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Neurological Examination of Sheep (*Ovis aries*) with Unilateral and Bilateral Quinolinic Acid Lesions of the Striatum Assessed by Magnetic Resonance Imaging

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Abstract

Acute toxic models of Huntington's disease (HD) and Parkinson's disease (PD) have been used extensively to study neuropathology and behavior in rodents and non-human primates, but not large animals. We have created an acute quinolinic acid (QA) model of HD in sheep (*Ovis aries*), first to investigate the clinical signs of ovine striatum pathology and second to assess the value of a veterinary neurological examination in the symptomology investigation. Sixteen sheep underwent two surgeries, four weeks apart, in which either QA or saline was infused into the left (unilateral) and then the right (bilateral) caudate nucleus. Neurological examinations were performed pre-surgically, two weeks after the unilateral surgery and eight weeks after the bilateral surgery. Examining veterinarians were blind to treatment group. Evidence of laterality and hind limb motor dysfunction was identified in the QA-lesioned sheep. The neurological examination identified clinical signs in two out of eight saline control sheep and four out of eight QA-lesioned sheep after the unilateral surgery and three out of eight saline control sheep and seven out of eight QA-lesioned sheep after the bilateral lesion surgery. There was no association between clinical profile and treatment group, or lesion size and location. While the neurological examination was moderately useful for identification of QA-lesioned sheep, it was not informative about lesion characteristics.

Key words

Basal ganglia, Excitoxicity, Putamen, Ventral striatum, Movement disorders, Locomotion

Introduction

Our knowledge of striatal function and dysfunction is derived from rodent, primate and human studies that have traditionally focused on the importance of the striatum in loss of motor control in basal ganglia disorders with characteristic motor symptomology such as Parkinson's disease (PD) and Huntington's disease (HD). However, the integrative role of the striatum with extensive motor, sensory, emotive and cognitive inputs suggests that pathology of this structure will have a broad clinical symptomology [1].

The striatum is comprised of the dorsal striatum (caudate nucleus and putamen) and ventral striatum (which includes the olfactory tubercule) [2]. While at first glance the striatum appears to be a homogenous structure, its

functional anatomy is complex and still not fully understood. The anatomical organization of the striatum includes a compartmentalization of neurones into matrix and striosome [3-5], afferent and efferent topographical regionalization [6, 7], and direct and indirect output pathways [8, 9]. Furthermore, our understanding of striatal functional anatomy is constantly being challenged and refined. For example, reclassification of striatal subdivisions has been suggested based on their interaction with cerebrocortical networks [10] and the role of the striatum has expanded from being simply part of the action selection cascade to one including monitoring and refining pre-selected actions [11]. Predicting a phenotype based on striatal pathology is therefore difficult, and many disorders that result in significant striatal pathology, including HD or PD, have complex and variable phenotypes [12, 13]. Extra-striatal pathology, particularly degeneration of the cerebral cortex, also contributes to symptomology seen in HD and PD [13].

Species variability in striatal function/dysfunction may affect translation of findings between species. For example, unlike humans, selective striatal neurodegeneration does not occur in most transgenic mouse models of HD [14], and motor symptoms in non-human primate models of PD require extensive dopamine depletion in the caudate nucleus and putamen. In idiopathic PD patients, dopamine depletion occurs primarily in the putamen [15]. Striatal function and dysfunction have recently been reviewed in detail [16-18].

The first aim of this study was to characterize the clinical manifestation of striatal lesions in sheep. Sheep have basal ganglia and other brain structures anatomically similar to primates and are increasingly being recognized as an important species for translational neurodegeneration research [19]. Functional anatomy of the basal ganglia and their outputs, however, is poorly characterized in sheep. As sheep are ungulate ruminant quadrupeds, their pyramidal and extrapyramidal pathways are likely to be different to those of rodents and primates, which utilize fine motor control to reach, grasp and climb. It thus seems unlikely that ruminants with striatal dysfunction will have a clinical presentation similar to that of rodents or non-human primates.

A standard veterinary neurological assessment has never been developed for rodents, and is not possible to conduct in non-human primate neurodegenerative models for reasons of safety. Rather, a battery of specific neurological tests is used to identify and characterize striatal dysfunction in these species, e.g. forelimb movement patterns, elevated body swing test and grip strength test in excitotoxin-lesioned rats [20-22], or staircase-based and object retrieval-detour tasks in non-human primates [23, 24]. There is, however, a standard veterinary neurological examination, that can be adapted for sheep. Therefore, the second aim in this study was to evaluate the usefulness of a neurological examination of sheep for diagnosing and characterizing the phenotype of sheep with quinolinic acid (QA) lesions of the striatum. QA is an excitotoxin that has been used extensively in rats [25-29], mice [30, 31], and non-human primates [23, 32, 33] to lesion the striatum and create an acute toxic model of HD.

QA is an n-methyl-D-aspartate (NMDA) glutamate receptor agonist that selectively spares nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase-positive neurons and axons of passage in a manner that resembles neuronal sparing seen in HD [34]. By performing a standard veterinary neurological examination on sheep with QA-induced striatal lesions, we were able to determine the value of the examination as part of a tool-kit for assessing neurological function in ruminant models of neurodegenerative disease. The data obtained enabled construction of a symptom profile for sheep with significant striatal damage.

Methods and Materials

Animals

Sixteen castrated-male, 18-month old, Merino-Border Leicester cross sheep (*Ovis aries*), weight range 58–65 kg, were sourced from a single property in Burra, South Australia. The sheep were transported to SAHMRI Gilles Plains, Australia, where all experimental procedures were conducted. The study was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013) and approved by the South Australian Health and Medical Research Institute (SAHMRI) and University of Adelaide Animal Ethics Committees.

