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Appraisal of state-of-the-art

Reducing suffering in experimental autoimmune encephalomyelitis (EAE)

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ABSTRACT

This report is based on discussions and submissions from an expert working group consisting of veterinarians, animal care staff and scientists with expert knowledge relevant to the field. It aims to facilitate the implementation of the Three Rs (replacement, reduction and refinement) in the use of animal models or procedures involving experimental autoimmune encephalomyelitis (EAE), an experimental model used in multiple sclerosis research. The emphasis is on refinement since this has the greatest potential for immediate implementation. Specific welfare issues are identified and discussed, and practical measures are proposed to reduce animal use and suffering. Some general issues for refinement are summarised to help achieve this, with more detail provided on a range of specific measures to reduce suffering.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an experimental model in which inflammation is induced in the central nervous system (CNS) by generating immune activity targeted at myelin. It is used as an animal model of multiple sclerosis (MS) and other diseases that involve demyelination, such as acute disseminated encephalomyelitis (ADEM; Sriram & Steiner, 2005).

EAE is recognised to have the potential to cause severe suffering in animals and is therefore a priority area for implementing all of the Three Rs. Whilst there are opportunities to replace, reduce and refine EAE, this report will focus predominately on refinement, as this has the greatest potential for immediate implementation. The potential for refinement will depend upon a number of factors, in particular on the precise scientific question that is being addressed, and on whether or not the adverse effects are a necessary component of the study. Whatever the ultimate requirements of the project, the approach to refinement should always involve careful consideration of what happens to the animal at each step of the study (including during husbandry and care, scientific procedures and adverse events), and implementation of the measures that can be taken to avoid or ameliorate any physical or psychological suffering.

2. MS and the EAE model

MS is a chronic inflammatory disorder of the CNS of unknown origin, associated with demyelination and axonal injury (Hu & Lucchinetti, 2009). It is one of the most common neurological disorders and causes of disability in young adults, involving unpredictable relapses and periods of remission, but inevitably it is a progressive disease which results in a significant deterioration in quality of life (Compton & Coles, 2008). MS is a severe disease and more effective treatments are needed (Orme, Kerrigan, Tyas, Russell, & Nixon, 2007).

The use of EAE as an animal model for MS is well established and the pathophysiology appears to be well understood. EAE can result in severe suffering in animals and there is thus a fundamental ethical dilemma associated with its use; MS is a disease that causes significant suffering in patients, so is it acceptable that research into MS also causes severe suffering in animals? This is a challenging question with no simple answer; it is clear, however, that wherever opportunities to avoid

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animal use and reduce suffering are available, they should be implemented.

It is important to note that EAE is not multiple sclerosis, which is a uniquely human disease. Although MS is poorly understood, with a complicated aetiology in terms of the autoimmune process, EAE seems to be useful in that it models many of the relevant immunological processes of MS and it has contributed to the scientific understanding of demyelination, autoimmunity, lymphocyte trafficking and the role of the blood brain barrier in CNS inflammation (Baker & Jackson, 2007; Sriram & Steiner, 2005).

EAE has been developed in a wide range of species including the rat, mouse, guinea pig, hamster, rabbit, dog, sheep, macaque and marmoset (Baxter, 2007). The most common species currently used in models of EAE are the rat and mouse (Table 1).

EAE is a spectrum of neurological disorders and many different antigens, species and strains are used in EAE studies (Vesterinen et al., 2010). Typically, EAE can be achieved by active induction or by adoptive transfer. Active induction involves animals being given a CNS antigen such as one of the following, in conjunction with an adjuvant: myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG) or S-100 protein. This results in the initiation of an immune response within the CNS, which leads to either a monophasic EAE, relapsing–remitting EAE or a chronic EAE depending on the antigen and the species or strain of animal used. In brief, the classical scenario for the development of EAE involves activation and expansion of peripheral antigen specific T-cells which enter the CNS and induce disease (Baker, Gerritsen, Rundle, & Amor, 2011; Batoulis, Recks, Addicks, & Kuerten, 2011; Denic et al., 2011; 't Hart, Gran, & Weissert, 2011). Adoptive transfer is established by administration of myelin-specific T cells from animals with actively induced EAE.

There is considered to be an unmet need for animal models of primary progressive MS disease, since this is one type of MS for which there are no effective therapies. The most commonly used models of EAE display acute monophasic disease, but chronic relapsing forms of mouse EAE (CR-EAE) have been produced by adapting the immunisation schedule and selection of the mouse strain used (Brown & McFarlin, 1981; Mokhtarian, McFarlin, & Raine, 1984; Zamvil et al., 1985). EAE in ABH Biozzi mice (Baker et al., 1990), in which the disease is 100% penetrant in the acute phase, is believed to have a high translational value (Weissert, 2012). This model is used to study the impact of the environment on lesion pathogenesis in the brain. The SJL-PLP model of EAE is less reliable with clinical signs appearing in 60% of animals, and with a variable time of onset and severity of signs.

