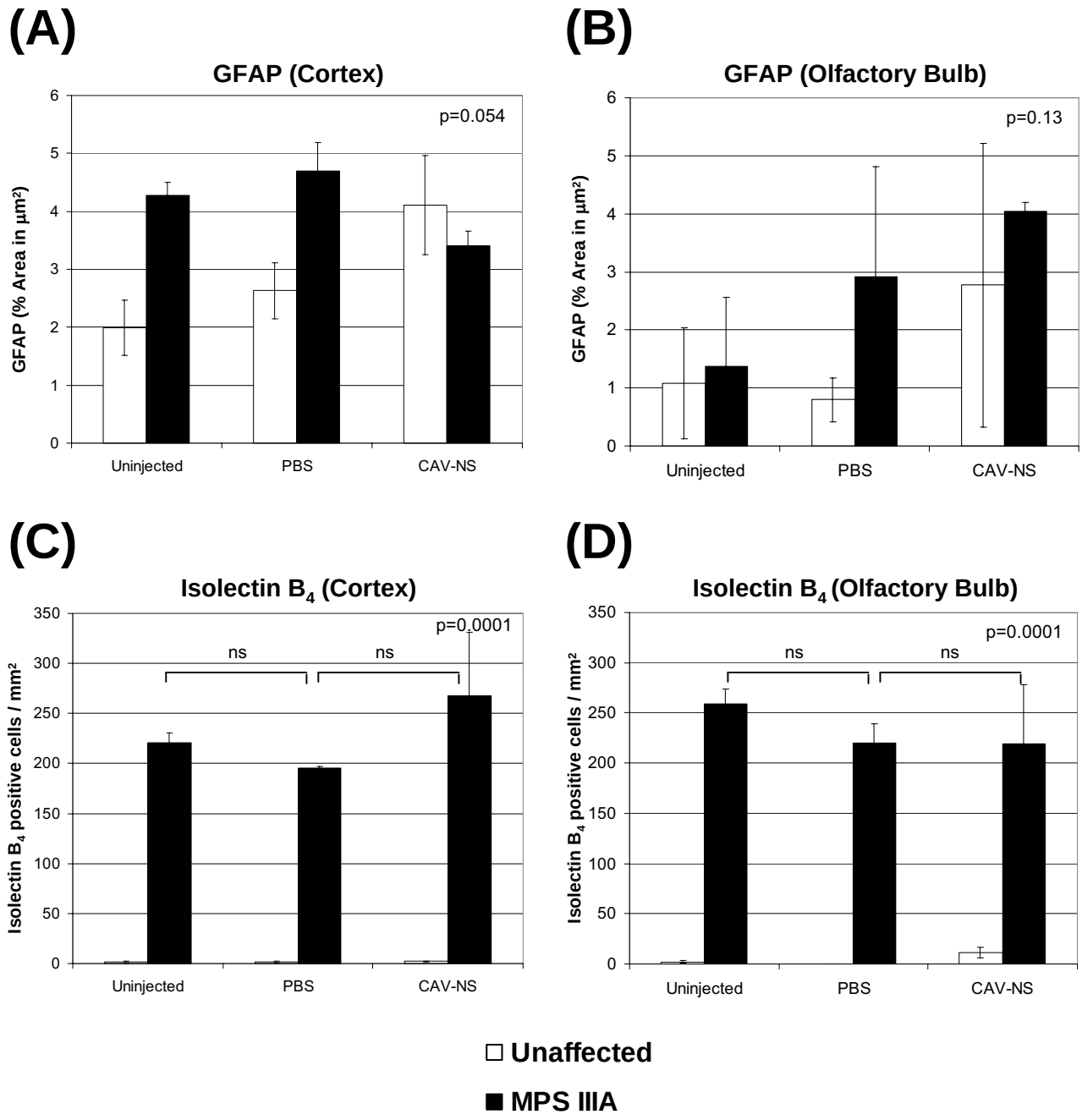


Figure 7.15: Quantitation of GFAP and isolectin B_4 immunostaining. (A, B) Astrocytes (GFAP-positive) and **(C, D)** activated microglia (isolectin B_4-positive) were immunohistochemically detected in paraffin-embedded brain sections from uninjected, PBS-treated or CAV-NS-treated mice sacrificed at 20-wks post-injection (n=2 mice/group; n=2 sections/mouse). The percentage of GFAP-expressing area or the number of isolectin B_4-positive cells per area was determined in the **(A, C)** cerebral cortex and **(B, D)** olfactory bulb using Metamorph imaging software. Error bars are ± 1 SEM and the ANOVA p-value for each area/immunostain is displayed. Selected post hoc comparisons are also indicated. ns, not significant.

to unaffected mice treated with PBS (2.63 ± 0.48%). The corresponding CAV-NS-treated MPS IIIA mice displayed less GFAP immunoreactivity (3.41 ± 0.25%) than both the PBS-treated MPS IIIA mice and the unaffected CAV-NS-treated mice. The differences in the percentage of GFAP expression were marginally statistically significant (ANOVA p=0.054).

The percentage of GFAP expression in the olfactory bulb was also assessed due to the high transgene expression observed in this structure at early time-points after treatment. No differences were detected between uninjected unaffected and MPS IIIA mice (unaffected 1.08 ± 0.95; MPS IIIA 1.38 ± 1.18%), thus complicating the interpretation of whether CAV-NS treatment had a therapeutic effect (**Fig. 7.15B**). However, in a trend similar to that observed in the cortex, GFAP expression in the olfactory bulb was elevated in PBS-treated MPS IIIA mice compared to PBS-treated unaffected mice (MPS IIIA 2.92 ± 1.89%; unaffected 0.80 ± 0.38%) and CAV-NS delivery at birth increased the percentage of GFAP staining in unaffected mice (2.77 ± 2.44%) compared to PBS-treated unaffected animals. In contrast to the pattern observed within the cortex, GFAP expression was not reduced in CAV-NS-treated MPS IIIA mice (4.04 ± 0.15%) compared to PBS-injected MPS IIIA mice (ANOVA p=0.13).

7.3.5.4. Isolectin B_4

The number of activated microglia (isolectin B_4-positive) was determined in the same subset of mice described in **Section 7.3.5.3.** Very few activated microglia were observed in the cortex or olfactory bulb of unaffected mice regardless of the treatment administered, with only occasional isolectin B_4-positive cells detected in the entire field of view (**Fig. 7.14**). In contrast, darkly stained, activated microglial cells were readily observed throughout the cortex and the olfactory bulb of MPS IIIA mice. The isolectin B_4-expressing cells were evenly distributed throughout both of these brain regions, with the cell morphology displaying dark cell bodies with short processes. Other areas of the brain were expected to also contain activated microglia but were not examined. There were no discernable differences between staining patterns in untreated MPS IIIA animals and those receiving PBS or CAV-NS at birth.

Activated microglial cells were quantitated in the cortex and the olfactory bulb (n=2 mice/treatment and genotype group; n=2 sections/mouse). A range of 1.2 ± 1.2 – 2.2 ± 0.5 activated microglial cells/mm^2 was measured in the cortex of unaffected mice (**Fig. 7.15C**). A 177-fold increase in cortical staining was observed in uninjected MPS IIIA mice (ANOVA p=0.0001). Similar patterns were observed in the cortices of PBS-treated and CAV-NS-treated MPS IIIA mice, with 116- and 120-fold increases in the number of activated microglia/mm^2, respectively. CAV-NS treatment at birth did not alter the density of cortical

activated microglia in MPS IIIA mice (PBS 195 ± 2.1; CAV-NS 267.6 ± 63.6; p>0.05 post-tests).

Likewise, very few activated microglia were observed in the granular cell layer of the olfactory bulb in unaffected mice (range 2.1 ± 1.2 to 11.1 ± 5.3; **Fig. 7.15D**). Uninjected MPS IIIA mice exhibited a 126-fold increase in isolectin B_4-expressing cells compared to uninjected unaffected mice (ANOVA p=0.0001). Administration of PBS or CAV-NS at birth to MPS IIIA mice did not have a significant effect on the number of activated microglia/mm^2 (220 ± 18.9 or 219.2 ± 59.1, respectively; p>0.05 post-tests).

7.3.6. *In Vitro* Cytotoxic Assessment of CAV-NS

7.3.6.1. NS Activity Time Course

Widespread and high-level GFP transgene expression was not maintained *in vivo* after CAV-NS delivery to newborn mice. An *in vitro* experiment was therefore designed to determine whether similar transgene kinetics were observed with the NS transgene using primary cultured neural cells from newborn mice (**Section 7.2.5**). These experiments could not be performed on samples collected from mice treated *in vivo* due to the undetectable NS activity in CAV-NS-injected brain homogenates. In dialysed conditioned media samples, NS activity was not detected above the assay background in any of the untreated wells (**Fig. 7.16**). Inoculation with CAV-NS at 1000 particles/cell resulted in high NS activity (375 ± 85 pmol/min/mg total protein) at 3-days after transduction. However, NS activity was reduced by 13% and 56% at 6- and 9-days post-inoculation, respectively, compared to the NS activity measured at 3-days post-transduction (ANOVA p=0.0001; post-test p=not significant at 6-days post-transduction; post-test p<0.05 (t=4.77) at 9-days).