Sheep were initially housed in a paddock where they were habituated to researchers and facility staff for six weeks. Three days prior to surgery, the sheep were penned individually in an indoor facility where they remained until they had fully recovered from the surgery, at which point they were group-housed in large outdoor pens for the remainder of the experiment. Sheep were fed meadow and lucerne hay for the duration of the experiment, with additional grain and Lauke Feedlot pellets (Lauke Mills, SA, Australia) when penned. Sheep were randomly assigned to either experimental or control groups. At the conclusion of the study, sheep were euthanised with an intravenous injection of 0.5 ml/kg of 325 mg/ml pentobarbitone sodium (Lethabarb, Virbac, Australia). After death, the heads were perfusion-fixed with 10% neutral buffered formalin before the brains were removed and placed in 10% neutral buffered formalin.

Chemicals and surgical method

Two surgical procedures, four weeks apart, were performed on each sheep. The surgery was based on a similar surgical procedure published in a study that injected an AAV9 vector into the brain of transgenic HD sheep [35]. Experimental sheep had QA infused into the caudate nucleus during each surgery. Control sheep received saline infusions *in lieu* of QA. The surgeon was blinded to the treatment group of the sheep. Determination of the dose was based on the dose range used in rodent and primate studies [27, 36-38]. A pilot study was performed to assess the effect of the chosen dose of QA in sheep (data not shown). In this pilot study, 10 sheep were given bilateral QA lesions, eight to twelve weeks apart and monitored for up to six months before being killed. Immunohistochemistry (see below) was performed on the

perfused brains of the pilot study sheep to assess the lesions. We aimed to find a dose that was large enough to cause a significant striatal lesion from a single dose but that did not evoke obvious clinical signs in the animals.

Immediately prior to surgery, QA (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) was dissolved in 1 M NaOH and neutralized to pH 7 with 0.1 M phosphate buffered saline (PBS). A contrast agent, dimeglumine gadopentetate (Magnevist, Bayer, Germany), was combined with the QA to make a QA solution (QA 180 mM, gadolinium 2 mM), stored in a BD 1 ml tuberculin syringe. The syringe was placed on ice in a lightproof polystyrene container until required during the surgery.

During the first surgery, QA (180 mM, 75 µL) or saline (75 µL) was infused into the head of the left caudate nucleus to create a unilateral (experimental) or sham (control) lesion. During the second surgery QA or saline (at the same concentration and volume as previous) was infused into the head of the right caudate nucleus to create a bilateral striatal or sham lesion. Initial rostral, lateral and ventral stereotaxic coordinates for targeting the caudate nucleus were based on cadaver surgeries with the coordinates being progressively refined after each stereotaxic surgical procedure. The stereotaxic coordinate range for targeting the head of the caudate nucleus was taken with bregma as 0,0,0: rostral 19 – 24 mm, lateral 4 – 6 mm, ventral 22 – 24 mm. General anesthesia was induced in the sheep using 5 mg/kg of 100 mg/ml ketamine hydrochloride (Ceva Animal Health Pty Ltd, Australia) and 0.4 mg/kg of 5 mg/ml diazepam (Pamlin, Ceva Animal Health Pty Ltd, Australia) administered via the jugular vein. Endotracheal intubation allowed sheep to be mechanically ventilated and anesthesia maintained using 2 – 2.5% isoflurane. A 4.5" 16-gauge catheter was inserted into the jugular vein and lactated Ringers solution (Hartmann's, Baxter Healthcare Pty Ltd, Australia) was administered at a rate of 10 ml/kg/hour. Sheep received 2 mg/kg of 50 mg/ml carprofen (Carprieve, Norbrook Laboratories Australia Pty Ltd) before being positioned in a stereotaxic frame (Kopf, model number 1630; Tujunga, CA, USA).

Sheep were given 3 g of 1 g cefazolin sodium (Cefazolin-AFT, AFT Pharmaceuticals, New Zealand) intravenously during surgery. Post-surgery, 22 mg/kg of 300 mg/mL procaine penicillin (Depocillin, MSD Animal Health, Australia) was administered daily for three days.

Sheep were placed in the sphinx position on a large elevated cylindrical pad; the ventrum of the sheep rested on top of the pad and the limbs extended down each side of the cylindrical pad, minimizing risk of pressure neuropathy or myopathy. The head was placed in a large animal stereotaxic frame. Ear bars and orbital notch prongs were adjusted such that a line between the ventral orbital rim and the horizontal canal of the external auditory meatus was parallel to the horizontal plane and perpendicular to the manipulator apparatus. The cranium was shaved and aseptically prepared. Sterile surgical techniques were maintained throughout the surgical procedure. A monopolar electrocautery was used to create a 3 cm curvilinear incision through the dermal and subcutaneous layers just caudal to the poll of the sheep's head. A rostral 3 cm incision was extended perpendicular to the curvilinear incision. The dermal, subcutaneous and periosteal layers were reflected to expose the skull. Bregma was identified and marked as a reference point using a surgical pen. A 3 – 4 mm burr hole craniotomy was performed to expose the dura using a Dremel 8220 drill (Dremel, Racine, WI, USA) at appropriate coordinates rostral to bregma and lateral to the midline. The manipulator was mounted on the Kopf stereotaxic frame at the predefined rostral and lateral coordinates and a convection-enhanced delivery (CED) cannula (MRI Interventions, Irvine, CA, USA) secured to the manipulator. The CED cannula was attached to a 25 cm Luer lock extension set that was secured to the syringe that contained the QA or saline. An infusion pump (New Era Pump Systems Syringe Pump NE-1000, Farmingdale, NY, USA) was used to purge air from the CED cannula and extension line. A 1.5 mm incision was made through the dura permitting the CED cannula to traverse the dura, while ensuring the dura closely adhered to the cannula to minimize cerebrospinal fluid leakage.