Although the Lewis rat is susceptible to MBP-induced EAE it is relatively resistant to EAE induced by systemic sensitisation with MOG antigen unless there is subsequent administration of pro-inflammatory cytokines. This has led to the development of 'focal' or 'targeted' EAE models where sub threshold systemic administration of MOG is followed, about 20 days later, by intracerebral injection of a combination of TNFα and INFγ into a specific brain region or into the spinal cord (Kerschensteiner et al., 2004; Serres et al., 2009a; Serres et al., 2009b; Tourdias et al., 2011). The subsequent EAE lesion is localised to the region of the CNS where the cytokines are injected; this is in contrast to the traditional EAE models where disseminated inflammatory CNS lesions are created. Targeted EAE lesions have been reported to be advantageous for behavioural assessment and for imaging (e.g. MRI) studies since they produce reproducible CNS inflammation to specific regions. This approach has the potential for Three Rs benefits if targeted EAE results in reduced animal numbers per study and for refined early humane endpoints. The use of focal EAE in conjunction with imaging techniques could also be a potential source of welfare benefit as long as the sensitivity of the imaging approach is sufficient to allow for a less severe EAE to be required.

EAE has two major roles: firstly it is used in basic research to understand the mechanisms underlying autoimmune disease in the CNS, such as demyelination, remyelination and inflammation. Secondly it is used in drug development to help identify new therapeutic agents that may be beneficial in MS and other similar disorders. In both cases, refinement of the procedures used to establish and monitor EAE is an important opportunity to reduce suffering and protocols should be reviewed regularly to ensure that the best practice is being implemented.

There is a bigger issue related to the translation of data resulting from EAE into clinical benefit, Vesterinen et al. (2010) identified 1717 compounds, in a systematic review of the literature, that have been reported to show benefit in EAE. Of these, only five treatments have successfully translated into the clinic and whilst all of these reduce relapse, they have no effect on disease progression. As outlined above, EAE is not a single experimental approach with a rigidly defined single phenotype. Selection of the appropriate model to test potential therapeutics is both highly important and challenging (Baker et al., 2011; Batoulis et al., 2011; Denic et al., 2011). What is certain, however, is that EAE is not a 'perfect' model of MS in humans. It may be prudent to select an EAE model that best allows the measurement of the involvement of a specific pathway of interest; one that the novel potential therapeutic of interest has been identified to modify. This then uses EAE as a mechanistic model, rather than a disease model (Hunter, 2011), which could, depending on the availability of biomarkers for treatment efficacy, allow for earlier humane endpoints to be used.

3. Effect of EAE on the animal

EAE affects many systems, both transiently and permanently. The experience of the individual animal depends upon the method of EAE induction and severity of the effects that are produced, how long the animal is kept and the number of relapses that occur. Effects can range from sub-clinical lesions to morbidity, and the death of the animal, in the worst case scenario. In some strains and species there can be up to 80% mortality, which is clearly a major ethical and welfare concern.

Table 1
Common EAE models used for laboratory research (Weissert, 2012).

Species	Strain	Induction	Disease type
Rat	Lew	MBP or MBP 68–88 peptide in CFA	Acute monophasic
Rat	DA	Spinal cord in IFA or MOG 1–125 in CFA or IFA	Relapsing/progressive
Mouse	PL/J	MBP, MBP Ac1–11 (or Ac1–9) in CFA + PT	Acute monophasic
Mouse	SJL	139–151 peptide in CFA + PT	Chronic relapsing
Mouse	C57BL/6	MOG protein or MOG 35–55 peptide in CFA + PT	Progressive or acute
Mouse	Biozzi ABH	MOG protein or MOG 92–106 peptide in CFA + PT	Chronic relapsing
Mouse	NOD	MOG 35–55 peptide in CFA + PT	Chronic progressive
Mouse	SJL	Theiler's murine encephalomyelitis virus	Relapsing progressive
Mouse	B10.PLH2^{u}	TCR^{MBP} transgenic	Spontaneous acute
Mouse	C57BL/6	TCR^{MOG} transgenic	Optic neuritis
Mouse	C57BL/6	TCR^{MOG}XIgH^{MOG} transgenic	Spontaneous opticospinal disease

CFA = complete Freund's adjuvant; IFA = incomplete Freund's adjuvant; PT = pertussis toxin.

Following initiation of EAE, there is an induction phase prior to the first presentation of clinical signs. During this phase the primary cause of suffering is the induction procedure itself; handling, restraint, injection site, injection volume and adjuvant use (see below) can all impact on welfare before any clinical signs related to EAE are manifest.

The first clinical signs of the acute phase of EAE occur after around 7 days and peak at around 10 to 12 days. When disease develops it may, depending on the species, strain and protocol, follow an acute clinical course followed by complete clinical recovery. Otherwise, animals may develop stable chronic neurological deficit, or even develop along a relapsing remitting clinical course during which episodes of clinical disease are separated by periods of clinical remission at intervals of 7 to 20 days.

Where initial recovery is not complete, the disease progression may be associated with the development of irreversible deficits, such as loss of tail tone or hind limb weakness due to failure of tissues to repair. Some animals develop weight loss, although some strains do not, but invariably animals will become hypo-motile and develop tail and limb paralysis. In most models there is a period of transient ascending paralysis moving from the tail to hind limbs and in some animals the forelimbs may become involved. This is due to a transient inflammation of the spinal cord, which causes various levels of reversible conduction block, demyelination and axonal and neuronal loss. This leads to sensory and motor disturbances of affected nerve tracts. Brain lesions can affect other outcomes such as visual disturbances. All of these effects have the potential to cause distress, for example due to anxiety and frustration at the reduced ability to move and neuropathic pain may also develop in these animals (Olechowski, Truong, & Kerr, 2009).

