An optimized and validated protocol for inducing chronic experimental autoencephalomyelitis in C57BL/6J mice

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\textbf{ABSTRACT}

\textbf{Background:} Myelin oligodendrocyte glycoprotein induced experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model of multiple sclerosis. However, variations in the induction protocol can affect EAE progression, and may reduce the comparability of data.

\textbf{Optimized method:} In the present study, we investigated the influence of the different components used for EAE induction in C57BL/6J mice on disease progression. MOG\textsubscript{35-55}-induced chronic EAE in C57BL/6J mice has been applied as a model to challenge optimal pertussis toxin (PTx) dosing, while considering variations in batch potency.

\textbf{Results:} We demonstrate that the dosage of PTx, adjusted to its potency, influences EAE development in a dose-dependent manner. Our data show that with our protocol, which considers PTx potency, C57BL/6J mice consistently develop symptoms of EAE. The mice show a typical chronic course with symptom onset after 10.5 ± 1.08 days and maximum severity around day 16 postimmunization followed by a mild remission of symptoms.

\textbf{Comparison with existing methods:} Previously studies reveal that alterations in PTx dosing directly modify EAE progression. Our present study highlights that PTx batches differ in potency, resulting in inconsistent EAE induction. We also provide a clear protocol that allows a reduction in the number of mice used in EAE experiments, while maintaining consistent results.

\textbf{Conclusion:} Higher standards for comparability and reproducibility are needed to ensure and maximize the generation of reliable EAE data. Specifically, consideration of PTx potency. With our method of establishing consistent EAE pathogenesis, improved animal welfare standards and a reduction of mice used in experimentation can be achieved.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that affects more than 2 million people worldwide (GBD, 2016). Being the most common cause for neurological disability of young adults and a precipitant of premature retirement, MS carries significant socioeconomic implications (Kobelt et al., 2017; Rommer et al., 2019). In general, disability in MS patients seems to be caused by an interplay between neuroinflammation and neurodegeneration, leading to the hallmarks of demyelination, inflammation, gliosis, and axonal loss (Dendrou et al., 2015). However, the pathophysiology is still not entirely understood. Therefore, animal models of...
optimal PTx dose adjustment for the consistent induction of chronic EAE, but they often lack sufficient details to enable reproducibility, thus limiting the comparability of EAE studies between research laboratories worldwide.

Crucially, the administration of PTx is frequently chosen at different concentrations across the various research groups, leading to inconsistencies in incidence, onset, and severity of the disease (Qin et al., 2017; Zhang et al., 2017; Langer et al., 2012). Of particular interest – and an often neglected concept – in this context is that individual batches of PTx can be immensely varied in their potency (Markey et al., 2019). In light of this, the present study is designed to establish an optimal PTx dose adjustment for the consistent induction of chronic EAE in C57BL/6J mice, while considering variations in potency and acting in strict accordance with animal welfare standards as well as the Basel declaration and abiding by the principles of replacement, reduction, and refinement (3R) (Balls et al., 1995; Abbott, 2010).

2. Material and methods

2.1. Study approval and design

All animal experiments were approved by the regulatory authorities for animal care and scientific use in North Rhine-Westphalia, Germany (81-02.04.2018, A382, 84-02.04.2016, A307, 84-02.04.2013, A142), and conducted in strict accordance with local laws and regulations, the recommendations of the ARRIVE guidelines (Animal Research: Reporting of IN VIVO Experiments) (Percie du Sert et al., 2020), the Basel declaration (Abbott, 2010), as well as the principles of reduction, refinement, and replacement (3R) (Balls et al., 1995). Animal numbers were determined by utilizing a power analysis to ensure proper statistical strength. Animals that were randomly assigned to each group before starting the experiment were evaluated daily in a blinded fashion.