A similar NS activity pattern emerged in the CAV-NS-treated cell extracts. Untransduced cell extracts had similar NS activity at all time-points (range of 37.1 ± 3.9 to 51.8 ± 11.5 pmol/min/mg total protein; **Fig. 7.16**). The highest NS activity was detected in CAV-NS-treated cells at 3-days post-transduction, with a 26-fold increase compared to untreated cells. However, whilst NS activity was elevated in comparison to untransduced cells (16-fold and 12.5-fold more NS activity in CAV-NS-treated cells compared to untransduced controls at 6- and 9-days post-transduction, respectively), NS activity was not maintained with time. A 38% and 52% reduction in NS activity was observed in treated cells at 6- and 9-days of culture compared to the activity measured after 3-days post-inoculation. However, this reduction in NS activity was not statistically significant.

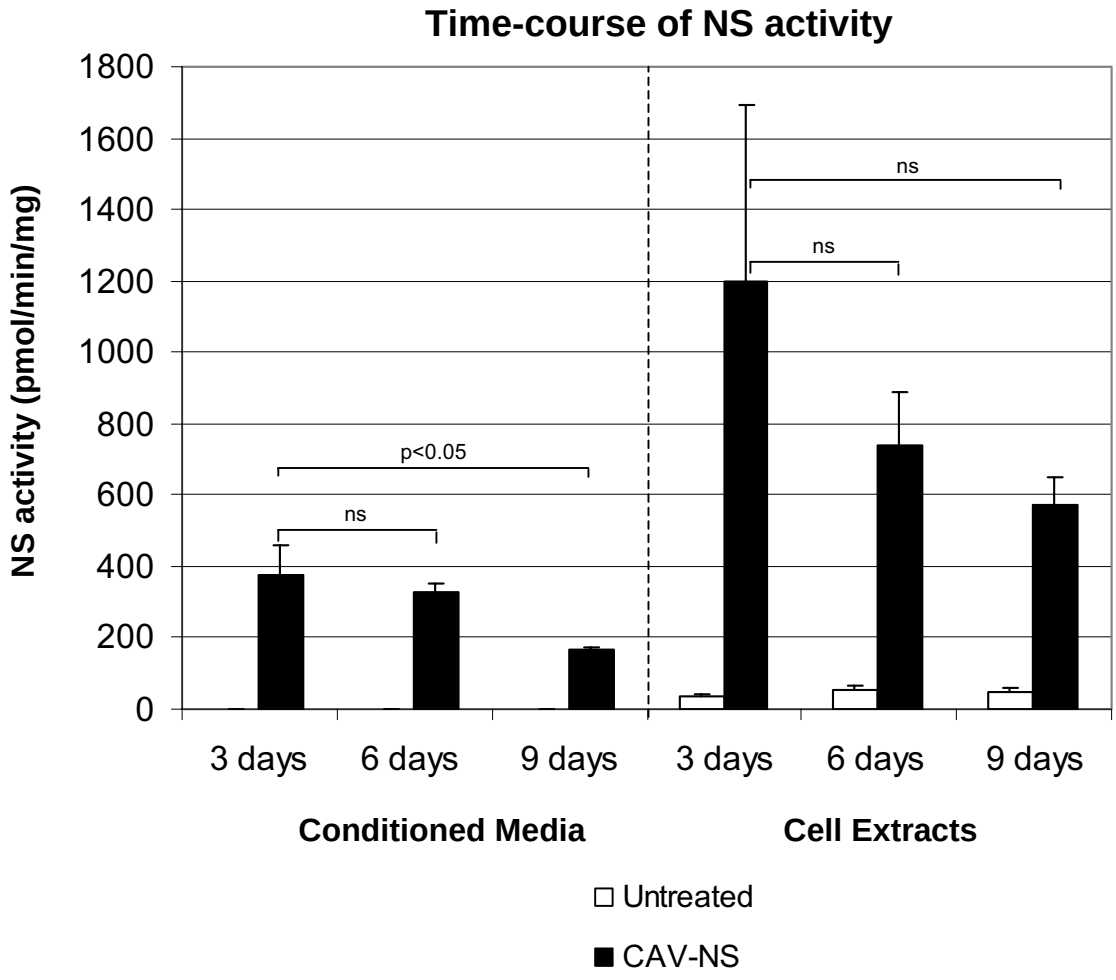


Figure 7.16: NS activity time course in primary neural cells. Primary neural cell cultures were harvested from unaffected, newborn mice and plated into 6-well trays. After 7 days, cells were mixed with CAV-NS (1000 particles/cell), or an equal volume of PBS as an untreated control, and cultured for 3-, 6- or 9-days post-transduction. For the latter 2 time-points, fresh media was applied every 3-days so that each media sample had 3-days of conditioning in total. At each time-point, conditioned media samples were collected, and cell extracts were harvested in 20 mM Tris/0.5 M NaCl (pH 7.0) and subjected to freezing/thawing to liberate the intracellular contents. After overnight dialysis in 0.2 M sodium acetate (pH 5.0), samples were assessed for NS activity using a tritiated tetrasaccharide substrate at 60°C and the activity normalised to total protein content. Error bars = 1 SEM.

7.3.6.2. CytoTox-One Membrane Integrity Assay

In an attempt to determine whether CAV-2 transduction was cytotoxic to primary cultures of mixed neural cells, the cellular membrane integrity at 2- and 7-days post-transduction was measured using the intracellular marker lactate dehydrogenase (**Section 7.2.6**). Lactate dehydrogenase activity is detected in conditioned media when cellular membranes are disrupted. Two different doses (100 and 1000 particles/cell) of CAV-GFP or CAV-NS were assessed. These particular time points were chosen to coincide with the ages at which neonatal mice were sacrificed.

In comparison to the cytotoxicity in the positive control that had been deliberately lysed with a detergent provided in the kit by the manufacturer (adjusted to 100%; **Fig. 7.17**), minimal cytotoxicity was observed in the untransduced samples at 2-days post-transduction in neural cells derived from newborn (3.2 ± 0.2%) or adult animals (3.0 ± 0.5%). CAV-GFP or CAV-NS transduction had a minimal effect on cytotoxicity at 2-days after inoculation (ANOVA p=0.11 adult; p=0.50 newborn) with the highest cytotoxicity observed in newborn-derived cells treated with 1000 particles/cell of CAV-GFP (3.9 ± 0.3%).

At 7-days post-transduction, the samples displayed higher lactate dehydrogenase activity (indicating increased cytotoxicity) compared to 2-days after inoculation, with newborn- and adult-derived, untransduced, primary neural cells displaying 7.4 ± 0.2% and 8.5 ± 2.1% cytotoxicity, respectively (**Fig. 7.17**). There was little difference between CAV-NS-treated, CAV-GFP-treated or untransduced treatments in the percentage of cytotoxicity measured. For each of the treatments, slightly more lactate dehydrogenase activity was measured in the newborn-derived samples compared to the adult-derived cells. Similarly, a slight increase in cytotoxicity was measured at the higher dose (1000 particles/cell) of CAV-GFP or CAV-NS compared to cells treated at 100 particles/cell but this was not statistically significant (p=0.21 adult; p=0.45 newborn).