Consideration needs to be given, for each study, to the extent of disease progression necessary in order to meet the aims of the study. For example, it may be possible to terminate the study at the point when the first clinical sign of CNS deficit is observed, so that tissue can be taken for histological analysis; this would reduce suffering by preventing the possibility of animals experiencing the more severe symptoms of EAE. Some scientific questions may require animals to experience a cycle of relapsing remitting EAE and this would result in a significant level of suffering for the animals involved. In cases such as these, the study should have a high scientific benefit, care must be taken to ensure that all procedures are refined wherever possible and that the study is appropriately powered (Vesterinen et al., 2010) to minimise the risk that it may need to be repeated. This issue has been highlighted recently by Scott et al. (2008) and Schnabel (2008) with regard to the use of the SOD-1 knock-out mouse model of amyotrophic lateral sclerosis for drug screening; they suggest that underpowered studies have led to a large number of false positive reports of efficacious drug treatments in the published literature.

4. Adjuvant usage in EAE

Adjuvants are used to amplify antigen specific immune reactions and are used in EAE to activate innate immune mechanisms that support the induction of autoimmune diseases ('t Hart et al., 2011). There are several adjuvants available and they all can, to varying degrees, cause inflammatory lesions, tissue necrosis and induce pain behaviours. The most commonly used is complete Freund's adjuvant (CFA), which is an emulsion of mineral oil (incomplete Freund's adjuvant; IFA) supplemented with inactivated *Mycobacterium tuberculosis* H37Ra. Adjuvant is usually given via the subcutaneous route but intraperitoneal and interplantar routes as well as injections into the base of the tail have been described (Leenaars et al., 1999; Stills, 2005). However, it has been reported that the welfare impact may be mitigated by using proper aseptic technique, careful preparation of the inocula and administering via the subcutaneous route with small injection volumes over multiple, well separated sites (Halliday, Artwohl, Hanly, Bunte, & Bennett, 2000).

Bordetella pertussis toxin (PTX) is also frequently administered in order to promote efficient induction of EAE. The mechanism of adjuvant activity in PT is not fully understood. It has been suggested that it may be due to increased permeability of the blood brain barrier, inhibition of G-protein signalling and signalling through TLR4 (Miller, Karpus, & Davidson, 2010). However, it is clear that PTX induces a significant augmentation of the leukocyte response within lymphoid organs (Vistica, McAllister, Sekura, Ihle, & Gery, 1986).

Careful consideration should be given as to which is the most suitable adjuvant for the study, taking into account both the animal welfare considerations and the desired immune response. Pilot studies may be helpful to select the optimum antigen/adjuvant combination for the species and strain of animal being used.

5. Potential for reduction of suffering through refinement of experimental procedures

European Directive 2010/63/EU (European Commission, 2010) requires consideration of the animal's life time experience, recognising all the potential sources of suffering. By separating out and addressing individually the various sources of pain, suffering and distress and predicting the overall impact on the animal in terms of the cumulative severity; the implementation of refinements to each component can reduce the level of suffering (Hawkins et al., 2011).

Table 2 describes the components of suffering in the EAE model and how each may be refined.

5.1. Housing and care

Animals with EAE are likely to have special husbandry needs. Highly debilitated animals will have limited ability to move around the holding cage and will have difficulty in feeding, drinking and maintaining body temperature. Appropriate standards of housing and care suitable for the species being used should be enforced with the following additional provisions, in addition to standard good practice.[1] The use of soaked food, fluid blocks or subcutaneous supplementation can aid in the control of the deterioration associated with the condition. Pre-feeding animals with high-energy supplement foods, in particular jelly and condensed milk, before they get sick, may be beneficial since sick animals may be reluctant to eat novel foods.

If an animal is completely non-ambulatory, careful consideration has to be given as to whether to euthanize the animal or to regularly hand feed in the hope that the animal will enter remission and recover. Providing support, including hand feeding or ensuring easy access to food and water, is common practice, because animals frequently do recover, in which case they can be used in the study instead of bringing naïve animals and inducing EAE in these. This is felt to reduce animal numbers and reduce the overall suffering caused by the study. However, this is not a straightforward choice, as there is evidence that EAE may induce neuropathic pain in animals (Lu et al., 2012; Olechowski et al., 2009) in which case allowing an animal to recover may actually contribute to the cumulative suffering of that individual. Difficult decisions such as these should be discussed with the establishment's ethics/animal care and use committee (which should ideally incorporate a veterinarian specialised in laboratory animal care) and appropriate behavioural monitoring regimes and humane end points should be defined prior to initiating a study.

Animals that are sick, especially those with hind limb paralysis, may lose body heat very easily and will benefit if housed with untreated animals since they can associate with other mice to aid thermoregulation. Animals should be weighed regularly, bladder function should be closely monitored as both urinary incontinence

[1] http://www.rspca.org.uk/sciencegroup/researchanimals/reportsandresources/housingandcare.

Table 2
The components of suffering in the EAE model and how each may be refined.