2.2. Mice

C57BL/6 J mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and kept in the central animal facility at Muenster University Hospital (ZTE, Muenster, Germany) in individually ventilated cages under standard conditions with a nest box and soft paper as nesting material per cage. After arrival, the mice were kept in the animal facility for at least 2 weeks before EAE induction was started. For immunization, 8–12-week-old male and female mice with an average body weight of 20 g were used throughout the study. All animals had access to food pellets (1324 M, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and water ad libitum. In addition, mice received a non-wetting hydration and nutrition replacement (Dietgel 31 M, Clear H2O, Portland, USA). In rare cases of small ulcers at injection sites, these were treated with antibiotic ointment (CutaMed topic, WDT, Garbsen, Germany).

2.3. Preparation of MOG35-55 emulsion

The following preparations were carried out on ice in a laminar flow cabinet under sterile conditions. For preparation of MOG35-55 peptide solution, lyophilized MOG35-55 (peptide purity > 99% measured by high performance liquid chromatography peptide sequence MEVGWYRSPFSRVVHLRNGK) was dissolved in 1x Dulbecco’s Phosphate Buffered Saline (DPBS; without calcium and magnesium chloride, Merck KGaA, Darmstadt, Germany) to achieve a final concentration of 10 mg/ml. The peptide solution was stored at −20 °C until further procedures. MOG I was obtained from Charité-University Hospital Berlin. MOG II was provided by Genosphere Biotechnologies (Paris, France). MOG III was purchased from sb-Peptide (Saint Égrève, France, former name Smart Bioscience). All three peptides were obtained as TFA salts with a purity of > 99% measured by high performance liquid chromatography. MOG I and III had a peptide content of 82% and TFA ion content of 18%, whereas MOG II had a peptide content of 70.92% and TFA ion content of 29.08%. For preparation of complete Freund’s adjuvant (CFA), 100 mg of heat-killed Mycobacterium tuberculosis (strain H37 Rv; Becton, Dickinson and Company (BD), Sparks, MD, United States of America (USA)) was dissolved in 10 ml of incomplete Freund’s adjuvant (IFA) (Merck KGaA, Darmstadt, Germany) to achieve a final concentration of 10 mg/ml. Heat-killed Mycobacterium tuberculosis is used for stimulation of the innate immune response. The CFA stock solution was stored at 4 °C. MOG35-55 emulsion, sufficient for the immunization of 10 mice, should be prepared freshly on the day of immunization. To obtain this emulsion, CFA stock solution (10 mg/ml) was at first diluted with IFA to a final concentration of 2 mg/ml and mixed thoroughly. Subsequently, 1100 µl CFA (2 mg/ml) was transferred into a 3 ml syringe with Luer Lock (Omnifix solo, B. Braun Melsungen AG, Melsungen, Germany). The same volume 1100 µl of MOG35-55 emulsion (2 mg/ml) was drawn in another 3 ml syringe with Luer Lock and both syringes were connected via a three-way stopcock (Discofix C, B. Braun Melsungen AG). Afterwards, both solutions were well mixed 1:1 to a final concentration of 1 mg/ml by pushing back and forth with an almost-closed three-way valve. By mixing thoroughly for at least five minutes, a white, viscous, and homogeneous emulsion was formed. Following this procedure, the emulsion was kept in the refrigerator for 60 min and remixed every 20 min. Prior to immunization, the emulsion was homogenized again by blending through the almost-closed three-way valve and then transferred into a 1 ml glass syringe (Sanitex, Kaunas, Lithuania) with a 0.55 mm hypodermic needle (B. Braun Melsungen AG), which were constantly kept on ice until their usage. Glass syringes were used due to a
reduced affinity of the emulsion to stick to glass surfaces compared to polypropylene syringes.

2.4. Preparation of PTx solution

On days 0 and 2 post-immunization, 50 ng, 100 ng, 150 ng, or 200 ng PTx (lot number 1001 with potency 0.6; Hooke Laboratories Inc., Lawrence, USA) in 100 µl DPBS was freshly prepared. Of note, potency of PTx varies among batches. For consistent EAE induction, it is of utmost importance to adjust the volume according to the potency of a given batch. The solution was mixed by inversion and stored permanently on ice.