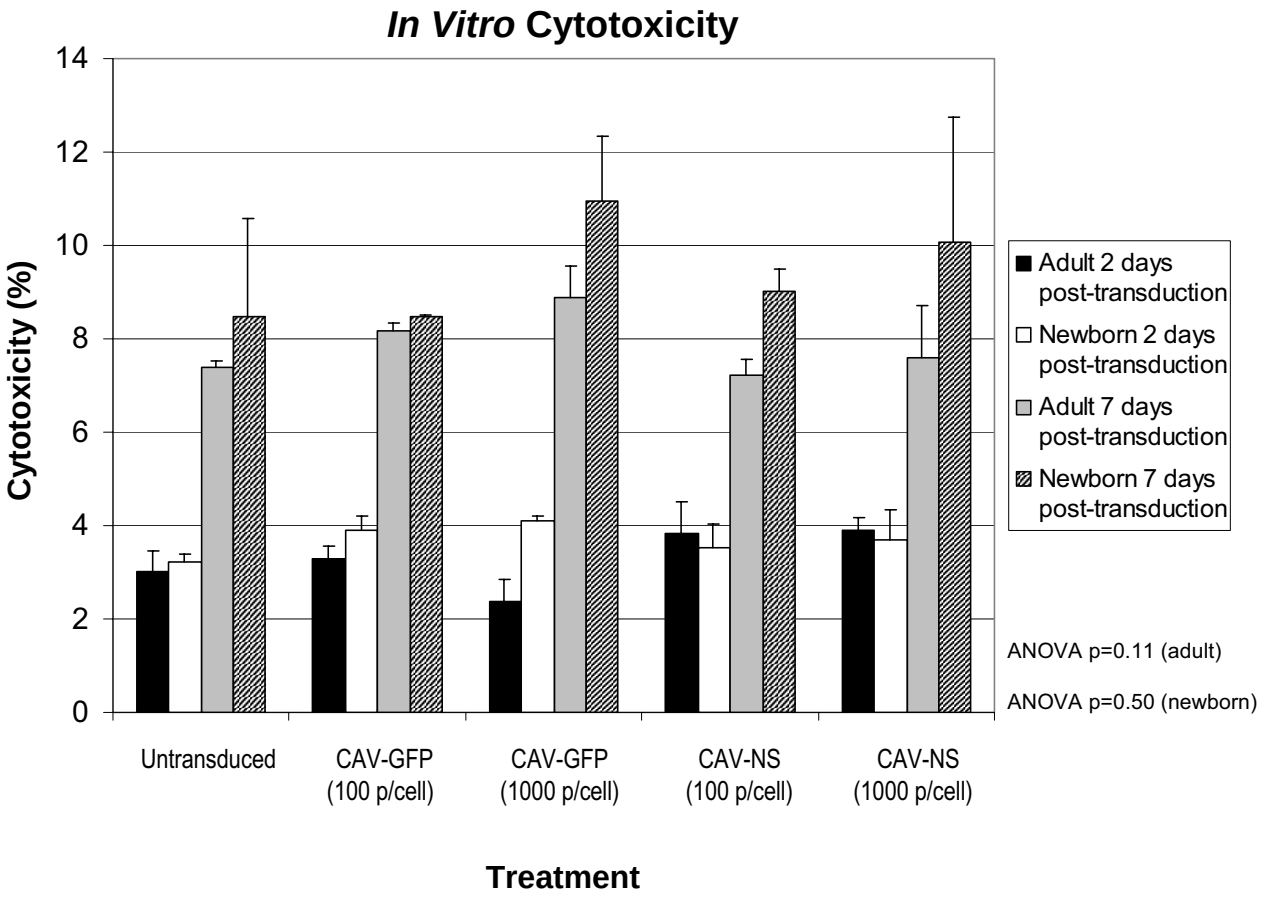


Figure 7.17: Cytotoxicity in transduced, primary neural cells. Primary neural cultures were derived from unaffected, newborn or adult mouse brains. After 7-days post-plating, cells were transduced with CAV-GFP or CAV-NS vectors at a dose of 100 or 1000 particles/cell or remained untransduced as a control. Two- or 7-days after transduction, the conditioned media was collected and the activity of the intracellular marker, lactate dehydrogenase, was determined as a measure of membrane integrity and cytotoxicity. Assay controls included cells that were deliberately lysed at harvest with a lysis buffer provided by the manufacturer, and media that had been cultured in the absence of cells (100% and 0% cytotoxicity, respectively). The results are the mean of 4 replicates + 1 SEM.

7.3.7. *In Vivo* Cytotoxic Assessment of CAV-NS via FISH

Method Development (Cell Smears and the XY18 Probe)

Fluorescence *in situ* hybridisation (FISH) was performed in CAV-treated human 293 cell smears to verify whether the signal generated by the probes was specific for the CAV-2 vector genome using fluorochrome-labelled pTG5412 (which could potentially detect the genome of any CAV-2 vector) and pCAV-NS (potentially with improved sensitivity due to the increased length of probe homology) DNA probes. The XY18 probe, specific for human chromosomes X, Y and 18, acted as a positive control.

Untreated and CAV-GFP-treated 293 cells were hybridised at 75°C for 10 min (**Sections 2.2.7.1** and **2.2.7.3**)**.** The pCAV-NS and pTG5412 DNA probes were comparable in signal localisation, intensity and in the degree of FISH-positive cells, with specific staining localised in discrete, punctate dots at the periphery of the cell nucleus. Whilst >95% of CAV-GFP-treated cells were GFP-positive, only a small fraction (~5%) of these cells displayed FISH-positive signal. The signal detection with the XY18 probe was much greater than that observed with the pTG5412 and pCAV-NS probes. No signal was detected in the untreated control hybridised with the pTG5412 or pCAV-NS probes.

The XY18 probe was also hybridised at 75°C to human skin biopsies to optimise the detection of DNA in paraffin sections for 5 or 15 min in the presence or absence of proteinase K digestion using a flat-bed PCR machine (**Section 2.2.7.3**). The most abundant and sensitive XY18 signal was detected with proteinase K pre-treatment for 15 min, indicating that DNA could be detected by FISH in paraffin-embedded tissues.

Genomic DNA Extraction and FISH Detection in Paraffin-Embedded Mouse Tissues

No detectable CAV-NS genome FISH signal was observed in paraffin-embedded mouse brain sections ($1x10^9$ CAV-NS particles/hemisphere, 1-wk post-injection) when hybridised at 75°C for 10 or 30 min with proteinase K pre-treatment. This tissue was selected as maximal GFP expression was observed in the contralateral, cryo-embedded brain hemisphere. Consequently, a method for extracting genomic DNA from PFA-fixed, paraffin-embedded tissue was developed to confirm that the CAV-2 genome was present in the tissue samples (**Section 2.2.1.1**). CAV-2 genome was only detected in brain tissues from mice treated at the highest dose ($1x10^9$ particles CAV-NS/hemisphere) at 2-days post-injection (**Table 7.4**).

Table 7.4: CAV-2 genome detection. The CAV-2 genome was detected in paraffin-embedded brain sections from mice injected with various doses of CAV-NS by: (a) PCR amplification of the CAV-2 genome or the murine NS as a DNA extraction control; or (b) localising the CAV-2 genome with a fluorochrome-labelled pCAV-NS probe via FISH. nd, not detected.

CAV-NS Dose (particles/hemisphere)		Time post-injection (n=2/group)		
		2-days	1-wk	2-wks
$5x10^6$	PCR	nd	nd	nd
	FISH	nd	nd	nd
$1x10^7$	PCR	Not assessed *(pups found dead)*	nd	nd
	FISH		nd	nd
$1x10^8$	PCR	nd	nd	nd
	FISH	nd	nd	nd
$1x10^9$	PCR	Present (n=2/2)	nd	nd
	FISH	Present	Present	nd
		Lateral ventricle (n=2/2)	Lateral ventricle (n=2/2)	
		Corpus callosum (n=2/2)	Corpus callosum (n=1/2)	
			Olfactory bulb (n=1/2)	

As a result, a biotinylated-CAV-NS probe was hybridised to CAV-2 genome-positive tissues (as detected by PCR amplification) and compared to the signal obtained with a fluorochrome-labelled CAV-NS probe to determine whether the signal could be amplified (**Section 2.2.7.4**). Two probe concentrations (3 or 6 μL/section) of biotinylated- or fluorochrome-labelled probe were hybridised for 30 min at 75°C or 78°C. FISH signal was detected in all samples, with discrete, punctate dots observed around the periphery of the nucleus (**Fig. 7.18**). Hybridisation at 78°C with 6 μL/section of probe produced the most sensitive signal detection, regardless of whether a biotinylated- or fluorochrome-labelled probe was used.

CAV-2 DNA was not detected in any of the mice treated with $5x10^6$, $1x10^7$ or $1x10^8$ particles of CAV-NS when assessed by the optimised FISH method (**Table 7.4**). In contrast, viral DNA was observed in both mice treated with $1x10^9$ CAV-NS particles/hemisphere at 2-days or 1-wk, but not at 2-wks post-delivery, with signal observed in the ependymal cell layer adjacent to the lateral ventricular space (**Fig. 7.18** and **Table 7.4**). In addition, CAV-2 DNA was observed within the corpus callosum of mice injected with 1 x 10^9 particles CAV-NS at 2-days- and 1-wk post-delivery. CAV-2 vector genome signal was also observed within the

