Evaluation of Disease Lesions in the Developing Canine MPS IIIA Brain

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Abstract Mucopolysaccharidosis IIIA (MPS IIIA) is an inherited neurodegenerative disease of childhood that results in early death. Post-mortem studies have been carried out on human MPS IIIA brain, but little is known about early disease development. Here, we utilised the Huntaway dog model of MPS IIIA to evaluate disease lesion development from 2 to 24 weeks of age. A significant elevation in primarily stored heparan sulphate was observed in all brain regions assessed in MPS IIIA pups ≤ 9.5 weeks of age. There was a significant elevation in secondarily stored ganglioside (GM3 36:1) in ≤ 9.5 -week-old MPS IIIA pup cerebellum, and other brain regions also exhibited accumulation of this lipid with time. The number of neural stem cells and neuronal precursor cells was essentially unchanged in MPS IIIA dog brain (c.f. unaffected) over the time course assessed, a finding corroborated by neuron cell counts. We observed early neuroinflammatory changes in young MPS IIIA pup brain, with significantly increased numbers of activated microglia recorded in all but one brain region in MPS IIIA pups ≤ 9.5 weeks of age (c.f. age-matched unaffected pups). In conclusion, infant-paediatric-stage MPS IIIA canine brain

exhibits substantial and progressive primary and secondary substrate accumulation, coupled with early and robust microgliosis. Whilst early initiation of treatment is likely to be required to maintain optimal neurological function, the brain's neurodevelopmental potential appears largely unaffected by the disease process; further investigations confirming this are warranted.

Abbreviations

CA3	Cornus ammonis region 3
CV	Coefficient of variation
FGF-2	Fibroblast growth factor 2
Ganglioside species	GD1, GM1, GM2, GM3, GT1
GC	Glucosylceramide
GlcSph	Glucosylsphingosine
LC	Lactosylceramide
LIMP-2	Lysosomal integral membrane protein-2
MPS-III A	Mucopolysaccharidosis type IIIA
NeuN	Neuronal nuclear antigen
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
SGSH	Sulphamidase
SM	Sphingomyelin

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Highlights

- Heparan sulphate accumulation (>17 -fold unaffected) is observed from 2 weeks of age in the canine MPS IIIA brain.

- Ganglioside GM3 accumulation (>twofold unaffected) is observed from 2 weeks of age in the canine MPS IIIA brain.
- Neuroinflammation is apparent in the canine MPS IIIA brain from 2 weeks of age.
- Canine MPS IIIA brain neurodevelopmental potential appears unaffected by the disease process.

Introduction

Mucopolysaccharidosis type IIIA (MPS-IIIA) is a lysosomal storage disorder that affects 1 per 114,000 live births in Australia (Meikle et al. 1999). MPS-IIIA is a generalised disease with clinical signs predominantly associated with the central nervous system, caused by insufficient sulphamidase (heparan-N-sulphatase; SGSH; EC3.10.1.1) activity and subsequent storage of heparan sulphate-derived oligosaccharides. This substrate itself is then believed to impede the final step in ganglioside degradation, with resulting secondary accumulation of gangliosides GM3 and GM2 (Schneider-Jakob and Cantz 1991). The role that these accumulating substrates play in phenotype expression is, at present, unknown.

Clinically, patients commonly present between 2 and 6 years of age after a period of normal development, with symptoms including hyperactivity, aggression, sleep disturbance, delayed development and loss of learned skills, followed by progressive neurodegeneration and a greatly shortened lifespan (Neufeld and Muenzer 2001). Effective treatment approaches are still being investigated and evaluated, and, to date, there is no widely available treatment.

Information regarding the time course of development of brain lesions in the human foetus is scant and variable (Greenwood et al. 1978; Ceuterick et al. 1980); thus study of animals with this disorder is essential for examining disease pathogenesis and evaluating potential treatments. Typically, these experiments have been performed in the mouse model of this disorder (Crawley et al. 2006). However, naturally occurring MPS-IIIA has also been observed in dog (Jolly et al. 2000), and use of this species would overcome the frequently questioned relevance of a small animal model.