A micro-manipulator was used to lower the CED cannula to the predefined ventral coordinate from the dural surface and the exposed skull covered with saline-soaked gauze. QA or saline infusion was started 5 minutes after the CED cannula was lowered to the ventral coordinate to allow tissue disrupted by the CED cannula to stabilize. QA or saline was infused at a rate of 2 µl/minute until the desired volume was completed. The CED cannula was left *in situ* for 10 minutes after the infusion ended before being slowly withdrawn over 10 minutes. Bone wax was used to seal the craniotomy after CED cannula removal. The wound was irrigated with saline and closed using monofilament absorbable suture. The sheep was removed from the stereotaxic frame and recovered from the anesthetic.

Magnetic Resonance Imaging

T1-MPRAGE sequences were performed on the sheep using a 3T Siemens Skyra scanner (Erlangen, Germany) at four time points; prior to the first surgery, seven days after the first surgical procedure, seven days after the second surgical procedure and twelve weeks after the second surgical procedure. Sheep were anaesthetized during each MRI. Anesthesia was induced with 20 mg/kg of 1000 mg/g thiopentone sodium (Ilium, Troy Laboratories, Australia), administered to effect in the jugular vein and maintained using 2% isoflurane. ITK-Snap software [39] was used to visualize and segment lesions visible on the T1-MPRAGE images and calculate lesion volumes.

Neurological examination procedure

Three neurological examinations were performed on the sheep; prior to the first surgery, two weeks after the first surgery and eight weeks after the second surgery. Two experienced veterinarians performed each examination. Both neurological examiners were blinded to whether a sheep was an experimental or control animal and did not see diagnostic MRI, however one veterinarian assumed primary responsibility for the sheep and therefore was not blind to the

planned proportion of sheep that received QA lesions or the surgical recovery. The surgical recovery was not discussed by the two veterinarians.

Neurological examination protocol

Neurological examinations were based on a standard canine veterinary neurological examination [40], modified to be suitable for sheep. Neurological examinations were performed using a systematic, repeatable method for consistency between examinations and sheep. All sheep recovered fully from surgery and appeared functionally normal prior to neurological examinations. The day before the neurological examination, the sheep were housed in individual pens in the testing area (Figure 1) to give them time to acclimatize to the testing area. Sheep were examined individually.

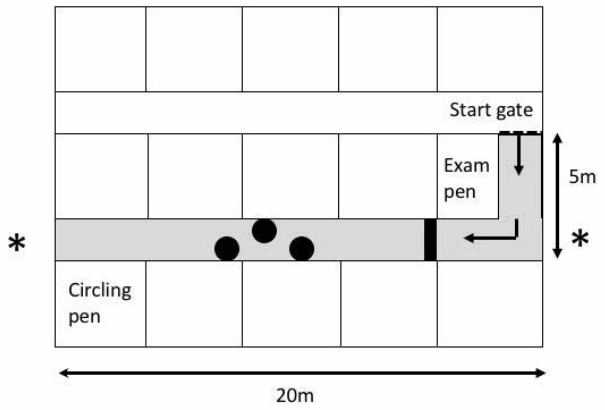

Figure 1: Diagram of the neurological examination facility. The solid black rectangle represents a 30 cm high, 40 cm wide step. The three solid black circles represent three 100 cm high, 60 cm wide bins. The * symbols indicate where observers stood as sheep navigated the L-shaped race (shaded gray) that began at the start gate (dashed line) and finished at the circling pen.

Initially a distance examination was performed with demeanor, behavior, gait (without exerting pressure to move) and posture of each sheep assessed in their individual pen. Gait was further evaluated using a twenty-five meter 'L' shaped race that led into a 4 meter by 4 meter square pen. The race contained a 30 cm high, 40 cm wide obstacle that traversed the race at the start of the long arm of the 'L' forcing the sheep to jump. Three cylindrical bins, 1 meter high by 0.6 meters wide were placed 5 meters past the jump in a pattern that forced the sheep to zig-zag. One examiner was placed at each end of the race to allow gait assessment from the front and back of each sheep as the sheep moved in a straight line and negotiated obstacles. The square pen was used to assess gait while circling the sheep clockwise and anticlockwise. Examination of the gait was used to detect evidence of paresis, ataxia, and hypo- or hypermetria.

After the distance examination was performed, each sheep was brought into a smaller 3 meter by 3 meter pen for the rest of the neurological examination. Pupil size and location, menace reflex, palpebral reflex, dazzle response, direct and indirect pupillary light reflex, nystagmus (presence of physiologic, absence of spontaneous), facial and pinnae sensation and response, facial symmetry, swallowing and tongue movement were evaluated to assess cranial nerves: II (optic), III (oculomotor), IV (trochlear), V (trigeminal), VI (abducens), VII (facial), VIII (vestibulocochlear), IX (glossopharyngeal), X (vagus) and XII (hypoglossal). Each cranial nerve assessment was described as either normal, decreased or absent.

Muscular tone and evidence of atrophy was evaluated by palpating the sheep while standing and exerting appropriate pressure over the front and hind limbs. Muscle tone was described as hyper, normal or decreased. Cutaneous trunci, tail and anal tone and the perineal reflex were also assessed while the sheep was standing and described as normal, decreased or absent.