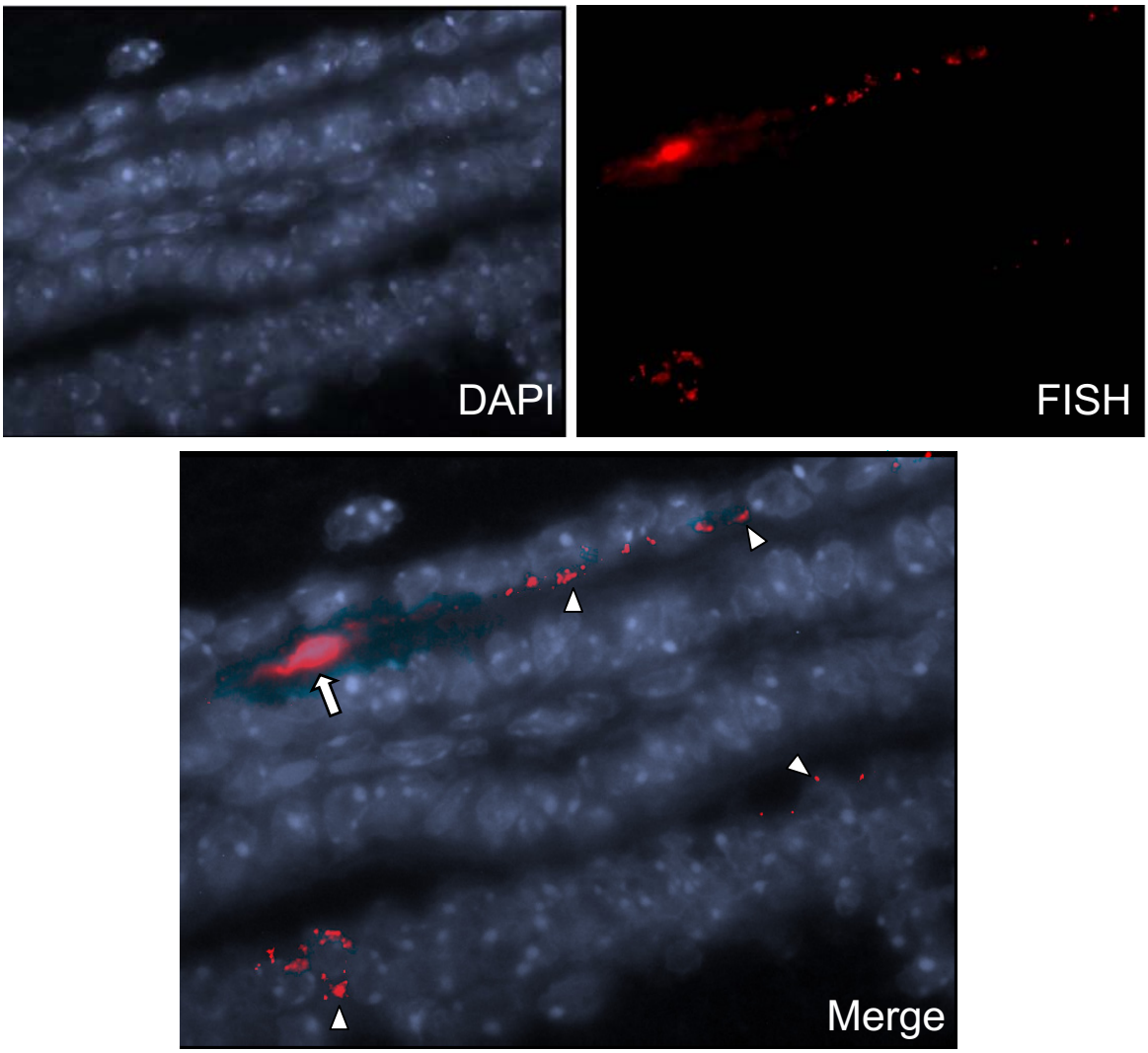


Figure 7.18: CAV-NS Vector Genome Detection by FISH. At various ages post-injection, mice were perfusion-fixed with 4% (w/v) PFA and the brain was divided along the midsagittal fissure. One hemisphere was embedded in paraffin wax, cut into 5 µm thick sections and the CAV-2 genome detected with a fluorochrome-labelled CAV-NS DNA probe using fluorescent *in situ* hybridisation (FISH; red signal, indicated with arrow-heads). Sections were counter-stained with the nuclear dye DAPI (blue). Representative images are displayed of the choroid plexus within the lateral ventricle from an unaffected mouse treated with 10^9 CAV-NS particles/hemisphere and sacrificed at 2 days post-injection. The area marked with an arrow is non-specific, autofluorescent signal.

olfactory bulb. However, by 2-wks post-injection no discernable CAV-2 genome signal was apparent in mice treated with CAV-NS, regardless of the dose administered.

7.4. DISCUSSION

The major aim of this study was to determine whether early intervention with CAV-NS gene therapy could ameliorate the MPS IIIA disease phenotype. Consequently, CAV-NS was injected into the lateral ventricles of newborn unaffected and MPS IIIA mice. Uninjected mice and PBS vehicle-treated mice were included as controls. The mice were sacrificed at various times post-injection to evaluate the biodistribution, amount of transgene expression and the effect on neuropathology using biochemical (HNS-UA storage) or histological analyses (lysosomal vacuolation, astrogliosis and activated microglia). In addition, mice underwent testing in a behavioural test battery to determine whether CAV-NS administration mediated improvements in the clinical symptoms of MPS IIIA mice. The effect of CAV-NS treatment on each of these aspects of MPS IIIA pathology will be discussed.

7.4.1. Biochemistry

Following CAV-NS delivery to newborn mice, limited NS activity and rhNS protein was detected in treated brain homogenates. The maximum rhNS expression mediated by CAV-NS was ~7.5 ng rhNS/mg total protein at 1-wk post-injection. In comparison, rhNS delivered intravenously to newborn MPS IIIA mice at 1 mg/kg resulted in rhNS concentrations of 180 and 45 ng rhNS/mg total protein in whole brains at 1- and 24-hrs post-injection, respectively (Gliddon and Hopwood, 2004). Weekly intravenous rhNS ERT at this dose improved the memory and spatial learning capabilities and reduced lysosomal storage vacuoles in the brains of MPS IIIA mice treated from birth (Gliddon and Hopwood, 2004).

Similar amounts of HNS-UA disaccharide storage were measured in the olfactory bulb and a hemi-coronal brain slice containing the injection site in MPS IIIA mice treated with PBS or CAV-NS. Although individual CAV-NS-treated MPS IIIA mice had reduced HNS-UA storage when compared to PBS-treated MPS IIIA animals, the intra-group variation (as well as the potential dilution of localised reductions in storage by untransduced regions, due to assessing the tissues in coronal brain slices) made it difficult to determine whether CAV-NS administration had mediated a subtle improvement in lysosomal storage.

7.4.2. Histology

As significant reductions in HNS-UA content were not observed in treated MPS IIIA mice, histological analyses were undertaken to examine whether localised correction of lysosomal storage was achieved after CAV-NS vector administration at birth. Three separate methods were employed to assess various components of MPS IIIA histopathology.

1) Vacuolation

The almost complete absence of storage vacuoles in the choroid plexus and ependymal cells of the lateral ventricle in CAV-NS-treated MPS IIIA mice suggested that the initial high burst of transgene expression, together with residual low-level transgene expression, was sufficient to delay/prevent vacuole formation at the injection site. In the absence of continued rhNS expression, storage vacuoles can re-accumulate in cortical cells approximately 6- to 8-wks after intravenous rhNS treatment in newborn MPS IIIA mice (Gliddon and Hopwood, 2004). Similarly, storage re-accumulates in adult MPS IIIA mice receiving intracerebral injections of purified rhNS, with substantial reductions in vacuolation in Purkinje neurons and hippocampal granule cells in animals injected 6-wks prior to sacrifice (compared to the vacuolation when treated >12-wks prior to sacrifice), when all treatment groups are euthanased at the same age (Savas *et al*, 2004).

CAV-NS-mediated decreases in the frequency of (and the number of vacuoles contained within) cortical, vacuolated cells in MPS IIIA mice demonstrated that this treatment effect was not restricted to the injection site. Further, reduced vacuolation at both the cortical surface as well as within deeper layers (further and closer to the injection site, respectively) implies that a treatment gradient effect was not present at 20-wks post-delivery. Unsurprisingly, the degree of correction was not as remarkable in the cortex (where transgene expression was only observed at 1-wk after CAV-NS delivery) as in the lateral ventricle injection site, where GFP expression was detected at every time-point assessed (7 time-points, ranging from 2-days to 20-wks post-injection).

2) Astrogliosis

Increased GFAP expression, as a marker of reactive gliosis, has previously been documented in the brainstem, superior colliculus, cortex, and the gracile and cuneate nuclei in MPS IIIA mice (Savas *et al*, 2004), and in the cortex and hippocampus of MPS IIIB mice (Li *et al*, 2002). Improvements in astrogliosis were observed in the cortex of CAV-NS-treated MPS IIIA mice (compared to MPS IIIA mice receiving PBS). However, significant

differences were not achieved in the cortex or olfactory bulb (p=0.054, 0.13), which may relate to the small sample size. In a study where mice were treated with purified rhNS at various stages of MPS IIIA disease progression, a re-occurrence of astrogliosis was observed in MPS IIIA mice treated 18-wks prior to sacrifice, whilst animals treated 6-wks before sacrifice displayed a nearly complete elimination of reactive gliosis (Savas *et al*, 2004). It is possible that greater improvements in reactive gliosis were achieved by CAV-NS treatment in MPS IIIA mice, but the treatment effect was lost soon after cessation of high transgene expression.