The onset of clinical disease in this model (ataxia, hypermetria and loss of learned behaviour) occurs at 1.5–2.5 years of age, with rapid progressive decline thereafter (personal communication; Neil Marshall, Massey University) (Yogalingam et al. 2002). Whilst accumulation of primary and secondary substrates in the dog cerebellum occurs by 10 weeks of age, and cerebellar Purkinje cell loss is evident by ~30 months, the effect of the disorder on the cerebrum is not well studied (Crawley et al. 2011; Hassiotis

et al. 2014), and a more detailed assessment of MPS-IIIA canine brain pathology is needed to provide a clearer picture about the initiation and progression of pathological disease. The aim of the present study was to evaluate developmental aspects of cerebral disease in canine MPS-IIIA. Given the initiation of newborn screening for this and other neurodegenerative lysosomal storage disorders in several sites around the world, our findings are important for both understanding the natural course of disease and guiding the initiation of treatments.

Methods

Animals

All breeding and procedures were undertaken with the approval of the Animal Ethics Committee of Massey University, New Zealand, and conformed to *The Code of Ethical Conduct for the Use of Animals for Teaching and Research* as approved under the *New Zealand Animal Welfare Act 1999*. All procedures conformed to the *Prevention of Cruelty to Animals Act 1985* and the *Australian code for the care and use of animals for scientific purposes*, 8th edition (2013). The dogs were bred, housed and maintained at a commercial research facility. Genotypes were established using previously described methods (Yogalingam et al. 2002). A brain was collected from both MPS-IIIA and unaffected cases at 2, 4 and 6 weeks of age. Archival tissues from animals euthanised at 9.5 weeks of age ($n = 1/\text{genotype}$) and 24 weeks ($n = 2$ MPS-IIIA; $n = 3$ unaffected) of age were also utilised. For details of sample collection and processing, see Supplementary Methods.

Determination of Total Heparan Sulphate

Total heparan sulphate was measured in 100 µg of freeze-dried total homogenate protein according to a previously published method (Trim et al. 2014), with slight modification (see Supplementary Methods). The level of heparan sulphate was normalised to a spiked internal standard.

Quantification of Lipids

Gangliosides (Supplementary Table 1) and other lipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), glucosylceramide (GC), lactosylceramide (LC), glycosylsphingosine (GlcSph) and sphingomyelin (SM)) were extracted from 500 µg of total protein using the Bligh-Dyer extraction protocol (Bligh and Dyer 1959) and measured using mass spectrometry (Supplementary Methods).

Histochemistry and Immunohistochemistry

All sections underwent batch-staining (Supplementary Methods). All imaging and analysis was performed with the operator blind to the genotype and age of the sample. A dog (Singer 1962) and sheep brain atlas (Michigan State University Brain Biodiversity Bank: <https://www.msu.edu/~brains/brain/sheep>) was used to define cerebellar or cerebral locations, respectively. Thresholding for total staining per area was undertaken for lysosomal integral membrane protein-2 (LIMP-2) and doublecortin. The number of activated microglia and neuronal nuclear antigen (NeuN)-positive cells was determined by manual counting.

Statistical Analysis

Data were examined using ANOVA with post hoc Bonferroni testing. $p < 0.05$ was regarded as significant.

Results

Quantification of Primary Heparan Sulphate Accumulation

At 2 weeks of age, heparan sulphate-derived disaccharides were elevated in all four regions of MPS IIIA central nervous system (compared to unaffected dog tissues; Fig. 1a–d), with accumulation of heparan sulphate to 24 weeks noted in the brain but not in the spinal cord. When the data from the four dogs aged ≤ 9.5 weeks of age is combined and compared with that from the four age-matched controls, significant differences in HS levels were apparent in all four brain regions ($p < 0.01$). With the exception of the spinal cord, all MPS IIIA dog brain regions exhibited a significant increase in HS levels with time ($p < 0.05$).

Quantification of Secondary Lipid Accumulation

Gangliosides

GM2 and GM3 36:1 gangliosides were the most abundant species found in the brain and spinal cord of dogs of both genotypes (Fig. 2a–h). On the whole, more GM3 was present than GM2. Significantly higher levels of GM3 were present in the cerebellum of MPS IIIA dogs ≤ 9.5 weeks of age c.f. unaffected dog brain (Fig. 2f). Hippocampus and cerebral cortex showed a similar trend. A significant increase in GM3 was noted in the cortex with time (Fig. 2b), and other brain regions showed similar trends. All MPS IIIA brain regions examined (but not the spinal cord) exhibited significant increases in GM3 by 24 weeks

of age (Fig. 2b, d, f). GM2 levels in MPS IIIA dog cerebral cortex were also significantly elevated by this age.