2.5. Induction of EAE

For immunization, animals were first anesthetized with isoflurane (1.5% isoflurane in 1 l/minute O2) in a red anesthesia chamber until general anesthesia was achieved. The red tint prevents the animals from seeing outside, creating a less stressful environment. Under isoflurane-induced anesthesia, 50, 75, or 100 µl of a 2 mg/ml MOG35-55 emulsion was administered into both flanks of the mice, proximal to the inguinal lymph nodes, by subcutaneous injections to induce EAE with 100, 150, or 200 µg MOG35-55 emulsion, respectively. 2 h after MOG35-55 administration, 100 µl of PTx solution was injected intraperitoneally (i.p.) via a needle with a diameter of 0.45 mm (BD). PTx was administered in the same way on day 2 after immunization. For this purpose, PTx solution was freshly prepared as described above. Of note, all animals were randomly assigned to each experimental group and were immunized alternately to minimize external influences. The application of the PTx solution was also performed alternately between the various treatment groups. For a visual illustration of the practical procedures of EAE induction, we would like to refer to an earlier study of our group (Bittner et al., 2014).

2.6. Evaluation of EAE

Clinical signs of EAE were monitored daily in a blinded fashion by two independent observers and translated into clinical scores as follows: grade 0, no detectable clinical signs; grade 1, partially limp tail; grade 2, paralyzed tail; grade 3, moderate hindlimb weakness; grade 4, complete hindlimb weakness; grade 5, mild paraparesis; grade 6, paraparesis, weakness in forelimbs; grade 7, heavy paraparesis or paraplegia; grade 8, tetraparesis; grade 9, quadriplegia or pre morbund state; grade 10, death. Onset of EAE was described as the relative body weight over time. Cumulative disease score was calculated as the sum of the daily clinical scores of each mouse during the EAE observation period and reported as an average within each group. Weight loss was calculated as the percentage change in daily weight compared with the initial weight on the day of immunization and displayed as the relative body weight over time.

2.7. Immunohistochemistry

After transcardial perfusion of mice with 40 ml DPBS at disease maximum, lumbar spinal cords were dissected in 10 µm-thin slices using a cryotome (Leica, Wetzlar, Germany). Sections were stained with hematoxylin and eosin (H&E) to assess the extent of inflammation or FluoroMyelin green (1:300; Thermo Fisher, Waltham, USA) for specific detection of myelin. In order to identify inflammatory infiltrates, slices were stained with hematoxylin at room temperature for ten minutes. After ten minutes of washing, the slides were stained with eosin for 20 s and washed again. Finally, slides were dehydrated using an ascending alcohol series (65%, 80% and 96% ethanol) with each two times for two minutes.

For the detection of demyelination, slices were dehydrated with DPBS for 20 min at room temperature. After dehydration, slides were stained with FluoroMyelin green (1:300) in DPBS for 20 min. In the next step, slides were washed four times with DPBS including 0.05% Tween-20 (Merck KGaA) for 10 min. For testing autofluorescence of the samples as well as sensitivity and affinity of the used antibodies, negative controls were obtained, and no specific signal was detectable. Following final washing, sections were mounted with Fluoromount-G, including 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher). Stained sections were examined by microscopy (Axio photot2, Zeiss, Oberkochen, Germany) and analyzed in a blinded manner using Image J software (National Institute of Mental Health, Bethesda, USA). To assess the degree of neuroinflammation, inflammatory and demyelinated areas were quantified and presented as the percentage of total area. For each animal, the arithmetic mean was calculated from five randomly selected coverslips with at least three slices per coverslip.