3) Microglial Activation

Activated microglia have also been implicated in the neurodegenerative process in mouse models of MPS I and MPS IIIB (Ohmi *et al*, 2003), Sandhoff disease (Wada *et al*, 2000), metachromatic leukodystrophy (Hess *et al*, 1996) and Niemann Pick type C disease (German *et al*, 2002). In the two areas examined in this study (the olfactory bulb and cerebral cortex), isolectin B_4-positive activated microglia were detected more frequently in uninjected MPS IIIA mice than in their unaffected counterparts. CAV-NS treatment did not influence the density of activated microglia in either of the structures assessed, suggesting that some pathological changes (e.g. activated microglia) may be more refractory to treatment than others (e.g. gliosis, vacuolation). Indeed, the inability of purified rhNS to treat another pathological change - ubiquitin-positive axonal spheroids - that are already present before the initiation of intracerebral therapy, supports this hypothesis (Savas *et al*, 2004). Alternatively, the amount of rhNS produced or the short duration of high-level transgene expression may have been insufficient to treat the microglial activation in MPS IIIA mice. Assessing the isolectin B_4 staining patterns of CAV-NS-treated MPS IIIA mice at earlier time-points after injection may clarify this situation.

Using additional immunohistochemical markers, such as HS, G_{M2} and G_{M3} gangliosides, cholesterol and ubiquitin (McGlynn *et al*, 2004; Savas *et al*, 2004), will aid in characterising the efficacy of CAV-NS therapy. Transmission electron microscopic analysis may also be beneficial in examining the effect of treatment (and the ultrastructure of the storage material within the inclusions) at various ages after CAV-2 delivery (Gliddon and Hopwood, 2004; Hemsley *et al*, 2007), particularly at the injection site and the olfactory bulb where high-level transgene expression were observed.

7.4.3. Possible Factors Influencing Transgene Persistence

Although GFP-expressing cells were observed at 20-wks post-injection, the transgene expression and distribution was greatly restricted compared to the peak expression at 1-wk after delivery. To verify whether NS activity also diminished with time after delivery, primary cell cultures derived from newborn mouse brains were inoculated with CAV-NS and NS activity was monitored over 9-days. NS activity was greatest at the first time-point assessed in both the cell extracts and conditioned media samples, and gradually lessened with time post-delivery, indicating that expression in both transgenes was reduced after delivery. Similar durations in transgene expression were observed in adult C57Bl/6 mice receiving ΔE1/E3-Ad vector into the cerebrospinal fluid, with β-galactosidase immunoreactivity peaking at 1- or 7- to 14-days post-delivery (for expression driven by the CMV or RSV promoter, respectively) and was essentially eliminated by 3-wks post-delivery (Christenson *et al*, 1998). Likewise, intra-striatal delivery of ΔE1/E3-Ad to adult mice conveys peak β-galactosidase expression at 10-days post-delivery, with considerable reductions in transgene activity by 4-wks post-injection in the absence of immunosuppression (Durham *et al*, 1997).

Potential factors that may have contributed to the transient expression mediated by CAV-NS may include transcriptional silencing of the CMV promoter (Brooks *et al*, 2004), the dilution of the CAV-2 genome during the growth of the mice, or loss of the transduced cells due to immune rejection of the cells (Michou *et al*, 1997; Lawrence *et al*, 1999), inflammatory processes resulting in toxicity to the transduced cells (Morral *et al*, 1998), or direct cytotoxicity resulting from the expression of the remaining CAV-2 viral genes in first-generation vectors (Driesse *et al*, 2000; Thomas *et al*, 2001a). In an attempt to elucidate which factors influenced the kinetics of transgene expression, several experiments were performed to confirm or exclude each hypothesis.

1) Transcriptional Silencing

CAV-2 genomic DNA was identified by PCR amplification of DNA extracted from paraffin-embedded brains or by direct visualisation using FISH, to determine whether the reduced transgene expression related to promoter silencing (where CAV-2 genomic DNA would be present and transgene expression absent) or a loss of transduced cells and viral genome (where both CAV-2 genome and transgene expression would not be detected). The CAV-2 genome was not detected after 2-days (PCR) and 7-days (FISH) post-delivery, suggesting that a loss of transduced cells (and thus vector genome), and not promoter silencing, was the likely contributing factor to declining transgene expression.

Alternatively, CAV-2 vector DNA may have persisted *in vivo* at concentrations below the limit of detection of these assays. Neuronal cell division in the adult mammalian brain is restricted to the dentate gyrus of the hippocampus, the olfactory bulb (with migrating cells originating from the subventricular zone) and, during early postnatal development, in the cerebellum (reviewed in Garcia-Verdugo *et al*, 2002). Consequently, stable expression from episomal (non-integrating) vectors such as CAV-2 may be achieved if delivered to post-mitotic neurons in fully developed animals (in the absence of immunogenic and inflammatory responses). However, as CAV-NS was administered to newborn mice, the CAV-2 episome was most likely divided between daughter cells during the normal growth and development of the treated mice to a degree that may have been undetectable.

2) Cytotoxicity

The absence of persistent CAV-2 genome at 7-days post-injection suggested that the transduced cells were eliminated. One mechanism by which this could occur is by cytotoxic responses against the transduced cells caused by the residual expression of CAV-2 viral genes (Morral *et al*, 1998) or by direct cytotoxicity against the virion capsid proteins (Yang *et al*, 1994; Easton *et al*, 1998). Even minor leakages of the late major promoter may be sufficient for the synthesis of cytotoxic viral proteins (discussed in Peltekian *et al*, 1997). Quantitation of a surrogate marker of cytotoxicity, lactate dehydrogenase activity, in primary neural cultures (where inflammatory and immunogenic responses are not confounding factors) was not significantly different between untreated and CAV-GFP- or CAV-NS-treated cells. This contrasts with the cytopathic consequences reported after intracerebral (Franklin *et al*, 1999; Thomas *et al*, 2001a) or intra-cerebrospinal fluid injection of first-generation Ad vectors into rats (Driesse *et al*, 2000; Tada *et al*, 2005), with reports of axonal degeneration, demyelination, dilated ventricles and a dose-dependent loss of glial, neuronal or ependymal cells at higher multiplicities of infection. Transgene-independent, acute cytotoxic responses have a threshold of 1 x 10^8 infectious units of ΔE1-Ad per injection in the rat parenchyma (Thomas *et al*, 2001a). This cytotoxicity can be eliminated by using lower doses of Ad or by heat-inactivating the virion particles before injection, suggesting that the expression of residual Ad genes contributes to this response (Thomas *et al*, 2001a).

Similarly, primary *in vitro* cultures of rodent neurons also display toxicity after Ad transduction, with morphological changes including shrunken cell bodies, fragmented neurites and reduced global protein synthesis rates apparent within 3- to 5-days after infection (Easton *et al*, 1998; Cregan *et al*, 2000). Older neurons (i.e. cultured for more days *in vitro* before

transduction) are more resistant to Ad toxicity than younger neurons but also exhibit cytotoxic effects with time in culture (Easton *et al*, 1998). These trends were confirmed in this study, with increased cytotoxicity (though not to a statistically significant degree) measured in newborn-derived compared to adult-derived neural cells.

3) Immune Responses

The absence of persistently high transgene expression was also potentially related to the clearance of transduced cells by innate and/or adaptive immune responses. The innate immune system generates transient, acute inflammatory responses against ΔE1-Ad, HD-Ad (Thomas *et al*, 2000) and biologically-inactive Ad (Byrnes *et al*, 1995), inducing the release of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 within hours of delivery (Cartmell *et al*, 1999). Intra-striatal injection of ΔE1-Ad is characterised by the infiltration of inflammatory cells such as $CD4^+$ and $CD8^+$ T lymphocytes, macrophages, neutrophils and natural killer cells as early as 6-hrs post-injection (Byrnes *et al*, 1995; Regardsoe *et al*, 2004), peaking at 6- to 7-days after injection (Zou *et al*, 2000; Regardsoe *et al*, 2004). The influx of cells is related to chemokine (e.g. RANTES, macrophage inflammatory protein-1β, monocyte chemoattractant protein-1 and –3) and IFN-related recruitment of cells into the transduced brain regions (Zirger *et al*, 2006a). In addition, local activation of astrocytes and microglia and an upregulation of MHC class I and II is also observed (Byrnes *et al*, 1996; Thomas *et al*, 2001a; Bhat and Fan, 2002). Acute brain inflammation is dose-dependent (Thomas *et al*, 2001a) and self-resolves approximately 2-wks after injection (Byrnes *et al*, 1995; Thomas *et al*, 2000).