There was little apparent difference in the amount of GM1 ganglioside in MPS IIIA and unaffected dog brain/cord (data not shown). No consistent difference was observed in the level of the 36:1 ganglioside species in the MPS IIIA or unaffected dog spinal cord; however, the 38:1 GM3 species was elevated in MPS IIIA compared to unaffected dog tissue (Supplementary Fig. 1). Finally, no consistent change in MPS IIIA versus unaffected tissues was noted in the GD/GT gangliosides (data not shown).

Other Lipids

The MPS IIIA dog cerebellum, but not other brain/cord regions, exhibited a change in lipid composition in the early post-natal period. The outcomes for the most abundant lipids in the cerebellum (GC, LC, PE and PI) are shown in Supplementary Fig. 2; data for other lipid species are shown in Supplementary Figs. 3, 4 and 5.

Unlike GC and GlcSph, which appear slightly elevated in MPS IIIA in the early post-natal period (Supplementary Figs. 2a and 3a–c, h), LC appears to be somewhat reduced in MPS IIIA cerebellum (compared with unaffected dog levels) from 6 weeks of age (Supplementary Figs. 2b and 3e–f). PE, PI and PS were elevated in MPS-III A compared to unaffected cerebellum, during the early post-natal period (approximately 4 to 9.5 weeks of age; Supplementary Figs. 2c–d and 4). No consistent differences in SM, PC or PG were noted in MPS IIIA and unaffected dog brain samples (Supplementary Figs. 3i, j and 5).

LIMP-2 Immunoreactivity

There was no change in the amount of LIMP-2 expression in a 2-week-old MPS IIIA brain (c.f. unaffected dog brain; Supplementary Fig. 6); however expansion in the late endosomal/lysosomal compartment in some regions of MPS IIIA dog brain was evident from approximately 4 weeks of age (Supplementary Fig. 6a–e). Statistical evaluation of the data from the four affected dogs ≤ 9.5 weeks of age (c.f. age-matched controls) demonstrated significant increases in LIMP-2 in part of the cerebral cortex (Supplementary Fig. 6a). MPS IIIA dogs aged 24 weeks exhibited significantly higher amounts of LIMP-2 reactivity in the hippocampus (c.f. unaffected dogs; Supplementary Fig. 6c), a trend also seen in other brain regions.

The reason one unaffected animal exhibited high LIMP-2 expression in the Purkinje cell layer of the cerebellum and the caudate nucleus head is not known, and aberrations were not noted in any other brain region/stain.

Fig. 1 Normalised heparan sulphate peak area in (a) cerebral cortex, (b) hippocampus, (c) cerebellum and (d) spinal cord taken from 2- to 24-week-old MPS IIIA and unaffected pups. Each data point reflects a single dog. Filled square 2-week-old pups, filled circle 4-week-old

pups, filled triangle 6-week-old pups, filled diamond 9.5-week-old pups, filled inverted triangle 24-week-old pups. *a.u.* arbitrary units. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Doublecortin Immunoreactivity

Doublecortin is a microtubule-associated protein expressed by neural stem cells and neuronal precursors (Horesh et al. 1999; De Nevi et al. 2013), necessary for early neuronal migration and cortical layer formation (Toriyama et al. 2012). Here we observed a 'peak' in doublecortin immunoreactivity at 2 weeks of age in all unaffected dog brain regions assessed (Supplementary Fig. 7). The level of staining dramatically declined by 24 weeks of age in most but not all areas, e.g. the dentate gyrus and the subventricular zone continued to express high levels of double-

cortin throughout. These areas maintain neurogenic potential into adult life.

At 2 weeks of age, select regions of the MPS IIIA dog brain appeared to display lower amounts of doublecortin immunoreactivity than the unaffected brain: anterior cerebral cortex, CA3, dentate gyrus and, to a lesser extent, the molecular layer of the cerebellum and caudate nucleus head. From 4 to 24 weeks of age, there was no consistent difference in the staining observed in MPS IIIA and unaffected brain, with the exception of the fasciculus subcallosus, where we observed maintenance of doublecortin reactivity compared to a large reduction in staining with age in unaffected dog brain.

Fig. 2 Quantification of GM2 36:1 and GM3 36:1 in 2–24-week-old MPS IIIA and unaffected dog brain (a–f) and spinal cord (g, h).