2.8. Immunological assessment of EAE

For purification of CNS-infiltrating cells, spinal cords were harvested after extensive transcardial perfusion with 40 ml DPBS. CNS tissues were cut by scissors into small pieces then further mechanically homogenized in DPBS with a syringe by drawing it up and drown, layered on a 30–50% Percoll (Merck KGaA) gradient and continuously centrifuged for 30 min at 1200 g without using the brake. For quantification of cell numbers isolated at the interphase, Calibrate beads (BD) were added to freshly purified cells prior to washing and staining. Afterwards, CNS infiltrating cells were stained with appropriate combinations of fluorochrome-conjugated antibodies from BioLegend (San Diego, USA) to the following CD3 in PC5.5 (clone 17A2), CD4 in Pacific blue (clone GK1.5), CD11b in Brilliant Violet 510 (clone M1/70), and CD45 in FITC (clone 30-F11). To inhibit Fc receptor binding, cells were preincubated with purified anti-CD16/CD32 antibody (BioLegend) for 5 min on ice. For cytokine analyses, cells were polyclonally re-stimulated for 6 h at 37 °C using a cell activation cocktail (BioLegend) in the presence of phorbol 12-myristate 13-acetate/ ionomycin, as well as brefeldin A to block cytokine secretion. Intracellular staining was performed with anti-IFN-γ in PE (XMG1.2, BioLegend) and anti-IL-17A in APC antibodies (eBio17B7, Thermo Fisher Scientific, former name eBioscience, Waltham, USA), after fixation/ permeabilization with Cyto-Fast Fix/Perm Buffer Set (BioLegend). Finally, stained samples were analyzed using a multi-color flow cytometer (Gallios, Beckman Coulter, Krefeld, Germany) using Kaluza software (Beckman Coulter). Cell viability was detected using a Zombie fixable viability dye (BioLegend) and cell doublets were excluded to guarantee single cell counting for analysis.

2.9. Statistical analysis

For each experiment, group sizes are given in the figure legends. Data were presented as the mean ± standard error of the mean and no data outliers were excluded. For comparison of EAE scores and body weights between groups, repeated-measures two-way analysis of variance (ANOVA) was performed followed by a Bonferroni post-hoc test. D’Agostino-Pearson omnibus was used to assess the normality of a dataset. In the case of multiple comparisons, Kruskal–Wallis test with Dunn post hoc analysis was used. Otherwise, pairwise comparisons between groups were conducted using the Mann-Whitney U-test. No data outliers were excluded. Data were analyzed using Prism 5.04 (GraphPad Software, San Diego, USA) and values of probability (P) < 0.05 were considered as statistically significant. The level of significance was labeled as NS (not significant), *P < 0.05, **P < 0.01, or ***P < 0.001.
3. Results and discussion

In our model, EAE was actively induced with murine MOG_{35-55} peptide, PTx as well as CFA-containing *Mycobacterium tuberculosis* in C57BL/6J mice that typically develop a chronic disease course. The onset of clinical symptoms occurs around day 10, and maximum severity is reached on day 15 post-immunization followed by moderate remission, as illustrated in Fig. 1A. Clinical signs of EAE were monitored daily and translated into clinical scores (Suppl. Table 1).

However, the administration of PTx is frequently chosen at different concentrations across various research groups, leading to inconsistencies in incidence, onset, and severity of the disease (Qin et al., 2017; Zhang et al., 2017; Langer et al., 2012). As it is already established that alterations in PTx dosage directly modify disease progression of EAE in C57BL/6 mice (Hasselmair et al., 2017), we chose to further investigate PTx dosing. We hypothesized that variations in PTx potency could be responsible for the described variances in disease severity. Our assumption is also based on the fact that lot-to-lot variations are further investigated the immune response. Specifically, we performed an experiment using different PTx dosing. After intensive research to obtain more precise information, we found another commercial source that provided the potency for each PTx batch since they thoroughly tested each lot in advance to determine its effectiveness. As shown in Supplementary Table 2, PTx can markedly differ in its potency between different batches.

Since former EAE studies did not consider the potency of the PTx batch used in experimentation, we performed experiments with increasing concentrations of the same PTx batch with a known potency without further modification of the induction protocol. Increasing doses of PTx subsequently led to a dose-dependent worsening of the clinical course of EAE (Fig. 1B). Accordingly, there was a dose-dependent increase of the cumulative EAE scores. Thus, compared with the cumulative scores of 2.22 ± 0.34 and 2.89 ± 0.33 using 50 ng and 100 ng of PTx respectively, the cumulative scores increased to 4.23 ± 0.5 in the 200 ng group (Fig. 1C). As a secondary marker of disease progression, weight loss was also accompanied with increasing PTx doses (Fig. 1D).