However, decreased expression mediated by ΔE1-Ad is not solely related to acute inflammatory responses, as stable and long-term transgene expression can be obtained after HD-Ad administration (where acute inflammatory responses are also generated) in the rodent brain (Thomas *et al*, 2001b). Rather, the reduction of transgene expression from the CNS is also influenced by adaptive immune responses such as the generation of CTL activity, antibody formation (reviewed in Christ *et al*, 1997; Schagen *et al*, 2004) and chronic inflammation (≥30-days post-delivery at doses $>10^8$ infectious units of ΔE1-Ad; Thomas *et al*, 2000). Transgene and/or Ad-specific CTL responses can be generated after peripheral delivery of Ad vectors to adult rodents (Yang *et al*, 1994; Dai *et al*, 1995; Michou *et al*, 1997; Chen *et al*, 2005). CTL responses can also be mounted after intravenous delivery of retroviral vectors to neonatal (2- to 5-day-old) MPS I kittens (Ponder *et al*, 2006). Whether CTL responses are generated against transduced cells after intraventricular delivery of CAV-NS to

neonatal mouse pups (and, if so, whether these responses were partially responsible for the loss of transgene expression) remains uncertain.

Antibody formation is another aspect of adaptive immunity. Antibodies specific for rhNS, GFP or CAV-2 neutralising antibodies were not detected in CAV-NS-injected mice at all doses assessed (as well as in PBS-treated and uninjected mice). This suggests that a humoral response was not generated by CAV-NS treatment in neonatal mouse pups. Adult mice injected peripherally (Dai *et al*, 1995; Vilquin *et al*, 1995; Michou *et al*, 1997) or intra-cerebrally (Kajiwara *et al*, 2000; Zirger *et al*, 2006b) with Ad vectors can generate humoral responses against the Ad capsid due to B lymphocyte interactions with Ad (discussed in Harvey *et al*, 1999 and references therein). In addition, humoral immune responses may be generated against the transgenic proteins encoded by Ad (Dai *et al*, 1995; Vilquin *et al*, 1995; Michou *et al*, 1997; Kajiwara *et al*, 2000; Jerebtsova *et al*, 2002). The immunogenicity of GFP has been thoroughly characterised with GFP-expressing cells inducing CTL responses in mice (Stripecke *et al*, 1999) and rhesus macaque monkeys (Rosenzweig *et al*, 2001). GFP-specific antibody production was also observed in 3 of 6 monkeys (Rosenzweig *et al*, 2001). These findings contrast with those of Gambatto *et al* (2000), where immune responses were not generated against GFP in C57Bl/6 mice when assessed *in vivo* (lack of extinction of transgene expression) or *in vitro* (CTL activity). Transgenic GFP mice and the C57Bl/6 parental strain display similar immunological profiles, also suggesting that GFP is not highly immunogenic (Kawakami *et al*, 1999). Whilst transgene-specific antibodies were not detected in our study, this may relate to the age at which treatment was administered rather than the immunogenicity *per se* of rhNS and GFP proteins (reviewed in Waddington *et al*, 2004), similar to previous reports in MPS VII mice (Kamata *et al*, 2003)

Chronic (long-lasting) inflammatory responses induced by the CAV-NS vector may have also eliminated CAV-NS-transduced cells. GFAP upregulation is observed within 3-days after administration of ΔE1-Ad to the rat striatum (acute inflammation) and, at high viral doses ($\geq 10^8$ infectious units/injection), chronic inflammation (e.g. $CD8^+$ T cell infiltrates, microglia/macrophage activation, MHCI upregulation) is still detectable 30-days later (Thomas *et al*, 2001a). Consistent with the latter findings, elevated GFAP immunoreactivity in the cortex of CAV-NS-injected, unaffected mice (compared to uninjected and PBS-treated unaffected animals) suggests that chronic inflammatory responses were induced by CAV-NS and were still present at 20-wks after vector administration. This same trend in GFAP immunostaining was evident to a lesser degree in the olfactory bulb. In all probability, the

extinction of high-level transgene expression mediated by ΔE1 CAV-NS was influenced by a combination of these (and potentially other) factors rather than by one sole factor.

7.4.4. Behavioural Phenotype Assessment of CAV-NS-Treated Mice

Two treatment effects were analysed in the behavioural test battery: firstly, whether the surgical procedures (cryo-anaesthesia/intraventricular injections) altered the behaviour of injected mice compared to uninjected control mice and, secondly, whether treatment with CAV-NS gene therapy normalised the disease phenotype in MPS IIIA mice. The results of the majority of the behavioural studies reported throughout this chapter were not statistically significant. Although the surgical procedures in anaesthetised/injected animals may explain the behavioural differences in the PBS- and CAV-NS-treated groups, the lack of separation between uninjected unaffected and MPS IIIA mice (at ages where discrimination between the disease groups has previously been observed) indicated possible underlying problems in the manner in which the tests were conducted. Whilst every care was taken to standardise the testing procedures, there are several possible explanations as to why the tests failed to discriminate between the two genotypes.

The long-term nature of this study may have resulted in the mice habituating to human handling, the behavioural testing room and the testing apparatus (for the tests performed twice; open field activity, motor function tests). In addition, the tests were conducted as a behavioural test battery in an attempt to examine multiple aspects of behaviour in what was considered to be in order from least to most invasive, similar to the rationale of other groups (McIlwain *et al*, 2001). This was also the chronological order in which pathological changes were observed (Hemsley and Hopwood, 2005). Consistent with other reports (McIlwain *et al*, 2001; Voikar *et al*, 2004), battery-testing appeared to influence the behavioural profile of the animals when compared to non-battery-tested mice (**Chapter 6**; unpublished observations, LDRU; Gliddon and Hopwood, 2004; Hemsley and Hopwood, 2005). For example, battery-tested mice appeared less anxious and fearful than (infrequently handled) non-battery-tested animals, as evidenced by the percentage of entries into open arms in the Elevated Plus Maze.

Exploratory and emotional behaviours are the most sensitive traits affected by prior training history in a test battery (Voikar *et al*, 2004). Battery-tested, male C57Bl/6 mice are less active (reduced path length and number of rears) than non-battery-tested animals when normalised for handling effects (i.e. non-battery-tested mice were removed from cages and transferred to the testing area an equal number of times as battery-tested mice; McIlwain *et al*,

2001; Voikar *et al*, 2004). Consistent with previous reports (LDRU unpublished observations, Hemsley and Hopwood, 2005; Crawley *et al*, 2006a), 10-wk-old uninjected MPS IIIA mice were less active than uninjected unaffected animals and statistically significant differences were not observed, potentially due to testing as part of a test battery. In addition, PBS- or CAV-NS-injected unaffected and MPS IIIA mice were less active than genotype-matched, uninjected animals, indicating that the surgical procedures altered the behaviour of injected mice. The reduced activity displayed in all groups tested in the open field at 19-wks (when compared to 10-wks) may be a habituation effect to the operator, testing arena and room, in combination with the influence of prior training in the test battery.

The emotional profiles of C57Bl/6 mice are also altered when assessed as part of a behavioural battery or in a naïve state, with significant differences in several anxiety-related parameters (latency to first open arm entry, the number of open arm entries, the percentage of open arm entries and the percentage of time spent on open arms; Voikar *et al*, 2004). In our study, uninjected test-battery mice displayed lower anxiety scores in the Elevated Plus Maze than non-battery-tested uninjected mice (entries into the open arms, time spent on the open arms and path length on the open arms). Also, battery-tested C57Bl/6 mice display decreased exploratory behaviour compared to non-battery-tested mice as measured by the path length and the total number of arm entries (this study and Voikar *et al*, 2004). The differences between the battery- and non-battery-tested mice in the open field activity and Elevated Plus Maze tests are hypothesised to relate to changes in the motivational state of the animals (Voikar *et al*, 2004).

Non-battery-tested, male "New York" MPS IIIA mice have unaltered gait lengths, decreased gait widths (≥ 15-wks of age), take longer to re-orient themselves in the negative geotaxis test (≥ 20-wks) and show reduced neuromuscular grip strength (≥ 20-wks) compared to their unaffected counterparts (Hemsley and Hopwood, 2005). In contrast, battery-tested, male congenic MPS IIIA mice display reduced gait lengths and widths, are not different in the negative geotaxis test and have reduced grip strength compared to unaffected animals (Crawley *et al*, 2006a). The discrepancies between these studies may reflect differences between the strains and training history of the tested mice. Whilst MPS IIIA mice in the current study were more likely to also have reduced gait width and take longer to re-orientate themselves in the negative geotaxis test than unaffected mice, the majority of the battery-tested mice from both genotypes passed the neuromuscular grip strength test at 19-wks of age. Superior motor co-ordination on the rotorod has previously been observed in battery-tested mice compared to naïve-tested animals (McIlwain *et al*, 2001; Voikar *et al*, 2004).