Tissue from the hippocampus of the unaffected 6-week-old dog was unavailable. Each data point reflects a single dog. Filled square

NeuN Immunoreactivity

Neuronal nuclear antigen (NeuN) immunoreactivity marks postmitotic neurons and is therefore a measure of mature neuron number (Fig. 3). In the subventricular zone of dogs of both genotypes (Fig. 3e), a slight but transient increase in NeuN staining was observed, i.e. from 2 to 9.5 weeks of age. There was no significant difference in NeuN reactivity in any brain region assessed, when MPS IIIA and unaffected dogs were compared. In both genotypes, caudate nucleus exhibited significantly fewer NeuN-positive neurons with age. This change was limited to unaffected animals in the dentate gyrus. As the MPS IIIA cerebellum does not exhibit Purkinje cell loss until ~30 months of age (Hassiotis et al. 2014), this region was not assessed here.

Neuroinflammation

The number of lectin-positive microglia with an amoeboid appearance indicative of activation was overall higher in the MPS IIIA dog brain, when staining was compared with that in the unaffected dog brain tissue (Fig. 4). Comparison of the data emanating from dogs aged ≤ 9.5 weeks of age indicated that MPS IIIA pup brain exhibited statistically significant increases in the number of activated microglia (c.f. age-matched unaffected dogs) in all regions except the dentate gyrus. The number of activated microglia in all brain areas examined was significantly higher in MPS IIIA versus unaffected dogs by 24 weeks of age.

Discussion

Understanding disease pathogenesis is necessary for the development of diagnostic and prognostic biomarkers and also serves to guide therapeutic intervention. There is a gap in our knowledge about the natural course of disease in MPS-IIIA, as early disease-related changes in the brain have not been well characterised, particularly with regard to human and large animal models of the disorder.

We report significant and early accumulation of both primarily and secondarily stored substrates in ≤ 9.5 -week-old MPS IIIA pup brain. Total heparan sulphate-derived disaccharides were elevated ~17- to 36-fold unaffected levels by 2 weeks of age, depending on brain area and secondarily stored substrates, e.g. GM3 ganglioside were ~two to ninefold normal levels at 2 weeks of age. All brain regions in young animals examined displayed statistically significant increases in HS, but only the cerebellum

exhibited statistically significant increases in GM3 in young pups. Cerebellar-related motor dysfunction is the first clinical change in these animals, readily apparent by ~2.5 years of age (Jolly et al. 2000).

Over the time course studied here, the amount of GM3 ganglioside accumulating in MPS-IIIA dog brain was greater than the amount of stored GM2, supporting qualitative observations made using immunofluorescence in the mouse model of this disorder (McGlynn et al. 2004). As anticipated, there was no significant change in the amount of GM1, GD1 or GT1 gangliosides in MPS-IIIA dog brain at any age, a finding also made in MPS-IIIA mice (Dawson et al. 2012). Previously unreported, however, is the finding of apparent reductions in some species of LC in developing MPS-IIIA brain. This outcome was noted in the MPS-IIIA dog cerebellum from ~6 weeks of age, and levels in other regions analysed were unchanged c.f. age-matched unaffected controls. A possible explanation is impaired degradation of GM3 and the subsequently reduced recovery of LC. Alternatively, increased GM3 synthesis (which utilises LC) may be occurring at the Golgi. The reduction in LC was preceded by a transient increase in its precursor GC and was accompanied by an elevation in PE, PS and PI, the latter two of which are known to significantly enhance the enzymatic activity of β -glucocerebrosidase, which catabolises GC (Dale et al. 1976).

Examination of the extent of endosomal/lysosomal compartment expansion by staining with LIMP-2 showed that organellar enlargement was greatest in the Purkinje cell layer of the cerebellum > hilus of the hippocampus > cerebral cortex > head of the caudate nucleus in MPS-IIIA dogs. Curiously, the molecular and granular cell layers of the cerebellum did not exhibit elevations in LIMP-2-reactivity, nor did the granule cell layer of the dentate gyrus or the CA3 region of the hippocampus. This observation suggests that there are cell type- or region-specific susceptibilities to substrate accumulation and resultant endosomal/lysosomal network expansion. In support of this hypothesis, differential expression of heparan sulphate glycoforms in the MPS IIIB mouse brain has been observed (McCarty et al. 2011), with antibodies to various sulphation domains found to react with heparan sulphate-positive inclusions in the cerebral cortex, thalamus and brainstem. Species differences precluded a similar evaluation in the dog tissues.

