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Practical approaches for refinement and reduction of animal experiments with bank voles in prion research

Praktische Ansatzpunkte zur Verbesserung und Verringerung von Tierversuchen mit Rötelmäusen in der Prionforschung

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Summary

The bank vole (Myodes glareolus) is by far not as common a laboratory rodent as mice or rats. Recently, however, bank voles have increasingly emerged as a powerful infection model for prion diseases such as human Creutzfeldt-Jakob disease (CJD) or chronic wasting disease (CWD) of cervids. The susceptibility of bank voles to infections with a variety of prion isolates from different host species will substantially facilitate research into prion diseases and their causative agents. To pro-actively promote a reduction of prion bioassays in bank voles against this background, we examined protein misfolding cyclic amplification (PMCA) using brain homogenate from normal bank voles as reaction substrate for its suitability as a possible in vitro alternative. PMCAs using bank vole brain homogenate as substrate (bvPMCAs) are known to be highly vulnerable to false-positive results. Such false-positive readouts predominantly result from inadvertent cross-contamination and unspecific seeding with minute amounts of prion agents such as 263K scrapie which are often present in prion research laboratories. For tackling this problem that potentially impedes a broader application of bvPMCA, we describe bvPMCA conditions that were ultra-sensitive to 263K scrapie prions and yet highly robust against inadvertent cross-contamination. With respect to refinement, we implemented refined housing conditions that prevented the occurrence of stereotypies in bank voles, and procedures which allowed a virtually stress-free handling when subjecting these animals to laboratory procedures such as weighing or inhalation of anaesthetics. These refinements can improve the welfare of bank voles in prion laboratories and other fields of research.

Keywords: Protein misfolding cyclic amplification (PMCA), 263K scrapie, stereotypy, housing, handling

Zusammenfassung

Die Rötelmaus (Myodes glareolus) ist bei weitem kein so häufig verwendeter Labornager wie beispielsweise Mäuse oder Ratten. Allerdings haben sich Rötelmäuse seit einiger Zeit zunehmend als leistungsstarkes Infektionsmodell für Prionkrankheiten wie die menschliche Creutzfeldt-Jakob-Krankheit (CJK) oder die Chronic Wasting Disease (CWD) der Hirsche herausgestellt. Die Suszeptibilität von Rötelmäusen für Infektionen mit einem breiten Spektrum unterschiedlicher Prionisolate aus verschiedenen Wirtsspezies kann erheblich dazu beitragen, die Erforschung von Prionkrankheiten und ihrer Erreger zu erleichtern. Um vor dem Hintergrund dieser Entwicklung proaktiv eine Verringerung von Rötelmaus-Infektionsversuchen in der Prionforschung voranzutreiben, haben wir die „Protein Misfolding Cyclic Amplification"-Technik (PMCA) mit Hirnhomogenat aus normalen Rötelmäusen als Reaktionssubstrat auf ihre Eignung als mögliche in vitro-Alternative untersucht. PMCAs mit Rötelmaus-Reaktionssubstrat (bvPMCAs, bv steht für engl. „bank vole") sind bekanntermaßen hoch anfällig für falsch-positive Ergebnisse. Diese beruhen vor allem auf Kreuzkontaminationen und unspezifischem „Seeding" mit Spuren von Prioninfektiosität, wie sie beispielsweise in Form von

263K Scrapie-Prionen häufig in Prionlaboren vorkommen. Um dieses Problem, das einer breiteren Nutzung der bvPMCA bisher potentiell im Wege steht, zu lösen, beschreiben wir Reaktionsbedingungen, unter denen wir bvPMCAs mit hoher Sensitivität für 263K Scrapie-Prionen frei von unerwünschten Kreuzkontaminationen durchführen konnten. Daneben stellen wir zur Verbesserung der Zucht, Haltung und Behandlung von Rötelmäusen Möglichkeiten für eine verbesserte Unterbringung und einen schonenderen Umgang vor. Diese erlauben es, das Auftreten von Stereotypien zu vermeiden, bzw. Rötelmäuse etwa beim Wiegen oder der Inhalation von Anästhetika weitgehend stressfrei zu halten. Die vorgestellten praktischen Ansätze zum Refinement lassen sich nicht nur in Prionlaboren sondern auch in anderen Forschungsbereichen nutzen.