Moreover, we found no sex-specific differences concerning clinical EAE symptoms, relative changes in body weight, and the day of disease onset (Suppl. Fig. 1). Of note, all immunized mice developed EAE symptoms regardless of the PTx dose used. Interestingly, not only was disease progression affected by the concentration of PTx, but also the onset of symptoms differed significantly across the experimental groups. Mice immunized with 200 ng PTx showed an early disease onset (8.38 ± 1.06 days). In contrast, mice receiving 150 ng or 100 ng PTx experienced first symptoms at 9.63 ± 1.06 or 10.5 ± 1.08 days post-immunization, respectively. Immunization with 50 ng PTx significantly delayed the onset of EAE symptoms to 12.1 ± 0.74 days (Fig. 1E). In the experimental groups immunized with 50 ng and 100 ng, there was no mouse needed to be excluded from the experiment. However, 12.5% of mice receiving 150 ng PTx and 75% of mice immunized with 200 ng PTx were excluded from the experiment due to the exclusion criteria for the purpose of animal welfare.

In addition to the clinical evaluation of the EAE course after PTx, we further investigated the immune response. Specifically, we performed flow cytometric analyses of peripheral immune cell infiltration into the spinal cord parenchyma, the site of the highest inflammatory activity in active EAE (Zhang et al., 2017). Consistent with the clinical exacerbations shown in Fig. 1B, the number of CD4^+^ T helper cells expressing cells, indicated the amount of infiltrated peripheral immune cells, also increased significantly in a dose-dependent manner from 2111 ± 1340 cells (50 ng) and 6451 ± 1194 cells (100 ng) to 8249 ± 1881 cells (150 ng) to 9083 ± 1634 cells (200 ng), each relative to 10,000 beads (Fig. 1F).

Interestingly, PTx also affected the adaptive immune response in a dose-dependent manner; we observed an increase of the proportion of infiltrated CD4^+^ T cells with higher doses of PTx (Fig. 1G). Further investigation of the inflammatory infiltrates revealed that clinical aggravation induced by higher doses of PTx was accompanied by a relative increase in infiltration of pro-inflammatory T helper (T_{H1}) cells (defined as CD4^+^ IFN-γ ^+^ T cells) and T_{H17} (defined as CD4^+^ IL-17A ^+^ T cells) cells into the CNS compartment (Fig. 1H and I). Thus, it appears that, in addition to a disturbance of BBB integrity, PTx leads not only to a pure increase in lesion load and cellular infiltration but also to an altered immune response with different composition of inflammatory infiltrates (Hofstetter et al., 2002; Hasselmair et al., 2017; Linthicum et al., 1982). This finding could have implications for the investigation of immunopathophysiological pathways and therapeutic targets.

In addition to the analysis of the CNS infiltration using flow cytometry, we also performed histological examination of the lumbar spinal cord, which is the main site of neuroinflammatory processes in active EAE (Rossi and Constanti, 2016). Consistent with the clinical course and the central immune cell invasion determined by flow cytometry, we also found a dose-dependent increase in the infiltration and demyelination in lumbar spinal cord sections at the disease maximum (Fig. 1J and K).