The basis for differential accumulation of substrates/lysosomal expansion is not known but may relate to the basal metabolic rate of neuronal subtypes/brain structures

Fig. 2 (continued) 2-week-old pups, filled circle 4-week-old pups, filled triangle 6-week-old pups, filled diamond 9.5-week-old pups, filled inverted triangle 24-week-old pups. The spinal cord was

prepared in a separate batch from the brain (hippocampus, cortex and cerebellum). * $p < 0.05$, ** $p < 0.01$

Fig. 3 Quantification of NeuN-positive cells in 2–24-week-old unaffected and MPS IIIA dog brain (a–g). Each data point reflects a

single dog. Filled square 2-week-old pups, filled circle 4-week-old pups, filled triangle 6-week-old pups, filled diamond 9.5-week-old

and/or their requirement for heparan sulphate. Glucose utilisation studies in adult rats and primates indicate that energy use is highest in cerebral cortex > caudate nucleus > thalamus > cerebellum (Sokoloff et al. 1977; Kennedy et al. 1978). In young (2–6-day-old) puppies, however, the reverse is observed with brainstem > cerebellum > thalamus > caudate > cerebral cortex (Duffy et al. 1982). Further exploration of the vulnerability of different brain regions to substrate accumulation in MPS-III A is warranted and will aid in the targeting of treatments.

Application of therapeutics will be challenging in any disorder in which neurodevelopmental processes are aberrant; therefore, we investigated whether neurogenesis in the young MPS-III A dog brain was impaired by the early and progressive intracellular accumulation of heparan sulphate. N-sulphated forms of this glycan have previously been shown to be associated with cellular proliferation in neurogenic regions of the brain, e.g. subventricular zone and rostral migratory stream (Mercier and Arikawa-Hirasawa 2012), and altered neurogenesis was reported in the MPS IIIB mouse brain (Li et al. 2002).

On the whole, neurodevelopmental potential as determined by doublecortin immunoreactivity in the brain does not appear to be significantly different between MPS-III A and unaffected dogs. An early but transient reduction in immunostaining was noted in the cerebellar molecular layer, CA3, dentate gyrus, caudate nucleus head and cerebral cortex in MPS-III A compared to unaffected dog brain. This may suggest slightly earlier or reduced neuronal dispersion and cortex lamination; however, there was no difference in NeuN-positive neuronal number in any of these regions of the MPS-III A dog brain at any time.

In contrast, the subventricular zone and fasciculus subcallosus exhibited elevated doublecortin levels from 4 to 9.5 weeks and 9.5 to 24 weeks of age (respectively) in MPS-III A compared to unaffected dogs. This result may relate to the increased level of fibroblast growth factor 2 (FGF-2) protein observed in homogenates of symptomatic-stage MPS IIIB mouse brain (McCarty et al. 2011), as infusion of this molecule increases neurogenesis in both young and older mice (Jin et al. 2003). Age-specific changes in FGF-2 mRNA expression in MPS IIIB mouse brain have also been reported (Li et al. 2002).

Alternatively, as doublecortin appears to direct neuronal migration by regulating the organisation and stability of microtubules (Horesh et al. 1999; Jean et al. 2012), this

outcome may suggest aberrations in microtubule structure/stability. The subcallosal fasciculus makes significant connections between the occipital and temporal and the frontal lobe and insula (Lockard 1977). Whether the increase in doublecortin in this structure is present at later ages remains to be discerned and would shed light on the significance of this result.

Microgliosis was apparent in the MPS-III A dog brain from approximately 2 weeks of age, and the number of activated cells continued to rise with age in all regions assessed. Of note is that activated microglia were observed in all brain regions investigated, including those devoid of aberrant LIMP-2 staining. This may suggest that substrate accumulation in these regions is occurring at levels below that required to initiate endosome/lysosome expansion or that extracellular activators (such as heparan sulphate, which is exocytosed or released from storing cells in a manner yet to be determined, making its way into the cerebrospinal fluid, blood and urine) are being detected by surveilling microglia. Whether the actions of these immune cells are beneficial or detrimental (or both) in the disease process has not been definitively defined.