Newborn MPS IIIA mice receiving cryo-anaesthesia and bilateral injections of PBS display impaired memory and spatial learning compared to PBS-treated unaffected mice at 20-wks of age (Fraldi *et al*, 2006). The cognitive deficits in MPS IIIA mice can be improved by treatment with an AAV vector encoding NS and SUMF1 (Fraldi *et al*, 2006). Based on these findings (and the data presented in **Chapter 6** and Crawley *et al*, 2006a), the mice in this study were assessed in the Morris Water Maze at 20-wks of age. Unexpectedly, the uninjected and PBS-treated MPS IIIA mice performed well in the acquisition phase. The major differences between the this study and the Fraldi *et al* (2006) study were that the mice in the AAV study were (a) not handled as frequently, (b) assessed in a less invasive behavioural battery (three open field activity tests at 10-, 15- and 18-wks and one gait assessment at 18-wks prior to Morris Water Maze testing at 20-wks), (c) were tested in the same order each day over 1-wk and (d) the experimenter was not blinded to the treatment/genotype of the mice during testing (Dr Kim Hemsley, personal communication). Battery-tested mice have been documented as being less proficient at using a spatially selective search pattern to locate the hidden platform in the probe phase of the Morris Water Maze compared to non-battery-tested mice (McIlwain *et al*, 2001). These observations do not support the data in this thesis, which showed that all groups were highly selective in searching the target quadrant during the probe phase. The week in which mice were tested affected the performance in the visible platform test and the cause/s of this variation is unclear.

For subsequent behavioural analyses, using fewer tests and not repeating the same test in a battery may provide phenotypic information that is more consistent with non-battery-tested animals. Home cage activity monitoring may provide data on the activity and sleep/wake patterns of unaffected and MPS IIIA mice in a less invasive manner than open field activity testing. Home cage activity differences have been reported in mouse models of MPS I (Jordan *et al*, 2005) and MPS IIIB (Cressant *et al*, 2004). Elevated Plus Maze testing should be the first component of the battery due to the sensitivity of this test to environmental and procedural manipulations (Voikar *et al*, 2004; Carobrez and Bertoglio, 2005). The cognitive function of MPS IIIA mice cannot be assessed via the Morris Water Maze after 25-wks of age due to statistically significant differences in swim speed between unaffected and MPS IIIA mice (unpublished observations, LDRU). Other memory and learning tests (e.g. fear conditioning, conditioned taste aversion or novel object recognition tests) may be valuable in assessing cognition in unaffected and MPS IIIA mice at older ages, where the disease phenotype may be more readily discriminated from wild-type mice.

7.4.4. Safety

Another important aspect of ΔE1 CAV-2 gene therapy is the safety of this treatment modality. The CAV-NS vector was tolerated well when administered under cryo-anaesthesia to neonatal mouse pups. The survival of 87% of CAV-NS-treated mice was comparable to uninjected and PBS-treated mice (93-94%) and to those detailed in **Chapter 5**. An increased incidence of hydrocephalus (3.7%) was observed in the cryo-anaesthetised/injected mice compared to the rates observed within the congenic mouse colony (3/1000 mice, 0.3%; unpublished LDRU records). Given that cerebrospinal fluid is produced in the epithelial cells of the choroid plexus (reviewed in Brown *et al*, 2004), it may not be surprising that damage results in the abnormal secretion/drainage of cerebrospinal fluid after injection into the lateral ventricle. Comparable incidences of hydrocephalus after cryo-anaesthesia/intraventricular delivery of cells or AAV vectors to newborn mice have been observed (unpublished observations, LDRU) suggesting that the hydrocephalus was influenced by the method of delivery rather than the CAV-NS vector.

ΔE1 CAV-2 treatment did not result in antibody production against the CAV-2 capsid, or rhNS or GFP transgenic proteins (compared to PBS-treated and uninjected mice). This suggests that humoral responses will not be generated against the HD CAV-2 vectors if they are delivered in the neonatal period.

7.5. SUMMARY AND CONCLUSIONS

Intraventricular administration of ΔE1 CAV-NS to newborn MPS IIIA mice resulted in transgene expression for >20-wks and mediated a reduction in both vacuolation and the reactive astrogliosis associated with the MPS IIIA disease process. However, the amount of NS generated by CAV-NS was insufficient to significantly alter several clinical parameters (as assessed by a battery of behavioural tests) as well as other pathological changes (HS-disaccharide storage, activated microglia).

The loss of CAV-2 vector genome and high-level transgene expression after approximately 1-wk post-injection was potentially caused by chronic inflammatory responses (as detected by elevated GFAP staining) against the ΔE1 CAV-NS vector. Other factors potentially played a less significant role (direct vector-mediated cytotoxicity) or were not responsible for the reduction in high-level transgene expression (transgene silencing, humoral responses). In conclusion, whilst improvements in some aspects of neuropathology were observed in treated MPS IIIA mice, early intervention with ΔE1 CAV-NS gene therapy was unable to significantly alter the course of MPS IIIA disease progression.

CHAPTER 8:
Conclusions and Future Work

8.1. INTRODUCTION

MPS IIIA is a severe and devastating disease and, at present, no effective treatment exists for these patients. At the commencement of this study, proof-of-principle ERT studies had demonstrated that if purified rhNS was delivered to newborn MPS IIIA mice via the superficial temporal vein, many aspects of disease progression, including functional changes, could be effectively ameliorated. Intravenously-delivered ERT requires regular infusions of recombinant enzyme and this enzyme is not efficiently delivered from the periphery to the CNS. Purified rhNS injected into the brain parenchyma or the cerebrospinal fluid can also mediate improvements in MPS IIIA mice. However, if a regular supply of rhNS is not maintained within the CNS, neurodegeneration continues to progress. Consequently, a number of strategies are being pursued to develop an effective and long-lasting therapy for MPS IIIA and other LSD that develop neuropathology.

Therefore, this study aimed to evaluate the safety and therapeutic efficacy of a gene therapy approach utilising a CAV-2 viral vector as a means of facilitating the expression of rhNS directly in the brain parenchyma of MPS IIIA mice.

8.2. SUMMARY

Before the efficacy of CAV-2 gene therapy in MPS IIIA could be investigated, a CAV-2 vector capable of expressing NS was first required. Consequently, the ΔE1 CAV-NS vector was generated and particles were then amplified, purified and titrated at high titres and infectivity. *In vitro* testing demonstrated that rhNS produced by CAV-NS could effectively reduce the sulphated GAG content of human MPS IIIA fibroblasts in a M6P-dependent mechanism. In addition, CAV-NS efficiently transduced primary neural cultures derived from MPS IIIA and unaffected newborn mouse brains.

Concurrently, preliminary *in vivo* hippocampal injections were performed on young, adult guinea pigs to evaluate the biodistribution and safety of CAV-GFP, a CAV-2 vector encoding the GFP marker gene, in a species which had not been assessed previously. GFP

was widely and strongly expressed throughout many regions of the guinea pig brain parenchyma without inducing CAV-2 neutralising antibody formation. In contrast, intra-thalamic injections in adult mice generated strong humoral responses against the CAV-2 capsid resulting in negligible transgene expression within the CNS.

To circumvent the vector-induced immunity, CAV-NS was bilaterally-injected into the lateral ventricles of newborn mouse pups at one of four doses. Widespread and dose-dependent transgene expression was observed throughout the brain parenchyma, with particularly strong expression in the choroidal cells and the ependymal layer of the lateral ventricle injection site and within the olfactory bulb. Transgene–specific (rhNS or GFP) or CAV-2 neutralising antibodies were not detected in uninjected, PBS-treated or CAV-NS-treated mice at significant concentrations.

Further studies in unaffected and MPS IIIA mice were conducted with 10^9 CAV-NS particles per injection, the most suitable titre determined in the preliminary experiments, with additional mice injected with saline vehicle or remained uninjected as controls. Several aspects of MPS IIIA behavioural phenotype were assessed in a behavioural test battery using previously established motor function and open field activity tests in conjunction with two tests that were characterised as part of this study: the Elevated Plus Maze (measuring anxiety-related behaviours) and the Morris Water Maze (cognitive function). Compared to PBS-treated MPS IIIA animals, CAV-NS treatment had no effect on the functional changes associated with MPS IIIA.

However, localised improvements in neuropathology were observed in CAV-NS-treated MPS IIIA mice, with a near complete absence of storage inclusions within the choroidal and ependymal cells of the lateral ventricle (the injection site) and reductions in vacuolation in cortical neuronal and glial cells. Likewise, CAV-NS treatment reduced the degree of reactive astrogliosis, but not activated microglia, in the cortex of MPS IIIA animals. These improvements in neuropathology were not detected in hemi-coronal brain slices using mass spectrometric detection of a HS-derived disaccharide, presumably due to the dilution of localised storage reductions by untreated brain areas. In closing, whilst ΔE1 CAV-NS gene therapy instigated at birth was able to ameliorate some aspects of neuropathology, this treatment was unable to significantly alter the MPS IIIA disease course, most likely due to insufficient NS enzyme production. Further, it is likely that transgene expression did not persist at high concentrations due to the induction of chronic inflammatory responses.