Apart from PTx dosage, we further elucidated the influence of varying amounts of MOG_{35-55} used for EAE immunization since former studies on MOG dosage in EAE did not consider the impact of PTx on disease progression. Dias et al., for instance, found no significant impact on clinical EAE symptoms by the administration of different MOG_{35-55} concentrations (Dias and De Castro, 2015). To this end, we performed EAE experiments induced by injection of 100 ng PTx with increasing concentrations of MOG_{35-55} peptide while leaving all other parameters of the induction protocol unchanged. Strikingly, the development of EAE symptoms was not affected by the amount of MOG_{35-55} used as immunization with 50 μg, 100 μg, 150 μg, or 200 μg MOG_{35-55} peptide did not induce significantly different disease progression (Suppl. Fig. 2A). In line with this, relative changes in body weight were not significantly affected by induction with varying quantities of MOG_{35-55} (Suppl. Fig. 2B). Accordingly, no dose-dependent increase in cumulative EAE scores was detected in these experimental groups (Suppl. Fig. 2C). Of note, initial symptoms appeared around day 11 post-immunization in mice immunized with 100 μg, 150 μg, or 200 μg MOG_{35-55} peptide. Mice receiving 50 μg MOG_{35-55} peptide tended to have a delay in disease onset, as the first symptoms could be observed 12.75 ± 1.71 days after EAE induction (Suppl. Fig. 2D). However, this delay was not significant.

Induction with different doses of MOG_{35-55} peptide had no influence on the disease incidence as all immunized mice developed clinical signs of chronic EAE. In summary, in line with the results presented by (Dias et al., 2015), we did not observe a dose-dependent effect of MOG_{35-55} on the EAE course. It seems that the presence of the autoantigen alone, rather than the quantity, is critical for disease progression.

Interestingly, apart from peptide purity of MOG_{35-55}, which is typically above 95%, its peptide fraction can also vary between different commercial sources due to the purification step after synthesis of MOG_{35-55} peptide (Boullierne et al., 2014). The peptide fraction reflects the percentage of MOG_{35-55} peptide present in the lyophilized mass and is at least 82%, together with a TFA ion content of 18% if TFA was used as counter ion during the synthesis. Boullierne et al. (2014) demonstrated that distinct ways of synthesizing MOG_{35-55} with a similar purity of >95% but diverse peptide fractions ranging from 45.4% to 72% may influence the severity of EAE due to higher TFA content. To exclude influences of MOG_{35-55} manufacturing, EAE was induced with MOG_{35-55} obtained from three different suppliers with similar peptide purity and peptide fraction. Induction with 100 ng PTx and the three different MOG_{35-55} led to similar EAE progression as shown in Fig. 1B for 100 ng PTx (Suppl. Fig. 3A). In line with the clinical course, relative changes in body weight (Suppl. Fig. 3B), cumulative EAE scores (Suppl. Fig. 3C), as well as days of disease onset (Suppl. Fig. 3D), did not differ across the experimental groups. Moreover, disease incidence was 100% in all groups, indicating a consistent and reproducible EAE induction.
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In summary, chronic EAE is a useful tool for studying neuroinflammatory processes and mechanisms underlying MS progression as well as for evaluating innovative therapeutic strategies. However, several variables have a significant influence on the course of chronic EAE in C57BL/6J mice, leading to varying outcomes and impeding the reproducibility of data between different research groups. This warrants a highly standardized procedure for the induction and monitoring of EAE to obtain reliable, comparable and, above all, reproducible results.

In this study, we highlighted the often-neglected detail that PTx batches vary in their potency, leading to inconsistent EAE induction. This is especially relevant given that usually the same absolute PTx amount is permanently used for EAE induction within a working group, irrespective of its potency. It is therefore highly important to consider the potency of the PTx in the EAE induction protocol. Otherwise, using the same absolute PTx amount, however of different potency, will result in massive variations in the clinical EAE course as well as the underlying neuroinflammatory mechanisms. Furthermore, we were able to show that the severity of the disease course as well as the onset of symptoms is strongly dependent on PTx dosage. Interestingly, a higher PTx dose not only led to increased permeability of the BBB, but also to an altered composition of the inflammatory infiltrates within the CNS. Thus, higher doses were associated with increased relative infiltration of pathogenic T helper 1 (Th1) and T helper 17 (Th17) cells. These PTx-dependent influences on the immune response must be considered when planning EAE studies, as they may have significant consequences when, for example, investigating immunomodulatory therapeutic approaches.