Schlüsselwörter: Protein misfolding cyclic amplification (PMCA), 263K Scrapie, Stereotypie, Unterbringung, Handling

Introduction

The bank vole (*Myodes glareolus*, formerly *Clethrionomys glareolus*) belongs to the family Cricetidae of the order Rodentia. It is a small rodent with a mouse-like appearance and can be relatively easy kept and bred in captivity. Bank voles are a rather uncommon rodent model that has recently attracted increased scientific interest (Larsen 2016). Traditionally, bank voles have been used in laboratory research for example on tuberculosis (Jespersen 1954), hantaviruses (Olsson et al. 2003, Voutilainen et al. 2015) and Ljungan virus-associated type 1 diabetes (Niklasson et al. 2003). During the past few years, bank voles have also emerged as a powerful infection model for prion diseases, or transmissible spongiform encephalopathies (TSEs; e. g. scrapie, bovine spongiform encephalopathy [BSE], chronic wasting disease [CWD], sporadic and variant Creutzfeldt-Jakob disease [sCJD and vCJD, respectively], or hereditary Gerstmann-Sträussler-Scheinker syndrome [GSS]).

The epidemic outbreak of BSE and the resulting emergence of vCJD in the United Kingdom in 1986 and 1996 (Wells et al. 1987, Will et al. 1996), respectively, led to a world-wide awareness of prions and prion diseases, and their potential hazards to animal and human health. Such awareness was revived during the past few years by the rapid spread of CWD in captive and free-ranging cervids in North America (Haley et al. 2015), the initial occurrence of CWD in 2016 in Europe (Benestad et al. 2016), and the unclear risks of zoonotic CWD transmission to humans via foodstuffs (Waddell et al. 2017). Prion diseases are by definition transmissible, at least within their natural host species. However, they are often difficult to model and to directly compare in laboratory animals such as mice. On the one hand, a variety of prion diseases transmit only very slowly, with incomplete attack rates or not at all to wild-type mice. On the other hand, transgenic mice that express the prion protein of a different species are often only susceptible to the prion diseases they have been genetically engineered for. The bank vole, in contrast, has been found to be susceptible to different prion diseases from different species including classical sCJD and atypical CJD (Nonno et al. 2006, Galeno et al. 2017) as well as GSS (Pirisinu et al. 2016) of humans, sheep scrapie (Di Bari et al. 2008), and CWD of cervids (Di Bari et al. 2013). In addition, further findings suggested the cellular prion protein (PrP^{C}) of bank voles to be a "universal acceptor" for prions (Watts et al. 2014). The bank vole´s susceptibility to infections with prions from classical and atypical forms of CJD, as well as with prions from GSS, CWD and other sources provides an unprecedented avenue to experimentally model and compare these diseases in a non-proprietary small wild-type rodent. Thus, the bank vole infection model meets a long-standing demand in prion research and is therefore likely to be increasingly used in the coming years. In a recent publication it has been even reckoned that "bank voles are currently poised to shape the future of research in the field of prion diseases" (Larsen 2016). Against this background we searched for practical approaches by which the use of bank voles for bioassays and other laboratory purposes can be refined and reduced in future prion research.

In order to promote reduction of prion bioassays in bank voles in the medium or long term we addressed the technique of protein misfolding cyclic amplification (PMCA). During the past few years PMCA has emerged as a very powerful alternative in vitro method for the highly-sensitive, cell-free detection and quantification of prions (Saborio et al. 2001, Castilla et al. 2006, Saa and Cervenakova 2015). The development of cell-free detection methods for prions that are on par with animal bioassays was hampered for a long period of time by the unconventional chemical composition and replication mechanism of prions. Prions are proteinaceous infectious particles without coding nucleic acids (Prusiner 1998, Colby and Prusiner 2011). As described elsewhere in more detail (Beekes und Wagenführ 2013), they consist essentially of a misfolded and aggregated isoform of the host-encoded prion protein (Prusiner 1982; 1998). The pathological conformers of the prion protein are referred to as PrP^{Sc} or PrP^{TSE} ("Sc" and "TSE" are acronyms for scrapie, and transmissible spongiform encephalopathy, respectively) (Prusiner 1998, Brown and Cervenakova 2005). The replication of prions exhibits strong parallels to the seeded growth of crystals, and is thought to occur by a mechanism of nucleation-dependent protein polymerisation (Come et al. 1993, Soto 2011). In the course of this process aggregates of PrP^{TSE} act as nuclei ('seeds') that recruit cellular prion protein and incorporate it, in a misfolded form, into their own oligomeric or polymeric structure. When PrP^{TSE} aggregates eventually fall apart into smaller units, this causes a multiplication of PrP particles with proteinaceous seeding activity, and thereby a further self-propagation of the pathological protein state. According to this concept, the self-replication of prions is mediated by their biochemical seeding activity, i. e. the ability to convert cellular protease-sen-

sitive prion protein into misfolded, aggregated and often Proteinase K (PK)-resistant PrP (PrPres).