Potential adverse effect or clinical sign	How this may be refined
Stress and discomfort or pain due to priming injection	• Refine handling and restraint, e.g. catching mice by cupping in the hands, or in their home cage tunnel, instead of by the tail. This method of capture is less aversive and induces less anxiety (Hurst & West, 2010) • Refine administration of the substance, e.g. formulate the substance and vehicle so as to be minimally irritant; select an injection site that will cause minimal pain and distress; use sharp needles of the narrowest possible gauge (Morton et al., 2001) • Do not inject into the foot pad or tail base • Consider the use of general anaesthesia if the animal will benefit
Possible reaction at injection site, causing irritation or discomfort	• Minimise injection volumes, use multiple, well separated sites if the subcutaneous route is used • Use the least irritant adjuvant • Provide analgesia
Surgery	• Use sterile technique and appropriate perioperative analgesia, minimise size of mini pumps if used
Paralysis, which may cause distress or anxiety: loss of tail tone, hind limb weakness, hypo-motility, limb paralysis	• Monitor urinary function, use manual expression of bladder if necessary (monitor carefully for signs of pain or distress following bladder emptying) • Ensure adequate refuges and nesting material provided • Group house with well animals to aid thermoregulation and comfort • Provide constant access to water and food placed in containers on the cage floor • Provide soaked food, fluid blocks, liquid nutrition or subcutaneous supplementation • Implement a humane end point appropriate to the study
Significant weight loss (e.g. up to 35%)	• Monitor body weight AND condition score more frequently • Provide constant access to water and food placed in containers on the cage floor • Provide soaked food, fluid blocks, liquid nutrition or subcutaneous supplementation. Feed by hand if necessary • Pre-feed animals with high-energy supplement foods, such as jelly and condensed milk • Apply humane end point appropriate to the study
Duration of acute clinical course, intensity of chronic neurological deficit or relapsing/remitting clinical course	• Use lower doses of antigen • Reduce all stressors • Reduce noise levels • Raise ambient temperature – use heating blankets, extra litter and nesting material, do not isolate sick animals – house with well animals, so they can huddle with the other mice to keep warm • Reduce study duration if possible • Apply a humane end point appropriate to the study

and urinary retention can occur in EAE. Manual bladder expression may be required until function returns and the animal may need to be cleaned regularly, but these care procedures will involve handling and restraint and may be stressful to the individual. Increased stress and glucocorticoid production can inhibit EAE and therefore it is important to remove stressors from the environment as far as possible (Mason, 1991). Rats and mice are both 'prey' species and inability to move may be highly distressing, so providing additional nesting material and appropriate refuges may reduce any distress.

5.2. Severity scoring and humane endpoints

In order to reduce severity in EAE, there is a need to develop and apply more humane endpoints. One approach that can be used to facilitate this is the use of welfare/clinical sign scoring systems. There are a number of scoring systems to assess the neurological effects of EAE so as to define an experimental end point, or the point when the pre-defined experimental outcome has been achieved; e.g. when animals exhibit full hind limb paralysis. This experimental endpoint is distinct from a humane endpoint, which is the pre-defined point when an animal is removed from a study for welfare reasons, e.g. if weight loss exceeds 25% for >24 h. The neurological deficit score systems, for defining experimental endpoints, do not take into account the effect of the disease on the general health and well-being of the animal. Depending on the specific scientific needs of the study, it may not be necessary to allow animals to develop full hind limb paralysis, in which case full or partial tail paralysis may be used as an earlier and more humane end-point. Typically, a simple clinical score system is used to monitor and quantify the disease course; for example a simple symptom score from 0 to 5 has been developed (Hashiba et al., 2011):

- Grade 0, no clinical sign
- Grade 1, decrease in tail tonicity
- Grade 2, hind limb weakness
- Grade 3, hind limb paralysis
- Grade 4, hind limb paralysis plus forelimb involvement
- Grade 5, moribund or dead

Neurological signs score systems allow for symptom-based humane endpoints to be implemented. For example, some researchers set a humane endpoint at grade 4, at which animals are humanely killed and tissue taken for further analysis. This may be over simplistic, however. Neurological deficits, described by such a score system, follow a predictable course but affected animals usually recover which makes defining a humane end point difficult. A neurological scoring system alone is too specific and needs to be combined with an assessment of general well-being to determine the humane end point, since some animals with severe neurological deficits maintain body condition, whereas some with relatively minor neurological disorders are sometimes generally much more affected and show signs of poor well-being (Wolfensohn & Lloyd, 2003). It may be more appropriate to use a more detailed scoring system (Table 3 and Fig. 1; adapted from Emerson et al., 2009), with additional welfare indicators, where the cumulative score can be weighted or un-weighted to account for the relative severity of the clinical signs being scored.

Including weight loss in the clinical score system is essential but potentially problematic, because in EAE models there can be quite large fluctuations in the weight of the animal. Simply assessing the weight loss is therefore an inadequate indicator of suffering. The weight loss is not just related to reduced feeding and/or drinking, but is often also associated with physical effects such as muscle mass loss, heat loss, increased urination or diarrhoea. Fluid administration, which is desirable to prevent or treat dehydration, will increase the weight or often temporarily reverse any weight loss that is occurring. Furthermore, weight loss over a long period of time may have far less effect on an animal then if the weight loss occurs over a very short period of time, especially if it is from muscle mass reduction. For these reasons it is better to use body condition assessment as well as weight loss, especially when defining and implementing humane end-points. It may

Table 3
An example of a more detailed clinical score sheet for assessment of disease severity in EAE mice.