Postural reactions were assessed with both examiners handling the sheep. Postural reactions were described as either normal, decreased or absent. The sheep were lightly restrained and not allowed to lean on the examiner or any object. Knuckling of the distal limb, crossover of fore and hind limbs, bilateral side-hopping and wheelbarrowing of fore and hind limbs was assessed. For the side-hopping assessment, one examiner supported the hind limb and abdomen while the other examiner supported the front limb and elevated the head and neck to the level of the horizontal plane. For the wheelbarrowing, the elevated limbs were held at the horizontal plane with one examiner holding each limb.

After assessment of postural reactions, the sheep were placed in lateral recumbency. Fore- and hind limb flexor withdrawal and hind limb patellar reflexes were evaluated as hyper, normal, decreased or absent. Presence or absence of a crossed extensor response was noted. The sheep was then allowed to stand with coordination of the standing process and time to stand evaluated as normal or decreased.

At the end of all the individual neurological examinations, the sheep were group housed in a 4 meter by 4 meter pen and behavior in a flock evaluated as either appropriate or inappropriate with any inappropriate behavior described.

Histology and post-mortem analysis of lesions

A midline sagittal incision was performed to split the perfused brain into two halves. A coronal slice was made through the striatum at the level of the optic chiasm. Sequential coronal blocks (5 mm) were cut rostrally and caudally from the initial incision. Gross anatomy of the coronal blocks was examined. Each block was then paraffin embedded. Sections (5 μm) were cut from the face of each block using a microtome. Sections were stained using haematoxylin and eosin. An avidin – biotin peroxidase technique was used for glial fibrillary acidic protein (anti-GFAP antibody, DAKO, 1/13000) immunohistochemical staining. Light microscopy was performed to examine the sections.

Results

Surgical recovery

After the left side (unilateral) surgery, one control and five QA-lesioned sheep developed hind limb paresis and

proprioceptive deficits. In four of the eight QA-lesioned sheep, clinical signs were mild, with a narrow, slightly crouched hind limb posture and crossing over of the hind limbs when standing or turning. One QA-lesioned sheep (QA8) developed mild – moderate hind limb paresis with ambulatory deficits when pressured to move, a slightly crouched hind limb posture and an inability to resist pressure over the hind limbs. Two QA-lesioned sheep with hind limb dysfunction (QA7, QA8), showed handling-induced orofacial dyskinesia and temporary inappetence. One of the QA-lesioned sheep with mild hind limb dysfunction (QA4) developed intermittent spontaneous anticlockwise circling, which was exacerbated by handling. Two other QA-lesioned sheep had reduced left pinna tone (QA4, QA5). All observable clinical signs resolved completely within one week, with sheep appearing normal under observation thereafter.

After the second surgery, one control (Control 8) and one QA-lesioned sheep (QA4) developed mild hind limb paresis and mild proprioceptive deficits. Two QA-lesioned sheep developed handling-induced orofacial dyskinesia and temporary inappetence (QA5, QA6), with one of the two sheep developing mild hind limb paresis and the other sheep developing a narrow hind limb stance with upright fetlocks, a wide–based forelimb stance, a very mild intention tremor and a tendency to circle left. All observable clinical signs resolved completely within one and a half weeks.

Neurological examination

Table 1 provides a summary of findings from the neurological examinations of the control and QA-lesioned sheep. There were no abnormalities detected during the pre-surgical neurological examinations of the sheep. Clinical signs were identified in two out of eight unilateral saline-injected (control) sheep and three out of eight bilateral saline control sheep during the post-surgical neurological examinations. Two control sheep had possible or very mild hind limb dysfunction identified during the unilateral examination, with one of those two sheep having a side preference during the bilateral examination. Two different control sheep had possible or very mild hind limb dysfunction identified during the bilateral examination. Per examination, clinical signs identified in control sheep were mild and limited in number, compared to the identification of multiple clinical signs in QA-lesioned sheep.

Three out of eight QA-lesioned sheep had evidence of hind limb dysfunction including reduced muscle tone, gait abnormalities and postural deficits during the unilateral examination, with one of those three sheep spontaneously circling to the right. The sheep that developed mild – moderate hind limb paresis and orofacial dyskinesia following unilateral surgery (QA8) was the most clinically-affected sheep with evidence of hind limb paresis during examination. Interestingly, the other sheep with post-surgical orofacial dyskinesia and hind limb dysfunction (QA7) had no clinical findings during the unilateral examination.

Seven out of eight QA-lesioned sheep had evidence of hind limb dysfunction and laterality during the bilateral examination, including circling, gait abnormalities, postural deficits and decreased hind limb tone. The most clinically affected sheep during the bilateral neurological examination was also the most clinically affected sheep during the unilateral neurological examination (QA8). The two sheep with the most severe clinical signs during surgical recovery after the second surgery were the least affected during the bilateral neurological examination. During the bilateral neurological examination, the sheep with upright hind limb fetlocks during surgical recovery (QA5) successfully jumped out of a holding pen when initially approached and only had a mildly abnormal narrow stance and a slow yet coordinated rise from lateral recumbency, while the other sheep with orofacial dyskinesia (QA6) had no clinical findings during the bilateral neurological examination.

Lesion location and volume

No lesions or evidence of sub-cortical structural abnormalities were visible on the MRI scan of any of the sheep prior to surgery or on any of the scans of the eight-control sheep after surgery. Of the eight QA-lesioned sheep, five had clearly visible bilateral striatal lesions, one had a large lesion in the right caudate nucleus and a small lesion in the left caudate nucleus, one had a large right caudate nucleus lesion only and one sheep had a small lesion in the left caudate nucleus. Five of the eight sheep (QA1, QA5 – QA8) also exhibited cortical hyperintensity with minor accompanying histological pathology. In all cases this appeared ipsilateral to the QA-induced striatal lesions and principally in the anterior insular cortex with inconsistent involvement of other structures in the frontal and temporal lobe.