Whilst our study is not the first to be undertaken in a large animal model of a lysosomal storage disorder, the evaluation of disease lesions at very early stages of development has been performed only infrequently. In the canine fucosidosis brain, astrocytosis was observed from 2 months of age (Kondagari et al. 2011); in an ovine model of neuronal ceroid lipofuscinosis, progressive primary substrate accumulation was noted in the midterm foetus (Jolly et al. 1989), astrocytic activation was detected 20 days prior to birth (third trimester foetus), and MHC class II immunoreactivity was prominent in microglia at 12 days of age (Kay et al. 2006); neuronal development was not significantly disturbed at early ages (Kay et al. 2006), which is consistent with our findings.

Finally, the development of cerebral pathology in MPS III A pups was assessed, and data from the 2-, 4-, 6- and 9.5-week-old pups has been grouped. We suggest that these animals are approximately equivalent to human infants, i.e. ~2, 6 and 9 months and ~1.3 years in humans, respectively, as determined by comparing dog to cat development and then cat to human development (Clancy et al. 2007). This equivalence is indicated behaviourally in the canine compared to human with respect to associative learning/classical conditioning responses, where a 1-week-old pup is

Fig. 3 (continued) pups, filled inverted triangle 24-week-old pups. The location of the brain regions assessed is shown in (h) and corresponds to (a–g) figure regions. The photos in (i, j) show NeuN

staining in the caudate nucleus head of 24-week-old unaffected and MPS III A dogs (respectively). * $p < 0.05$

Fig. 4 Determination of the number of activated microglia in various regions of the 2–24-week-old dog brain (a–g). Each data point

reflects a single dog. Filled square 2-week-old pups, filled circle 4-week-old pups, filled triangle 6-week-old pups, filled diamond

as developed as a 2-month-old human infant. Comparison of physical capacity indicates that a 1-week-old pup, e.g. supporting the forelimbs, is equivalent to a 6-month-old human infant (Wood et al. 2003). Crawling commences up to 3 weeks of age in pups and occurs by 9 months in humans; walking commences from 3 to 4 weeks of age in pups and occurs around 13 months in humans (Wood et al. 2003).

The findings reported here are pertinent, given that newborn screening is now being established in several sites around the world, enabling early diagnosis and, if warranted or possible, early initiation of treatment. Our data indicates that early neurodevelopment is normal in the MPS IIIA brain and the neurogenic potential is retained; further evaluations are warranted however, given the limited number of samples available to this study. Should the nature and rate of human MPS IIIA brain pathology development mirror that observed here, early disease lesions are potentially reversible. Nevertheless, treatment should be started as early as practical post-diagnosis, in order achieve the best outcomes for the patient.

Synopsis

The infant-paediatric-stage mucopolysaccharidosis IIIA canine brain exhibits substantial and progressive primary and secondary substrate accumulation, with inflammatory changes that extend beyond substrate-accumulating brain regions; therapeutic intervention should occur at or soon after birth for the best outcome.

Contributions of Individual Authors

LKW carried out the experiments, analysed and interpreted the data and wrote the manuscript. NRM, RDJ, PJT, SKD and MFS carried out the experiments and interpreted the data. KMH designed the study, interpreted the data and wrote the manuscript.

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Competing Interest Statement

Leanne K. Winner declares that she has no conflict of interest.

Neil R. Marshall declares that he has no conflict of interest.

Robert D. Jolly declares that he has no conflict of interest.

Paul J. Trim declares that he has no conflict of interest.

Stephen K. Duplock declares that he has no conflict of interest.

Marten F. Snel declares that he has no conflict of interest.

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Ethics Approval

All institutional and national guidelines for the care and use of laboratory animals were followed.

Documentation of Approval from the Institutional Committee for Care and Use of Laboratory Animals (or Comparable Committee)

The Massey University Animal Ethics Committee approved ethics application MUAEC 10/93 which states within: 'Pups may be retained for breeding, used in other projects, re-homed or euthanased. Affected pups, and where indicated appropriate controls, will be grown within the existing colony. Any pups destined for clinical trials will be covered by a new animal ethics application. Pups/dogs not used in the longer term trials may be euthanased at varying ages to obtain tissues for biochemical and histological studies'.

Fig. 4 (continued) 9.5-week-old pups, filled inverted triangle 24-week-old pups. The location of the regions assessed is shown in (h) and corresponds to (a–g) figure regions. Images in (i, j) show lectin

staining in the caudate nucleus head of 24-week-old dog unaffected and MPS IIIA dog brain (respectively). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

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