Starting date																	Nonweighted			Weighted factor 1				Weighted factor 2				
Animal #		Day (after encephalitogen injection)															Separate totals	Grouped totals	cumulative	Weighting factor	Separate totals	Grouped totals	Cumulative	Relative severity	Weighting factor	Separate totals	Grouped totals	Cumulative
Category	Clinical signs	7	8	9	10	11	12	13	14	15	16	17	18	19	20													
	None																		99	0.0			46.7	0.0	0			73.6
Weight	Loss≥0.4 g first day; ≥0.1 g thereafter			1	1	1	1	1									5	5		1.0	5.0	5.0		1.0	1	5.0	5.0	
Skin	Piloerection		1	1	1	1	1	1	1	1	1	1	1	1	1		13	21		0.5	6.5	10.5		1.0	0.5	6.5	10.5	
	Matted fur				1	1	1	1	1	1	1			1			8			0.5	4.0				0.5	4.0		
Tail	Loss of tone in distal half of tail or in tail segment			1	1	1	1	1	1	1	1	1	1				10	26		0.3	3.3	8.6		1.5	0.5	5.0	13.0	
	Loss of tone in entire tail					1	1	1	1	1	1						6			0.3	2.0				0.5	3.0		
	Diminished lifting or diminished curling of tail				1	1	1	1	1	1	1	1	1	1			10			0.3	3.3				0.5	5.0		
Bladder	Incontinence				1	1	1	1	1								5	5		1.0	5.0	5.0		1.5	1.5	7.5	7.5	
Righting	Difficulty righting when placed on back			1	1	1	1	1	1	1	1	1					9	16		0.5	4.5	8.0		2.0	1	9.0	16.0	
	Inability to right within 5 s after placed on back				1	1	1	1	1	1	1						7			0.5	3.5				1	7.0		
Gait	Clumsy				1	1	1	1	1	1	1	1	1				9	18		0.3	3.0	5.9		2.0	0.67	6.0	12.1	
	Dragging 1 hindlimb					1	1	1	1	1							5			0.3	1.7				0.67	3.4		
	Dragging 2 hindlimbs					1	1	1	1								4			0.3	1.3				0.67	2.7		
Paresis	Reduced range of forelimb abduction when placed on back					1	1	1	1								4	6		0.5	2.0	3.0		2.5	1.25	5.0	7.5	
	No forelimb abduction when placed on back						1	1									2			0.5	1.0				1.25	2.5		
Advanced signs	Side resting position							1									1	2		0.3	0.3	0.7		3.0	1	1.0	2.0	
	Near complete or complete plegia							1									1			0.3	0.3				1	1.0		
	Rapid, slow or deep breathing																0			0.3	0.0				1	0.0		

This table (adapted from Emerson, Gallagher, Marquis, & LeVine, 2009) shows hypothetical data from an EAE study in mice where the 'clinical symptom' score is derived from a 5-point scale, an unweighted clinical score chart or from a weighted clinical score chart. The data are from days 7 to 20 following initiation of EAE. The 5 point scale is an indication of neurological deficit reflected by physical deficiency. The scale is as follows: 0, no clinical sign; 1, decrease in tail tonicity; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis plus forelimb involvement; and 5, moribund or dead. The non-weighted scale scores a series of clinical signs individually, this avoids the assumption that the appearance of a sign is dependent on the presentation of one lower down on the scale. The weighted scale uses the same list of signs but grades them with relative severity. Whilst the 5-point scale is useful as a measure of disease progression for scientific purposes, it has less value as a measure of welfare and suffering. The clinical score systems, both weighted and unweighted offer a more welfare-specific measure of disease progression and, in conjunction with a clear welfare protocol with strict humane endpoints tailored to accommodate the scientific objectives, can be used to reduce suffering. The weighted scale offers the most nuanced approach because clinical signs are given a relative severity score. This can be useful as long as the relative severity assigned to each is an accurate reflection of the actual suffering experienced by the animal.

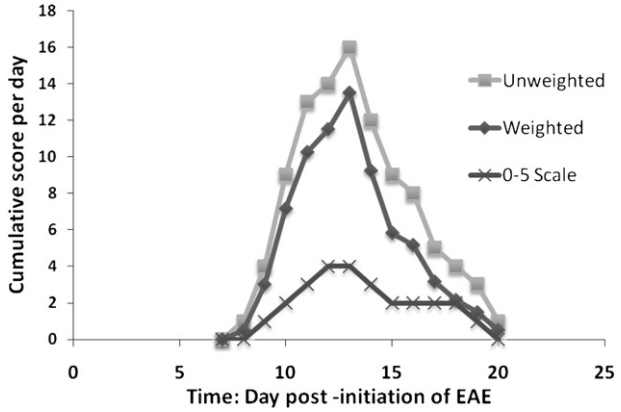

Fig. 1. Hypothetical data taken from Table 3 score sheet data for assessment of disease severity in EAE mice. This figure (adapted from Emerson et al., 2009) illustrates the daily scores from hypothetical animals with EAE, The values were generated by using three different scoring scales (from Table 3). The unweighted and weighted scales offer different information than the 0–5 scale since the clinical score table does not assume that each sign is preceded by all of the earlier signs in the scale. Additionally, the weighted scale takes into account the relative severity of each of the clinical signs being monitored.

be suitable to establish a 'dual humane end-point' for weight loss with two alternative limits; a maximum weight loss limit for the whole duration of the protocol (e.g. 35%) and a lower limit, such as 15 to 20% over a shorter period (e.g. over 2 to 3 days) (Ullman-Cullere & Foltz, 1999).