The head of the caudate nucleus was the predominant structure lesioned in all sheep, with inconsistent involvement of the putamen, ventral striatum and cortex. Table 2 shows lesion volume and location for individual sheep identified

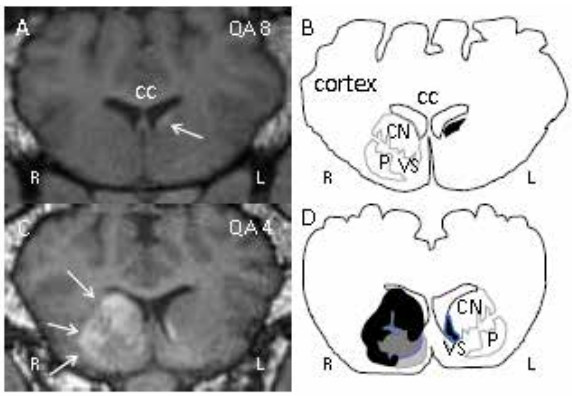

Figure 2: Comparison of the largest and smallest QA lesion by MRI one week after surgery. Coronal slice comparing the smallest (**A**, **B**, top row, first lesion in the left striatum) with the largest QA lesion (**C**, **D**, second lesion in the right striatum) one week after lesion surgery. Images in A and C are from T1-MPRAGE MRI scans. White arrows point to the hyperintensity of the QA lesion. **B** and **D** are stylized cartoons of the sections in **A** and **B** with the lesions shaded in black/grey. Approximate locations of caudate nucleus (CN), putamen (P) and ventral striatum (VS) are indicated in grey in the cartoons. The lesion is confined to the CN in QA8. In QA4, the lesion is visible in the CN, P and VS on the right side. There is also some hyperintensity visible in the CN and VS on the left side from the first lesion in this sheep. cc=corpus callosum.

Table 1: Neurological examination results for individual sheep.

Animal ID	Pre-surgical	Two weeks after the left side surgery	Eight weeks after the right side surgery
QA1	nad	nad	Spontaneous slow moderately-tight circling left
			Narrow hind limb stance
			Hind foot placement frequently rotated when standing
			Occasional overextension left hind when circling
			Side-hopping mild delay left hind
			Slow coordinated rise from lateral recumbency
QA2	nad	nad	Reluctant to circle to left
			Mild decreased muscle tone left hind
QA3	nad	Occasional right hind limb scuff and knuckling when circling	Reluctant to circle to left
		Narrow hind limb stance	Occasional overextension right hind when circling
			Incorrect correction of cross over right hind
			Slow hind limb wheelbarrow
			Collapsed once on forelimb wheelbarrow
			Slow coordinated rise from lateral recumbency
			Mild right hind muscle atrophy
			Mild decreased muscle tone both hind limbs
QA4	nad	Spontaneous moderately-fast tight circling right	Spontaneous moderately-fast tight circling right
		Reluctant to circle left	Very reluctant to circle to left
		Narrow hind limb stance	Side-hopping mild delay left and right hind
		Hind foot placement frequently rotated when standing	Decreased hind limb muscle tone
		Failure to correct left or right hind limb crossover placement	
QA5	nad	Decreased left ear tone and sensation	Mild narrow hind limb stance
			Slow coordinated rise from lateral recumbency
QA6	nad	nad	nad
QA7	nad	nad	Spontaneous moderately tight circling right
			Reluctance to circle left
			Narrow hind limb stance
			Hind foot placement frequently rotated when standing
			Failure to correct hind limb crossover placement
QA8	nad	Narrow hind limb stance	Spontaneous moderately tight circling right
		Slightly crouched in hind limbs	Narrow hind limb stance
		Occasional crossing over of hind limbs when circling	Hind foot placement frequently rotated when standing
		Slow coordinated rise from lateral recumbency	Side-hopping mild delay left and right hind
		Very mild reduced muscle tone hind limbs	Decreased hind limb muscle tone
		Repeatable collapse on hind limb wheelbarrow	Occasional crossing over of hind limbs when turning
		Failure to correct hind limb crossover placement	Occasional overextension of hind and stepping on forelimbs
Control 1	nad	nad	nad
Control 2	nad	nad	nad
Control 3	nad	nad	nad
Control 4	nad	Slightly crouched in hind limbs	Reluctant to circle to left
Control 5	nad	Occasional mild hind limb bunny hop gait	nad
Control 6	nad	nad	Very mild upright hind limb posture
Control 7	nad	nad	Possible / very mild left and right hind side-hop delay
			Possible / very mild hind limb wheelbarrow delay
			Left fore flexor withdrawal decreased response
Control 8	nad	nad	nad

nad= No abnormalities detected

by MRI. Figure 2 shows MRI images of the sheep with the largest striatal lesion visible on MRI (QA4), compared to that with the smallest striatal visible lesion (QA8). Atrophy of the affected caudate nucleus with concomitant enlargement of the lateral ventricle is evident on the final MRI in all lesioned sheep with the morphology of the lateral ventricles in control sheep unchanged (Figure 3).