The identification of seeding-active prion protein as the self-replicating principle of prions eventually gave rise to the development of PMCA (Saborio et al. 2001), which mimics, in a cyclic process and accelerated mode, nucleation-dependent PrP polymerisation in the test tube as described elsewhere (Boerner et al. 2013). In principle, PMCA cycles consist of two phases. In the first phase, PrP seeds such as PrP^{TSE} from humans or animals are incubated in normal brain homogenate containing an excess of PrP^{C} to induce the growth of PrP aggregates. In the second phase, the reaction mixture is exposed to ultrasound. The ultrasonic treatment fragments grown PrP oligo- or polymers into smaller units, which in turn provides new seeding-active particles for further aggregate growth. Thus, over consecutive PMCA cycles the number of seeds increases and accelerates the replication of the pathological protein state (Castilla et al. 2006). This effect can be enhanced by "serial PMCA" in which reaction mixtures are periodically passaged into fresh normal brain homogenate after a defined number of PMCA cycles (Bieschke et al. 2004, Castilla et al. 2005). Technical advancements of the PMCA technology, referred to as quantitative PMCA, and the additionally established real-time quaking-induced conversion assay (RT-QuIC, an alternative PrP seeding assay) now allow the direct titration of the seeding activity of several different prion strains in vitro (Chen et al. 2010, Wilham et al. 2010, Pritzkow et al. 2011, Makarava et al. 2012).

Therefore, bioassays in bank voles could be substantially reduced if the sensitivity of this infection model to prions from sporadic and genetic human TSEs could be transferred to PMCA using brain homogenate from bank voles as PrP^{C} substrate. For cervid CWD such transfer has been already reported (Di Bari et al. 2013). However, PMCA with bank vole brain homogenate as reaction substrate (bvPMCA) is known to be highly vulnerable to false-positive results (Cosseddu et al. 2011). This is not least due to the fact that bvPMCAs, including those to be used for prions of sporadic CJD or genetic human TSEs, can be efficiently seeded by a broad range of prions of different origin (Cosseddu et al. 2011). Cross-contaminations with hamster-adapted 263K scrapie prions provide a particular challenge in this context. This is because 263K scrapie can cause ultra-efficient PrP^{TSE} amplification in bvPMCA (see results), and has long since been used in many prion laboratories as a model TSE agent for many purposes in basic and applied prion research, including the development and improvement of PMCA itself (Saborio et al. 2001, Chen et al. 2010, Wilham et al. 2010). Therefore, 263K scrapie prions potentially constitute a highly disruptive factor for bvPMCA in TSE laboratories and may thereby impede a broader application of this technique.

In a pioneering approach Cosseddu et al. (2011) defined rigorously controlled reaction conditions for bvPMCA that allowed the reliable exclusion of interfering cross-contaminations with a bank vole-passaged prion strain (termed v586) of spontaneous origin and extremely efficient amplification activity in PMCA. Based on their findings the authors suggested comprehensive safeguards for bvPMCA. However, the exclusion of cross-contaminations with 263K scrapie prions was not directly examined in that study. Against this background, we aimed at the definition of as simple as possible bvPMCA conditions that achieved a similar sensitivity for 263K scrapie as for v586 prions (in terms of the amount of infected brain tissue needed for a positive test result), and simultaneously proved robust against 263K scrapie cross-contaminations. A systematic analysis of crucial PMCA steps allowed us to identify such conditions, which we think will help to advance bvPMCA as a viable alternative to bank vole bioassays for animal and human prions.

As to refinement, we focused on the problem of stereotypies to which bank voles kept in captivity are particularly prone (Ödberg 1987, Cooper 2010), and on the improvement of stress-free handling when subjecting bank voles to laboratory procedures (e. g. weighing or inhalation of anaesthetics). The refinements we achieved in this direction can be used to improve the welfare of bank voles when using these animals in prion laboratories as well as other fields of research.