A simple neurological symptom score system, as outlined above, can therefore be useful if it forms part of an integrated behavioural assessment that includes regular and thorough monitoring of animals with EAE (this may include monitoring outside of 'normal' working hours during critical phases). An example of such a system is given below; each of the conditions in the list is a potential humane end point[2] such that animals displaying any of these indicators would be humanely killed:

- bilateral forelimb paralysis (grade 4) lasting longer than 24 h
- complete hind limb paralysis with or without forelimb involvement; without any indication of a reduction in clinical score for up to 5 days
- self mutilation (e.g. by chewing digits or tail)
- clinical signs of intercurrent disease (i.e. shivering, hunching, listlessness) other than Grade 3 (hind limb paralysis)
- arthritis, which is an unwanted side effect in response to adjuvant and/or antigen
- failure to spontaneously recover from EAE after around 4 weeks post-induction (EAE should be self-limiting)
- failure to eat or drink for >24 h
- weight loss of >35%

EAE is unique in its combination of clinical manifestations. There should be effective communication between animal care, veterinary and research staff (in conjunction with the local ethical or animal care and use committee if appropriate) to ensure that clear humane endpoints are defined. The system for scoring and monitoring animals should be clear and unambiguous, with the necessary tools for decision making to prevent crisis management, and systems must be set up to take appropriate action. This requires training of animal care staff in the likely adverse events to be encountered and methods to deal with them. It must be clear who has responsibility for decision making and adequate training should be provided for all involved. It is good practice regularly to review the actual levels of suffering and fate of the animals, with a view to developing the implementation of the Three Rs and disseminating good practice.

6. Potential for replacement and reduction

There are already many in vitro assays for investigating immune function, blood–brain barrier function, neurodegeneration, and myelination, including cell lines and brain slice cultures that can be used to replace some in vivo studies of MS and similar disorders. For example, a recent publication (Zhang, Jarjour, Boyd, & Williams, 2011) described a new in vitro method using brain slices from neonatal mice, which retain the three dimensional architecture of the brain and in which myelination, demyelination and remyelination of axons can be quantitatively assessed. This model was validated with a number of factors known to modulate remyelination in vivo and was shown to correlate exactly with the in vivo data. The authors suggest that this model could be used to filter potential therapies designed to promote remyelinisation prior to performing in vivo studies, thereby reducing animal use. Further study and validation could increase the translational predictability of this model and may replace the need for EAE to test potential new drugs that modulate myelination. Although the technique still uses animal tissue, the suffering involved in the conventional EAE model is prevented.

There have been technical advances in imaging techniques that have the potential to reduce the number of the animals used. For example, two-photon microscopy enables the movement of cells around the immune system to be studied in vivo, which will allow the steps preceding development of neuroinflammation, demyelination and axonal loss to be studied in detail prior to development of disease. Magnetic Resonance Imaging (MRI) with contrast agent allows the temporal and spatial quantification of changes in disease progression in chronic-relapsing EAE. Whilst imaging techniques may allow for earlier detection of EAE-related neurological changes which should result in earlier humane end points, it should be recognised that repeated general anaesthesia, required for repeated imaging, can have a significant impact on cumulative suffering and the harms and benefits should be carefully considered (Hawkins et al., 2012).

EAE is, as mentioned above, a spectrum of neurological disorders achieved through a diverse number of experimental approaches. New EAE models are being developed and these may prove to yield more translational results (with regard to human disease) than many of the current models (Sriram & Steiner, 2005). The use of traceable myelin-responsive T cell receptor (TCR) transgenic T cells, passive transfer models and advanced ex vivo analyses all contribute to the ability to generate meaningful data on the basic mechanisms of disease and the mode-of-action of candidate compounds. Moreover, these technologies allow a tiered approach to studies. For example, a test compound can first be used in less severe immunisation experiments not leading to EAE, and on-target effects assessed using ex vivo analysis of transferred TCR transgenic T cells. Upstream of this, predictive in vitro assays are being developed and validated, to allow moderate-throughput screening assays. These would be of use for compound re-profiling studies to filter compounds prior to testing in animal models. The net effects of these new opportunities could be that fewer EAE studies per candidate molecule may be needed which could lead to reductions in animal numbers. However, there is always the possibility that the same number, or a greater number, of animals may be used in a drug development programme, due to the selection of a greater number of 'higher quality' candidate drug molecules.

7. Communicating and disseminating good practice

Dissemination of good practice requires the combined and coordinated efforts of researchers, ethical review committees, journal editors, referees, funders and national professional bodies. This requires a

[2] It should be noted that the endpoints listed above represent an upper limit of acceptable suffering under current UK legislation and that this reflect the severity of the EAE model. Ideally, a humane endpoint should correspond to the earliest point at which action can be taken to ameliorate suffering. Further mechanistic understanding of the pathophysiology of EAE and the application of refinement should result in humane endpoints with a much lower level of suffering. This is one of the key aims of the expert working group.

collective understanding of sources of suffering in experiments that use animals, how to identify when these occur and what to do to reduce suffering. Guidance documents from national regulatory authorities as well as from scientific animal welfare and Three Rs organisations are useful resources to achieve this, but a collective effort from researchers to report and promote refinement advances is essential if the maximum benefit is to be achieved for animals used in research and testing.

All information relevant to animal welfare should therefore be included in publications arising from work using procedures involving EAE. This would increase the implementation of refinement advances and reduce needless repetition of animal studies. The ARRIVE guidelines (McGrath, Drummond, McLachlan, Kilkenny, & Wainwright, 2010) set out a framework for the minimum content that a published article should contain in this regard (these include: welfare-related assessments and interventions that were carried out prior to, during, or after the experiment, any adverse events and any implications from the work that may have wider Three Rs benefits). These guidelines have been reviewed, from a MS-research perspective, with specific regard to EAE and a revised framework suggested (Amor & Baker, 2012).