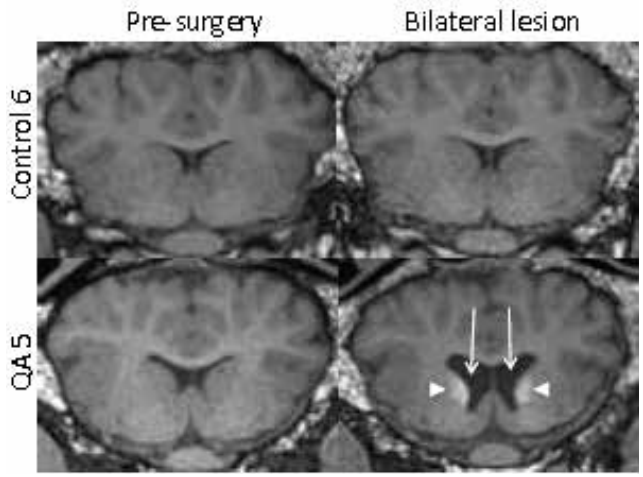

Figure 3: Ventricular enlargement is seen after QA lesions. Examples of MRI T1-MPRAGE coronal slices showing enlargement of the lateral ventricles (white arrows) and corresponding atrophy of the caudate nucleus in a QA-lesioned sheep with bilateral lesions. Lesions are visible as hyperintensity in the caudate nucleus (white arrow heads in lower right image). No change in ventricle volume is seen in the matched control saline-treated (Control 6) sheep. Images are taken from scans conducted at two time points; pre-surgery and 12 weeks after the second lesion (bilateral lesion).

Gross pathological, histological and immunohistochemical examination of QA-lesioned brains revealed that all sheep had striatal lesions as indicated on MRI. Lesions were typical of those described in other species. That is, they were characterized by reactive gliosis with a core devoid of neurons surrounded by a penumbra with heterogeneous graduated neuronal damage (data not shown).

There was no clear relationship between the neurological examination findings and striatal lesion size. One sheep with comparatively severe hind limb dysfunction, evident during both post-surgical neurological examinations, had only a small lesion evident on MRI (QA8, Figure 2). The sheep with upright fetlocks during surgical recovery had the largest lesions evident on MRI (QA5, Figure 3), yet was one of the least clinically affected sheep on neurological examination. Table 2 provides a subjective ranking of striatal lesion volume per sheep and neurological examination findings. There was also no relationship between neurological examination findings and striatal lesion location. Two sheep (QA4, QA5) had large bilateral lesions affecting the caudate nucleus, putamen and ventral striatum. Despite lesioning of similar structures, their clinical presentation was different, with one of the two sheep (QA4) presenting with milder clinical signs during surgical recovery yet significantly more clinical findings during the neurological examination. Two sheep (QA2, QA3) had caudate nucleus-only lesions on both sides with only one of the two sheep (QA3) displaying clinical signs during the unilateral neurological examination. Of the two sheep, the same sheep (QA3) displayed significantly more clinical findings during the third neurological examination (after both sides had been lesioned).

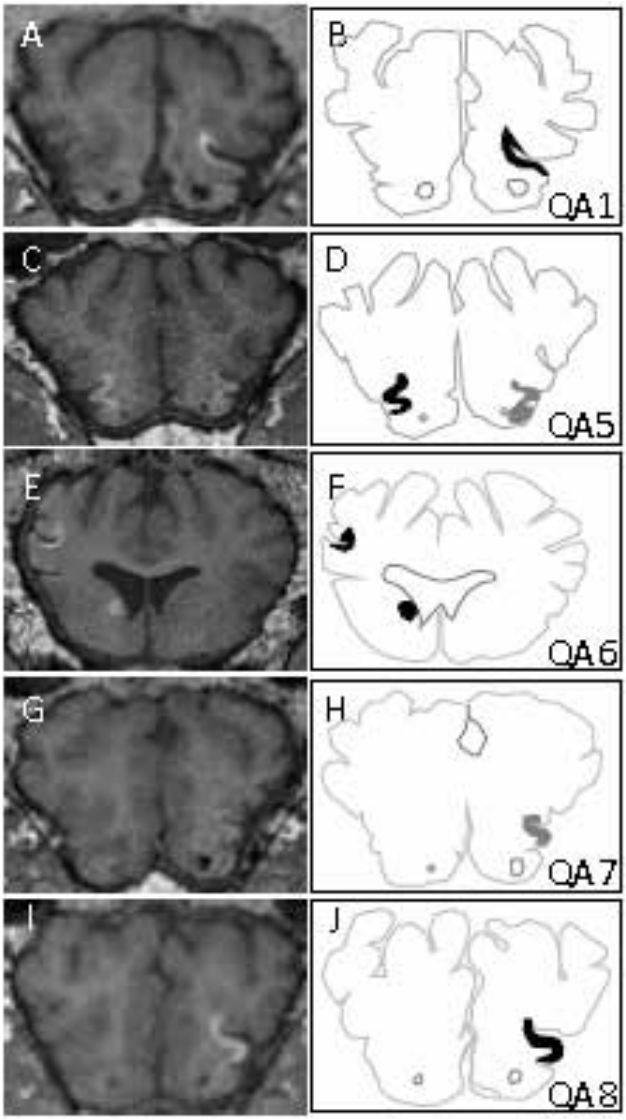

Figure 4: Pathology of the cerebral cortex is seen in addition to QA lesions of the striatum. Examples of MRI T1-MPRAGE coronal slices (**A**, **C**, **E**, **G**, **I**) from each animal showing hyperintensity of the cerebral cortex. Hyperintensity was on the side ipsilateral to the striatal injection site (that is not necessarily in the section). The section from each animal was chosen to show the highest intensity of cortical hyperintensity. **B**, **D**, **F**, **H** are stylized cartoons of the sections, with regions of hyperintensity shaded in black/grey.