Besides PTx concentration, other factors are known to influence the development of EAE but might be difficult to maintain equally between different laboratories. Weight is one such factor and has a major impact on the course of EAE in C57BL/6J mice, as mice with an initial body weight below 18 g developed a more severe course of EAE with increased weight loss compared to mice with a body weight above 20 g. (Taireshri et al., 2005). In contrast, obese C57BL/6J mice receiving a high-fat diet revealed an exacerbated EAE course accompanied by more severe pathological changes within the spinal cord than mice fed with a normal diet (Ji et al., 2019). In addition, a high-salt diet also leads to an aggravation of the clinical course of MOG<sub>35-55</sub>-induced EAE in C57BL/6J mice (Kleinentiefeld et al., 2013; Jorg et al., 2016; Kremenetsov et al., 2015). Another EAE study demonstrated that eight-month-old male C57BL/6J mice have a delayed disease onset with more acute symptoms and without remission compared to six- to eight-week-old mice, due to age-related changes within the immune cell compartment (Matejuk et al., 2005). Another critical factor is the animals’ sex, as female mice of various strains showed enhanced susceptibility to EAE compared to males (Pappenfuss et al., 2004; Rahn et al., 2014; Butterfield et al., 1999; Palazynski et al., 2004), whereas this is controversial as differences were not apparent when comparing female and male C57BL/6J mice for developing chronic EAE (Hasselmann et al., 2017; Pappenfuss et al., 2004; Palazynski et al., 2004; Okuda et al., 2002). Other factors influencing EAE progression are the microbiome of the gut (Gandy et al., 2019; Lee et al., 2011; Cignarella et al., 2018) and viral (Chen et al., 2017; Mathur et al., 2017) or bacterial infections (Edwards et al., 2015). Interestingly, even the early handling of mice immediately after birth affected the susceptibility of male mice to EAE, indicating that modulations of mother-infant interactions may presumably lead to an altered EAE course. (Columba-Cabezas et al., 2009).

These issues concerning the comparability and validity of EAE results due to inconsistent induction protocols also contribute to problems in the translation of pre-clinical insights. Thus, unfortunately, many therapeutic approaches that show promising results in EAE studies ultimately fail due to a lack of efficacy in human MS trials (Huntemann et al., 2021; Rolfs et al., 2020). More uniform standards regarding the protocols of EAE induction as well as the experimental conditions could help to ensure that only compounds with more valid pre-clinical data find their way into clinical trials. Given the need for a standardized procedure, this study provides a structured EAE induction protocol. The establishment of this standardized EAE induction protocol has been enabled by the extensive testing of PTx lots for potency by the manufacturer Hooke Laboratories Inc., which is essential for consistent, reproducible induction. Beyond the pure testing of the clinical course, we here investigated the underlying autoimmune neuroinflammatory processes by evaluating the CNS invasion of peripheral immune cells using flow cytometric examinations as well as histological analyses.

Considering the results of this study, we recommend a PTx dose of 100 ng with respect to lot number 1001. Using this dose, we observed a sufficiently strong EAE course with an average onset of symptoms between day 10 and 11 after immunization and a disease maximum around day 16. Furthermore, we detected an appropriate degree of CNS immune cell infiltration at the disease peak by flow cytometric as well as histological examination at this dose (Fig. 1). On the other hand, in the experimental group receiving 100 ng PTx, no mouse had to be removed from the experiment due to the exclusion criteria. Considering that none of the mice showed a relative body weight below 80% compared to baseline and none had a critically severe disease course, we recommend a dose equivalent to 100 ng PTx of lot 1001 since this dose seems to maintain the balance between disease severity and aspects of animal welfare. Nevertheless, given the multitude of factors influencing the EAE course, it is advisable for each working group to test the PTx of a known potency under the individual laboratory conditions in order to titrate the absolute PTx amount to the desired EAE severity. Based on this titration, a constant EAE induction can subsequently be achieved by adjusting the PTx amount to the respective potency.