Researchers collaborating with others who are working within a less well developed culture of animal welfare have a responsibility to disseminate good practice as widely as possible, in order to reduce the harms to animals and promote better scientific practice. This can be achieved by sharing detailed protocols, offering training and by emphasising the impact of improved welfare on the quality and relevance of experimental data.

8. Summary

Multiple sclerosis is an incurable, severe disease and new treatments are needed. However, there is considerable debate as to the suitability of current animal models of MS for pre-clinical efficacy assessment of novel therapeutics. Careful consideration must be given to the use of alternatives to living animals, and for both scientific and welfare reasons experimental procedures involving animals should be refined to ensure that suffering has been minimised. This report gives some practical refinement approaches that can be used to reduce suffering in EAE. The authors hope that these refinements will be taken up, used and further developed by researchers working in this field.

Authors' contributions

This manuscript represents the outcome of discussions from an expert working group (EWG) assembled by SW on behalf of the RSPCA as part of its work towards ending severe suffering for animals in research. PH, DA, CC, SL, ML, H-MV and GW were all members of the EWG, Norman Flynn (Home Office, UK) was an observer. The manuscript was written by SW, PH and EL and was edited and revised by the EWG.

References

Amor, S., & Baker, D. (2012). Checklist for reporting and reviewing studies of experimental animal models of multiple sclerosis and related disorders. *Multiple Sclerosis and Related Disorders*, *1*, 111–115.

Baker, D., Gerritsen, W., Rundle, J., & Amor, S. (2011). Critical appraisal of animal models of multiple sclerosis. *Multiple Sclerosis Journal*, *17*, 647–657.

Baker, D., & Jackson, S. (2007). Models of multiple sclerosis. *Advances in Clinical Neuroscience & Rehabilitation*, *6*, 10–12.

Baker, D., O'Neill, J. K., Gschmeissner, S. E., Wilcox, C. E., Butter, C., & Turk, J. L. (1990). Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *Journal of Neuroimmunology*, *28*, 261–270.

Batoulis, H., Recks, M. S., Addicks, K., & Kuerten, S. (2011). Experimental autoimmune encephalomyelitis — Achievements and prospective advances. *Acta Pathologica, Microbiologica et Immunologica Scandanavica*, *119*, 819–830.

Baxter, A. G. (2007). The origin and application of experimental autoimmune encephalomyelitis. *Nature Reviews Immunology*, *7*, 904–912.

Brown, A. M., & McFarlin, D. E. (1981). Relapsing experimental allergic encephalomyelitis in the SJL/J mouse. *Laboratory Investigation*, *45*, 278–284.

Compton, A., & Coles, A. (2008). Multiple sclerosis. *The Lancet*, *372*, 1502–1517.

Denic, A., Johnson, A. J., Bieber, A. J., Warrington, A. E., Rodrigues, M., & Pirko, I. (2011). The relevance of animals models in multiple sclerosis research. *Pathophysiology*, *18*, 21–29.

Emerson, M. R., Gallagher, R. J., Marquis, J. G., & LeVine, S. M. (2009). Enhancing the ability of experimental autoimmune encephalomyelitis to serve as a more rigorous model of multiple sclerosis through refinement of the experimental design. *Comparative Medicine*, *59*, 112–128.

European Commission (2010). DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* (L276/33).

Halliday, L. C., Artwohl, J. E., Hanly, W. C., Bunte, R. M., & Bennett, B. T. (2000). Physiologic and behavioural assessment of rabbits immunized with Freund's complete adjuvant. *Contemporary Topics in Laboratory Animal Science*, *39*, 8–13.

Hashiba, N., Nagayama, S., Araya, S., Inada, H., Sonobe, Y., Suzumura, A., et al. (2011). Phenytoin at optimum doses ameliorates experimental autoimmune encephalomyelitis via modulation of immunoregulatory cells. *Journal of Neuroimmunology*, *233*, 112–119.

Hawkins, P., Burn, C., Deacon, R., Dennison, N., Heath, M., Mackin, P., et al. (2012). Report of the 2011 RSPCA/UFAW Rodent Welfare Group meeting — Harms and benefits of new technologies. *Animal Technology and Welfare*, *11*, 95–104.

Hawkins, P., Morton, D. B., Burman, O., Dennison, N., Honess, P., Jennings, M., et al. (2011). A guide to defining and implementing protocols for the welfare assessment of laboratory animals: Eleventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. *Laboratory Animals*, *45*, 1–13.

Hu, W., & Lucchinetti, C. F. (2009). The pathological spectrum of CNS inflammatory demyelinating diseases. *Seminars in Immunopathology*, *31*, 439–453.

Hunter, A. J. (2011). Have animal models of disease helped or hindered the drug discovery process? *Annals of the New York Academy of Science*, *1245*, 1–2.

Hurst, J. L., & West, R. S. (2010). Taming anxiety in laboratory mice. *Nature Methods*, *7*, 825–826.

Kerschensteiner, M., Stadelmann, C., Buddeberg, B. S., Merkler, D., Bareyre, F. M., Anthony, D. C., et al. (2004). Targeting experimental autoimmune encephalomyelitis lesions to a predetermined axonal tract system allows for refined behavioral testing in an animal model of multiple sclerosis. *American Journal of Pathology*, *164*, 1455–1469.