Finally, the veterinary neurological examinations were unable to identify a phenotype associated with the cerebral cortex pathology (Figure 4). Lesioning of the striatum was the most important factor for the development of clinical signs detectable using a veterinary neurological examination. During the neurological examination after the first surgery, four QA-lesioned animals had clinical signs detected, with hind limb dysfunction identified in three of those four animals (QA2, QA3, QA4), and decreased ear tone and sensation identified in one animal (QA5). None of the three sheep with hind-limb dysfunction had cortical pathology in addition to striatal pathology. QA5 had additional cortical pathology,

Table 2: Location and volume of striatal lesions visible on a T1-MPRAGE MRI with a subjective ranking of QA lesion volume and neurological examination severity for comparison.

ID	Side	Location	Volume of striatal lesion visible				
			on MRI (mm^3)			Subjective Ranking	
			1 week	4 weeks (left only)	8 weeks (right) 12 weeks (left)	Combined lesion volume	Neurological exams
QA1	Left	VS, IC, GR, Ci	0	0	0	2	4
	Right	CN, P, GR, Ci	985		166		
QA2	Left	CN	523	149	6	5	2=
	Right	CN	290		31		
QA3	Left	CN	528	199	16	4	6
	Right	CN	146		19		
QA4	Left	CN, P, VS	672	484	48	7	5
	Right	CN, P, VS	1559		270		
QA5	Left	CN, P, VS, IC, GR	910	861	353	8	2=
	Right	CN, P, VS, IC	1527		491		
QA6	Left	CN	40	14	10	3	1
	Right	CN, P, VS, IC, GR, OF, Sy, Si	522		166		
QA7	Left	CN, P, VS, IC	761	377	118	6	7
	Right	CN, P	830		210		
QA8	Left	CN, VS, IC	11	10	0	1	8
	Right	-	0		0		

[1]CN: caudate nucleus; P: putamen; VS: ventral striatum; O: olfactory tract and bulb; IC: insular cortex; GR: gyrus rectus; Ci: cingulate gyrus; OF: orbital-frontal gyrus; Sy: sylvian gyrus; Si: sygmoideus gyrus

[2]Subjective Ranking: Combined lesion volume, 1= smallest, 8=largest; Neurological exam, 1=least affected, 8=most affected.

however the clinical signs exhibited by QA5 are consistent with a recognized stereotactic frame complication due to the ear bar-induced facial nerve inflammation rather than cortical dysfunction. During the neurological examination after the second surgery, clinical signs were found in seven out of eight sheep. Three of the five sheep with pathology of the cerebral cortex (QA6, QA8, QA5) incurred the lesions during the second surgery (QA5 developed cortical pathology after both surgeries, ipsilateral to the surgeries). No clinical signs were detected during the neurological examination of QA6. QA8 and QA5 had evidence of hind limb dysfunction, as seen prior to the cortical pathology and also in sheep without cortical lesions.

Discussion

In this study we evaluated the usefulness of a veterinary neurological examination for identifying and characterizing clinical signs in sheep with striatal lesions, principally affecting the caudate nucleus. All animals had lesions of the striatum while a smaller number showed additional hyperintensity of the cerebral cortex. The results suggest that a neurological examination is a reasonable method for detection of sheep with bilateral striatal lesions, though it is unreliable for identification of sheep with unilateral striatal lesions. The neurological examination was poor at identifying behavioral changes relating to rostroventral and rostrolateral cortical pathology and poor at characterizing the magnitude or extent of striatal or cortical pathology.

Therapeutic research ideally targets pre-clinical or early clinical disease phases. We created a degree of striatal and cortical pathology that produces a normal-appearing phenotype, unless the sheep was interrogated or provoked. Careful neurological evaluation identified a characteristic phenotype associated with bilateral striatal lesions. This consisted of mild hind limb paresis, mild hind limb proprioceptive deficits and evidence of laterality with either spontaneous or handling-induced rotation in one direction or reluctance to turn in one direction.

There were a number of clinical signs identified in control sheep, which may be due to pathology associated with the saline infusion or may (more likely) reflect the subjective nature of the veterinary neurological examination. Clinically evident unilateral QA-lesioned and bilateral QA-lesioned sheep had the same characteristic phenotype, with the phenotype being more pronounced in bilateral-lesioned sheep. Four sheep frequently rotated one or both hind limbs when standing. The rotation occurred without muscle contracture and appeared to be proprioceptive rather than dystonic. There was no evidence of the spontaneous chorea, dyskinesia or dystonia that has been reported in primates [36, 41]. Spontaneous chorea, dyskinesia and dystonia in primates typically resolved over time. Failure to identify these motor abnormalities in sheep

during the neurological examinations may reflect the delay between surgical lesioning and neurological examination. Lack of spontaneous chorea, dystonia or dyskinesia in our study may also reflect our choice of dose of QA. We chose a dose that would produce sheep with subtle symptomology reflecting an early stage of HD, and this may not be large enough to cause chorea, dystonia or dyskinesia.

Two sheep had mild cranial nerve deficits after the unilateral surgery, specifically reduced pinna tone. CNVII (the facial nerve) disease can result in reduced pinna tone and can occur secondary to middle ear inflammation [40]. Pinna tone was normal in these sheep at the final neurological examination. We believe the cranial nerve deficits were a surgical complication resulting from ear bar placement and not part of the pathological striatum phenotype in sheep.