Materials and Methods

Breeding, animal housing, management and humane euthanasia of bank voles

We established and propagated a colony of bank voles (Bv109M, homozygous for methionine at codon 109 of PrP) (Di Bari et al. 2013) for prion research projects starting with breeding pairs kindly provided by the laboratory of Prof. Umberto Agrimi (Istituto Superiore di Sanità, Viale Regina Elena, 299 – 00161 Rome, Italy). The breeding of bank voles has been approved by the competent animal protection authority in Berlin (Landesamt für Gesundheit und Soziales [LAGeSo], Berlin, Germany; current approval number: IC 114 – ZH 22), and the bank voles were bred throughout monogamously. When possible, the bank voles were kept in small single-sex groups of up to four animals following weaning. However, in the case of social incompatibilities (e. g. aggressive behaviour of males, or of females kept in breeding cages), individual animals were single-housed. The housing of bank voles followed the Directive 2010/63/EU of the European Parliament and of the Council, the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123), and the German Animal Welfare Act. Further guidance was obtained from a review by Cooper (2010) on the care and management of voles. Health monitoring of our bank vole colony was performed according to the recommendations of the Federation of European Animal Science Associations (FELASA) (Mähler et al. 2014). Quarterly microbiological monitoring revealed positive findings of *Pasteuralla pneumotropica, Helicobacter* spp., *Spironucleus* spp. and *Trichomonas* spp. which did not compromise the suitability of bank voles as donors of PMCA substrate.

Initially, we kept our bank voles in transparent 1290D Eurostandard Type III cages (425 mm length (L) x 276 mm width (W) x 153 mm height (H), base area: 820 cm^2; Tecniplast S.p.A., IT). Chipped wood was used as litter (Lignocel 3-4S; Rettenmaier & Söhne GmbH + Co KG, DE), and paper wool (Nisti, article number 33800; Claus GmbH, DE) and nestlets (article number H4201; ssniff Spezialdiäten GmbH, DE) were provided as nesting material and for environmental enrichment. However, for refinement of the housing conditions Type III cages were eventually replaced by substantially larger transparent 2000P cages (612 mm L x 435 mm

W x 216 mm H, base area: 2065 cm^2; Tecniplast S.p.A., IT). In addition, we further enriched the environment in the cages of the bank voles with the following articles: GLP mouse mazes and GLP mini mouse mazes (Des. Res.™, product codes 110686 and 110688, respectively; Claus GmbH, DE), play tunnels (article number H0528-151; ssniff Spezialdiäten GmbH, DE; article number 3084030; Zoonlab GmbH, DE), polycarbonate bottles (article number ACBT0252, Tecniplast S.p.A., IT), wood bricks (article number H0234-NGM; ssniff Spezialdiäten GmbH, DE), as well as mouse igloos, crawl balls and fast-tracs for mice (article numbers 13100, 13121 and 13150, respectively; Plexx B.V., NL) (see also Fig. 1).

Furthermore, we established handling procedures in order to reduce stress as far as possible during the routine handling and clinical monitoring of the bank voles over long periods of time, or when subjecting the animals to laboratory procedures. One key element of these procedures was the accommodation, from birth, of the bank voles to individual experienced caretakers and veterinarians, who handled the animals as a basic principle strictly in a calm routinely manner. The handling of bank voles was predominantly performed by female personnel. Another crucial step addressed the potentially stressful grasping or picking up of the bank voles, which we tentatively replaced by luring the animals with feed (sunflower seeds) into polycarbonate bottles (article number ACBT0252, Tecniplast S.p.A., IT), e. g. for weighing or humane euthanasia. For such handling of bank voles bottles were used without a cap. Humane euthanasia was performed by transferring the bottle with the animal inside into an inhalation chamber that contained a preformed isoflurane atmosphere. Although not mandatory, we reported euthanasia of normal bank voles from our colony to the competent animal protection authority in Berlin (LAGeSo; Registration Numbers T 0286/09, T 0300/15).

Protein Misfolding Cyclic Amplification

Preparation of amplification substrate

After euthanasia of bank voles aged between 55–60 days with isoflurane, brains were dissected immediately after transcardial perfusion with a buffer solution containing EDTA as previously described (Pritzkow et al. 2011), and either directly used for the preparation of normal brain homogenate (NBH) or stored at 80°C until further processing. 10% (w/v) NBH in PMCA conversion buffer adjusted to pH 6.9 (CB 6.9) was prepared as PMCA substrate from freshly dissected or frozen bank vole brains according to Pritzkow et al. (2011).