With respect to MOG<sub>35-55</sub> peptide, we did not observe any differences between the products of different suppliers. Furthermore, the
disease severity does not seem to correlate with the amount of MOG35-55 applied. Based on previous studies (Hassellmann et al., 2017; Bittner et al., 2014), we recommend a dose of 200 µg MOG35-55 per mouse for reasons of comparability. It is important to mention that we observed a consistent disease course under these conditions. With regard to consistent EAE induction, it should also be noted that we did not detect any sex-specific differences. However, in earlier studies, a differential susceptibility to EAE depending on the animals’ sex was detected in some trials, e.g. in SJL mice after EAE induction with proteolipid protein (Bebo et al., 1996). With regard to MOG35-55-induced chronic EAE in male and female C57/B6J mice, however, there is considerable evidence that the influence of the animals’ gender on the progression of EAE is limited further supporting our observations (Papenfuss et al., 2004; Rahn et al., 2014; Butterfield et al., 1999; Palaszynski et al., 2004; Okuda et al., 2002).

By ensuring a consistent EAE induction, a reduction in the number of animals in EAE studies might be achieved. Regarding the reduction in the number of animals used, it is also important to note that we observed an incidence of 100% in all experimental groups regardless of the PTx dose. In contrast, Hassellmann et al. (2017) reported a disease incidence of 100% only at a dose of 500 ng. However, despite a similar induction with two injections of PTx, disease incidences of only 80% and 60% incidence were detected when using PTx doses of 350 and 250 ng, respectively (Hassellmann et al., 2017). Interestingly, also the onset of symptoms was significantly delayed in all groups compared to the disease courses of this study. Possible reasons for these differences could therefore involve the already discussed variations in the potency of PTx (Suppl. Table 2). Potentially, PTx with a much lower potency was used for the induction of EAE in that study contributing to the observed differences. Therefore, in turn, underlines that adequate reporting is required to understand deviating results of EAE experiments. Based on the ARRIVE (Animals in research: Reporting in vivo experiments) guidelines, Baker and Amor (2012) have published a proposal for minimal reporting standards. These standards include important information on experimental study conditions, such as basic information on the animals’ strain and age, husbandry conditions as well as randomization. Concerning animal welfare, researchers face increasing requirements as defined by the European Parliament and the Council directive 2010/63/EU “on the protection of animals used for scientific purposes”. Therefore, as well as the principles of the Basel Declaration (Abbott, 2010) – reduction, refinement and replacement (Balls et al., 1995) – more uniform and consistent EAE induction conditions are urgently needed in order to reduce the numbers of mice being used in EAE. This requires higher standards for comparability and reproducibility, as provided by this study.

CRediT authorship contribution statement

N.H.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. A.V.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. L.G.: Data curation, Investigation, Methodology, Writing – review & editing. S.G.M.: Conceptualization, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. S.E.: Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: N.H., A.V., L.G., A.M.H. and S.E.: None. S.G.M. received honoraria for lecturing and travel reimbursement for attending meetings from Almirall, Amicus Therapeutics Germany, Bayer Health Care, Biogen, Celgene, Diamed, Genzyme, MedDay Pharmaceuticals, Merck Serono, Novartis, Novo Nordisk, ONO Pharma, Roche, Sanof-Aventis, Chugai Pharma, QuintilesIMS, and Teva. His research is funded by the German Ministry for Education and Research (BMBF), Bundesinstitut für Risikobewertung (BfR), Deutsche Forschungsgemeinschaft (DFG), Else Kröner-Fresenius Foundation, Gemeinsamer Bundesausschuss (G-BA), German Academic Exchange Service, Hertie Foundation, Interdisciplinary Center for Clinical Studies (IZKF) Muenster, German Foundation Neurology and Alexion, Almirall, Amicus Therapeutics Germany, Biogen, Diamed, Fresenius, Medical Care, Genzyme, HERZ Burgdorf, Merck Serono, Novartis, ONO Pharma, Roche, and Teva.

Data Availability

All data are available on request from the authors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jneumeth.2021.109443.

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