Laterality can result from differences in dopamine levels between the two striata of an individual [42]. Laterality, either spontaneous or induced, is a variable clinical feature in rodents and primates with excitotoxic lesioning of the striatum [23, 43, 44]. Lower limb motor dysfunction, especially dystonia, has been identified in primates with excitotoxic lesions of the striatum [45, 46] however paresis of the lower limbs has not been reported previously. Postural deficits are consistent with lesions of the extrapyramidal tract and are common in basal ganglia disorders like HD and PD [47, 48].

The neurological examination was an unreliable modality for detection of unilateral striatal lesions with clinical signs detected in only four out of eight sheep after unilateral surgery, despite lesions being evident on MRI. The difficulty of unilateral striatal lesion detection in sheep is consistent with other species: Non-human primates typically appeared unaffected or display mild, transient clinical signs following unilateral excitotoxic lesioning [36, 41, 43] necessitating the use of dopaminergic agonists to induce clinical signs; while rodents with unilateral QA lesions of the striatum required sophisticated quantitative behavioral tests principally involving complex reaching tasks [22, 49] for reliable detection.

Rodents and primates display bilaterality in their extrapyramidal pathways with approximately 10-20% of basal ganglia output neurons projecting to the contralateral thalamus [50, 51]. The crossover of basal ganglia output neurons allows the unaffected basal ganglia to compensate for and mask clinical symptomology following a unilateral striatum lesion [52]. While the bilaterality of extrapyramidal pathways in sheep is unknown, the assumption of crossover in the efferent pathways of the basal ganglia of sheep, as in rodents and primates, would mean the unaffected basal ganglia could compensate for and mask unilateral striatal lesions, potentially necessitating a more sensitive method of detection of striatally-related neurological dysfunction than allowed by the veterinary neurological examination used in this study. The neurological examination detected all sheep with well-developed bilateral-lesions evident on MRI, though no clinical signs were detected in the sheep that had a large lesion in the right caudate nucleus and a small lesion in the left caudate nucleus. The ability of the neurological clinical examination to detect bilateral striatum lesions likely reflects a loss of bilateral pathway compensation.

Due to the difficulty associated with stereotactic placement in the sheep brain [53], delivery of QA to and pathology of extra-striatal sites was anticipated, as occurs in similar primate studies [23, 36, 54]. The inability of the neurological examination to detect cortical pathology in the QA-lesioned sheep likely reflects the exact structures lesioned, the mainly unilateral nature of the cortical pathology and associated plasticity of the brain following injury [55, 56] and the motor bias of the veterinary neurological examination. The anterior insular cortex was the principal cortical structure lesioned in those sheep with cortical pathology, with variable lesioning of the gyrus rectus, orbital-frontal cortex, anterior cingulate gyrus, lateroventral sygmoideal cortices and lateroventral sylvian cortex. These cortices have a wide range of prescribed functions [57-62], however they tend to be non-motor functions. The primary motor cortex is located within the frontal lobe and so medial and posterior to the structures lesioned (https://msu.edu/~brains/index.html).

The relationship between the proportion of a sheep's total striatal area that was-lesioned and the associated symptomology was poor, even in bilaterally-lesioned sheep. For example, one sheep had the largest lesion in both the left and right striatum yet was one of the least clinically affected of the bilaterally-lesioned sheep, while another sheep with similar sized lesions displayed marked laterality and evidence of hind limb paresis. Non-human primate studies have found that the association between lesion size and clinical signs is also not straightforward and that very large lesions involving 60% of the striatum or greater can paradoxically be associated with less choreic movement than smaller lesions [63]. The four-week delay between the left and right striatal lesions may have allowed resolution of acute reversible neuropathology on one side at the time of second lesion and the opportunity for brain plasticity mechanisms to reduce symptomology [64, 65].

The region of the striatum consistently lesioned was the head of the caudate nucleus, with inconsistent involvement of the putamen and ventral striatum. No association was identified between lesion location and the clinical profile. Non-human primate studies have found that lesion location is important for the development of dyskinesia and rotational behavior in primates, with putamen lesions associated with motor dysfunction [23, 36]. Assuming sheep display similar topographical regionality as primates, more extensive lesioning of the sheep putamen may result in a greater range and severity of clinical signs detectable by neurological examination. Notably, the topography of the striatum is heterogeneous and includes a large number of functional subdivisions [66]. Thus, even slight variation in lesion placement or size between striatae may impact different functional sub-divisions, potentially resulting in different symptomology.

The histological and immunohistochemical examination findings are consistent with QA-induced changes in the striatum of rodents and non-human primates [25, 33, 67, 68]. Ventricular enlargement and corresponding striatal atrophy were visible in the MRI of QA-lesioned sheep. There are some excellent papers that provide immunohistochemical

characterization of the QA-lesioned rodent and primate brains [25, 33, 67, 68]. There is no evidence to suggest that the effect of QA in the sheep brain would be different from that of the other species. Because we did not find a correlation between neurological signs and lesion size, we did not conduct a full histological analysis.

In summary, this study evaluated the use of a veterinary neurological examination as a method of phenotype identification and pathology characterization in sheep with significant excitotoxic lesions of the striatum and cortex, principally affecting the caudate nucleus of the striatum. A phenotype was identified consisting of mild hind limb motor dysfunction and laterality, however the diagnostic sensitivity of the veterinary neurological examination was moderate. The phenotype appeared to be associated with the striatal lesions, with it being more evident after bilateral striatal lesioning. Characterization of the proportion or region of the striatum and cortex lesioned was not possible in this study using a standard veterinary neurological examination. To ensure we can comprehensively evaluate striatal symptomology and pathology in sheep, more specific neurological tests of striatal dysfunction need to be developed than those currently available.

Conflict of Interest

The authors have no conflict of interest.

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