Leenaars, P. P. A. M., Hendriksen, C. F. M., de Leeuw, W. A., Carat, F., Delahaut, P., Fischer, R., et al. (1999). The production of polyclonal antibodies in laboratory animals. The Report and recommendations of ECVAM workshop 35. *Alternatives to laboratory animals*, *27*. (pp. 79–102).

Lu, J., Kurejova, M., Wirotangseng, L. N., Linker, R. A., Kuner, R., & Tappe-Theodor, A. (2012). Pain in experimental autoimmune encephalitis: A comparative study between different mouse models. *Journal of Neuroinflammation*, *9*, 233.

Mason, D. (1991). Genetic variation in the stress response: Susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. *Immunology Today*, *12*, 57–60.

McGrath, J. C., Drummond, G. B., McLachlan, E. M., Kilkenny, C., & Wainwright, C. L. (2010). Guidelines for reporting experiments involving animals: The ARRIVE guidelines. *British Journal of Pharmacology*, *160*, 1573–1576.

Miller, S. D., Karpus, W. J., & Davidson, T. S. (2010). Experimental autoimmune encephalomyelitis in the mouse. *Current Protocols in Immunology*, *88*, 15.1.1 (Supplement).

Mokhtarian, F., McFarlin, D. E., & Raine, C. S. (1984). Adoptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. *Nature*, *309*, 356–358.

Morton, D. B., Jennings, M., Buckwell, A., Ewbank, R., Godfrey, C., Holgate, B., et al. (2001). Refining procedures for the administration of substances; Report of the BVAAWF/FRAME/RSPCA/UFAW joint working group on refinement. *Laboratory Animals*, *35*, 1–41.

Olechowski, C. J., Truong, J. J., & Kerr, B. J. (2009). Neuropathic pain behaviours in a chronic-relapsing model of experimental autoimmune encephalomyelitis (EAE). *Pain*, *141*, 156–164.

Orme, M., Kerrigan, J., Tyas, D., Russell, N., & Nixon, R. (2007). The effect of disease, functional status, and relapses on the utility of people with multiple sclerosis in the UK. *Value in Health*, *10*, 54–60.

Schnabel, J. (2008). Standard model. *Nature*, *454*, 682–685.

Scott, S., Kranz, J. E., Cole, J., Lincecum, J. M., Thompson, K., Kelly, N., et al. (2008). Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotrophic Lateral Sclerosis*, *9*, 4–15.

Serres, S., Anthony, D. C., Jiang, Y., Broom, K. A., Campbell, S. J., Tyler, D. J., et al. (2009). Systemic inflammatory response reactivates immune-mediated lesions in rat brain. *The Journal of Neuroscience*, *29*, 4820–4828.

Serres, S., Anthony, D. C., Jiang, Y., Campbell, S. J., Broom, K. A., Khrapitchev, A., et al. (2009). Comparison of MRI signatures in pattern I and II multiple sclerosis models. *NMR in biomedicine*, *22*, 1014–1024.

Sriram, S., & Steiner, I. (2005). Experimental allergic encephalomyelitis: A misleading model of multiple sclerosis. *Annals of Neurology*, *58*, 939–945.

Stills, H. F. (2005). Adjuvants and antibody production: Dispelling the myths associated with Freund's complete and other adjuvants. *ILAR Journal*, *46*, 280–293.

't Hart, B. A., Gran, B., & Weissert, R. (2011). EAE: Imperfect but useful models of multiple sclerosis. *Trends in Molecular Medicine*, *17*, 119–125.

Tourdias, T., Hiba, B., Raffard, G., Biran, M., Nishiguchi, T., Aussudre, J., et al. (2011). Adapted focal experimental autoimmune encephalomyelitis to allow MRI exploration of multiple sclerosis features. *Experimental Neurology*, *230*, 248–257.

Ullman-Cullere, M. H., & Foltz, C. J. (1999). Body condition scoring: A rapid and accurate method for assessing health status in mice. *Laboratory Animal Science*, *49*, 319–323.

Vesterinen, H. M., Sena, E. S., Ffrench-Constant, C., Williams, A., Chandran, S., & Macleod, M. R. (2010). Improving the translational hit of experimental treatments in multiple sclerosis. *Multiple Sclerosis*, *16*, 1044–1055.

Vistica, B. P., McAllister, C. G., Sekura, R. D., Ihle, J. N., & Gery, I. (1986). Dual effects of pertussis toxin on lymphoid cells in culture. *Cellular Immunology*, *101*, 232–241.

Weissert, R. (2012). Experimental autoimmune encephalomyelitis. *Experimental autoimmune encephalomyelitis — Models, disease biology and experimental therapy* (pp. 3–20). : In Tech.

Wolfensohn, S., & Lloyd, M. (2003). A handbook of laboratory animal management and welfare. (3rd ed.). : Wiley-Blackwell.

Zamvil, S., Nelson, P., Trotter, J., Mitchell, D., Knobler, R., Fritz, R., et al. (1985). T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. *Nature*, *317*, 355–358.

Zhang, H., Jarjour, A. A., Boyd, A., & Williams, A. (2011). Central nervous system remyelination in culture — A tool for multiple sclerosis research. *Experimental Biology*, *230*, 138–148.