8.3. CONCLUSIONS AND IMPLICATIONS

The major conclusion from this work is that ΔE1 CAV-NS gene therapy mediated localised improvements in several aspects of neuropathology but was unable to significantly alter the progression of MPS IIIA disease. At the beginning of this project, this was the first gene therapy study to be undertaken in MPS IIIA. Intraventricular administration of CAV-NS gene therapy to newborn mice was well tolerated with no gross side-effects observed in treated mice. Significant antibodies against the rhNS, GFP or CAV-2 capsid proteins were not measured in CAV-NS-treated mice, suggesting that this treatment did not induce antibody-mediated immune responses. The absence of humoral and other responses against CAV-NS is of particular importance as this suggests that administration of HD CAV-2 vectors will also be well-tolerated in future *in vivo* studies and is unlikely to limit the efficacy of therapy.

The continued expression of the GFP transgene (and presumably the rhNS transgene) for at least 20-wks after CAV-NS delivery considerably reduced lysosomal storage inclusions at the injection site and reduced astrogliosis in the treated MPS IIIA mouse CNS. In addition, at the peak level of expression, GFP was observed in many brain regions, located both within the vicinity of, and at distant locations from, the lateral ventricle injection sites. Taken together, these results suggest that if the CAV-2 system could be directed to generate persistent and high-level NS expression in all of the areas exhibiting transgenic protein at peak expression, this treatment modality may be useful in mediating widespread neurological improvements in the murine MPS IIIA model.

At 20-wks post-injection, GFP expression was restricted to the lateral ventricle, most likely due to chronic inflammatory responses against the CAV-NS vector. These findings correlate closely with the inflammatory and immunogenic responses observed after human Ad delivery to the rodent CNS which, in retrospect, is not surprising given that both the canine and human Ad are foreign antigens to the murine CNS. As ΔE1 and subsequent generations of Ad vectors have been extensively evaluated in rodent and larger animal models, we can be guided by these findings to devise suitable immunosuppression and/or immunomodulation strategies (to address the stability of transgene expression) and further improve the CAV-2 vector design. The latter will be discussed in greater detail in **Section 8.4**.

8.4. FUTURE DIRECTIONS

For MPS IIIA and other inherited disorders of metabolic disease, widespread and high-level expression of the therapeutic gene are required *in vivo* for extended periods of time. The extinction of transgene expression due to inflammatory responses against the transduced cells limited the therapeutic application of ΔE1 CAV-NS gene therapy in MPS IIIA mice. Consequently, to improve transgene longevity, future work should be directed at reducing the toxicity and inflammation associated with CAV-NS via the use of HD CAV-2 vectors expressing NS (Soudais *et al*, 2004; reviewed in Palmer and Ng, 2005 and references therein). Indeed, gene expression mediated by human and canine HD Ad vectors has exceeded 2.5-years and 1-year, respectively, after a single delivery in immunocompetent mice (Kim *et al*, 2001; Soudais *et al*, 2004). HD Ad have improved transgene persistence compared to first-generation Ad in the brain, liver and muscle (Morral *et al*, 1998; Zou *et al*, 2000; Bilbao *et al*, 2005) and retain many of the advantageous features of first-generation (CAV-2) vectors such as high yields of at least $2x10^{11}$ particles/mL, reduced risk of insertional mutagenesis due to the vector genome persisting as a non-integrative episome, high-level gene expression, as well as the preferential transduction of neurons and widespread transport of the virion particles via axoplasmic transport (Soudais *et al*, 2004).

In addition, the larger cloning capacity of HD vectors (at least 34 kb for CAV-2 vectors; Soudais *et al*, 2004) allows greater flexibility in the design of the vector sequences and can potentially allow the delivery of multiple transgenes and other elements that may enable the regulation of transgene expression. For instance, the Tet-ON regulatory system that has been developed for human HD Ad could be incorporated into the vector design, whereby the addition of tetracycline results in the reverse tetracycline-responsive transcriptional activator binding to the promoter to activate transcription (Berenjian and Akusjarvi, 2006). The GFP marker gene is currently required for HD CAV-2 vector preparation and has the potential to be neurotoxic (Liu *et al*, 1999) and immunogenic (Stripecke *et al*, 1999). The Tet-ON system driving the *in vivo* expression of GFP could be activated preceding sacrifice of the animal, thus allowing the easy identification of transduced cells whilst avoiding/reducing potential negative effects associated with GFP expression.

Future CAV-2 vector designs should also consider the incorporation of the SUMF1 gene, which catalyses the essential conversion of a sulphatase active site cysteine to a C_α-formylglycine residue for the catalytic activity of all sulphatases, including NS (Cosma *et al*, 2003; Dierks *et al*, 2003). Recent gene therapy studies conducted in mouse models of MPS IIIA (Fraldi *et al*, 2007) and metachromatic leukodystrophy (Takakusaki *et al*, 2005; Kurai *et*

al, 2007) have demonstrated that SUMF1 co-expression significantly increases the sulphatase enzyme activity.

In addition, other promoters driving the expression of NS should be assessed to determine which provides the most prolonged and high-level transgene expression within the CNS. Gerdes *et al* (2000) have demonstrated that high-level expression in the CNS can be achieved from low doses of Ad when strong promoters (e.g. major immediate early murine CMV) are utilised. This is also advantageous as the low viral doses do not promote cellular inflammation (Gerdes *et al*, 2000). The human CMV promoter used in the ΔE1 CAV-NS vector has been reported to have shorter-lived transgene expression than the RSV promoter when ΔE1/E3-Ad was delivered to the cerebrospinal fluid of C57Bl/6 mice (Christenson *et al*, 1998). Another strong candidate promoter is the neuron-specific human synapsin I promoter, where transgene expression mediated by ΔE1-Ad at 9-months after intra-hippocampal delivery was equivalent to the expression driven by the CMV promoter at 3-days post-injection (Glover *et al*, 2003).

Once the optimal HD CAV-2 vector design has been determined, the therapeutic efficacy of the HD CAV-NS vector should be compared to the biodistribution and the NS transgene activity/protein concentration mediated by the ΔE1 CAV-NS vector. Alternative injection sites should be evaluated, such as intra-parenchymal injections into areas of pathology (e.g. the hippocampus or cerebellum) or extensive neural networks (e.g. the thalamus), or delivery directly into the cerebrospinal fluid via the cisterna magna (a less invasive method of delivery). If HD CAV-2 vectors are demonstrated to be less toxic and less inflammatory than first-generation CAV-2 vectors, similar to the data obtained with HD Ad, multiple injection sites or increased viral doses per site may be attainable. Also, as pathology is observed in the somatic tissues of MPS IIIA mice (e.g. hepatosplenomegaly, distended bladders) the most widespread/long-lived CNS delivery method could be assessed in combination with intravenous infusions of HD CAV-NS.

At present a newborn screening program for MPS IIIA has not been implemented and consequently the majority of MPS IIIA children have established pathology at diagnosis. It will be interesting to see whether pathology in MPS IIIA mice can be reversed or delayed with HD CAV-NS gene therapy at various ages throughout development, including *in utero* delivery. Ubiquitin-positive axonal spheroids present at 6-wks of age in MPS IIIA mice are resistant to intracerebral ERT, suggesting that there may be irreversible pathological changes and that treatment should be initiated as soon as possible (Savas *et al*, 2004). The potential also exists for HD CAV-2 vectors to be administered multiple times in the absence of

immunosuppressive regimes (Koehler *et al*, 2006). It will be essential to determine whether a single administration of HD CAV-NS will be sufficient for life-long NS expression or whether multiple doses (and potentially immunosuppressive schedules) are required.

In conclusion, ΔE1 CAV-NS gene therapy was able to mediate widespread and high-level transgene expression after delivery to the newborn murine brain. Although transgene expression was still detected at 20-wks after administration, this treatment did not significantly impact on the clinical progression associated with MPS IIIA, presumably due to the short-lived nature of high-level transgene expression *in vivo*. The data presented in this thesis have expanded our understanding of the murine MPS IIIA phenotype and demonstrated some of the challenges associated with CAV-2 - and other viral vector-based gene therapy - that must be resolved before CAV-2 gene transfer becomes a viable treatment for neurological LSD.

Publications Resulting From Work Described In This Thesis

Poster presentation at the 8th International Symposium on Mucopolysaccharide and Related Diseases in Mainz, Germany (2004). Lau, A.A., Hemsley, K.M., Skander, N., Kremer, E.J., Hopwood, J.J. Canine adenoviral vectors: A preliminary study of an alternative gene delivery system for alpha-mannosidosis.

Poster presentation at the 9th International Symposium on Mucopolysaccharide and Related Diseases in Venice, Italy (2006). Lau, A.A., Hemsley, K.M., Kremer, E.J., Hopwood, J.J. Gene transfer in the MPS IIIA mouse model using canine adenoviral vectors.