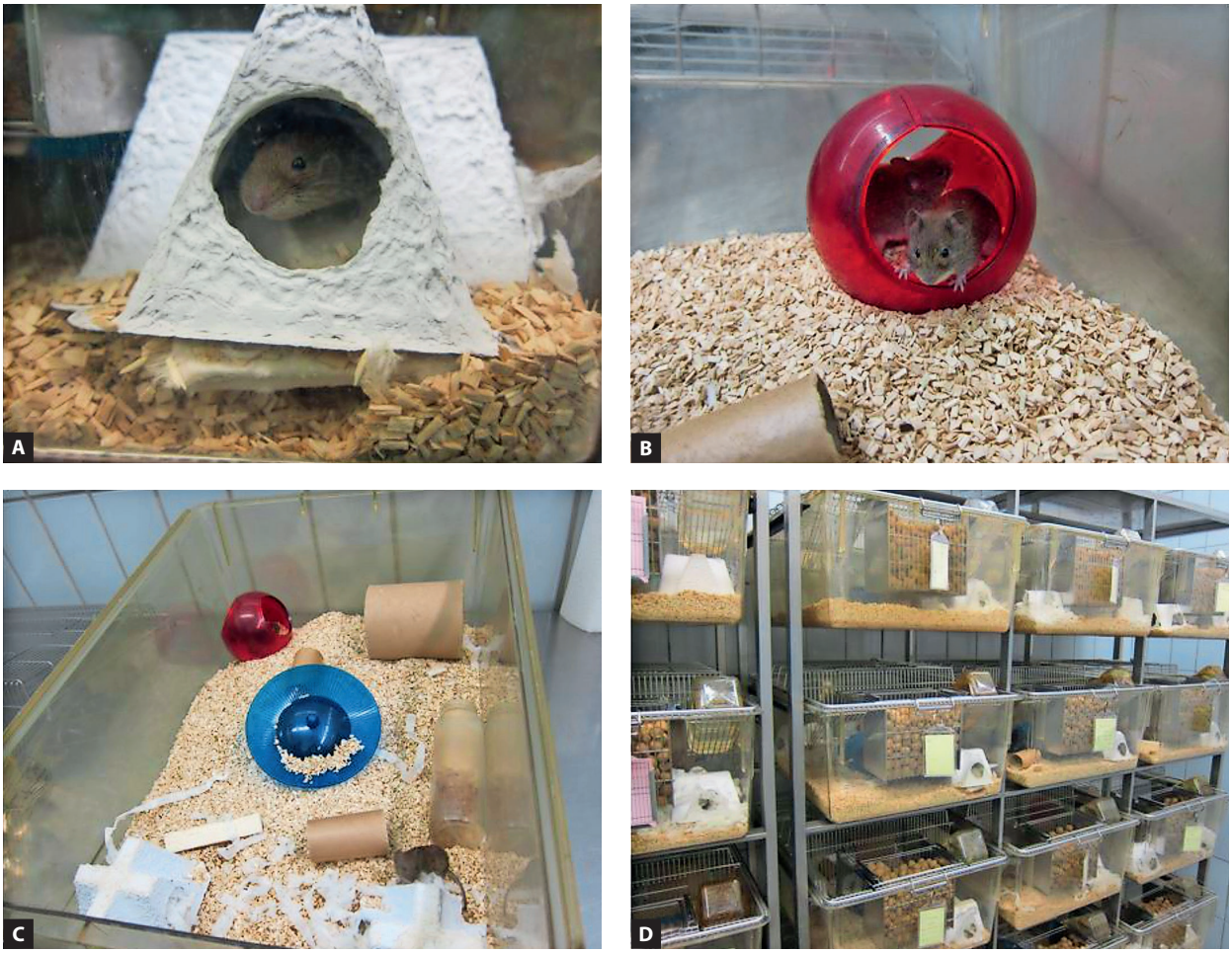

FIGURE 1: *Photographic illustration of the housing of bank voles in large cages with diversely enriched environments. A – Bank vole looking from inside a mini mouse maze. B – Two bank voles inside a crawl ball. C – Overview of enrichment items typically present in the cages of our bank voles. The picture shows two mini mouse mazes, a red crawl ball, three play tunnels of different diameters and lengths, a polycarbonate bottle, a blue fast-trac for mice, a wood brick, and white pulp strips. D – Partial view of the colony room with cages placed along one wall.*

Preparation of PMCA inoculates
263K scrapie hamster brain tissue was taken from the brain sample stock of our laboratory (Boerner et al. 2013). 10% (w/v) scrapie brain homogenate (SBH) from such tissue was prepared, and SBH dilutions in NBH (pH 6.9) containing final concentrations of 10^{-10}, 10^{-11} or 10^{-12} g/10 µl of homogenized 263K scrapie brain tissue were produced as previously described (Pritzkow et al. 2011).

Amplification of PrP misfolding and aggregation
PMCA was performed according to the protocol of Pritzkow et al. (2011) with the following modifications. 20±2 mg of glass beads (diameter 0.5–0.75 mm, Carl Roth, DE) each were filled into 0.5 ml Eppendorf safe lock tubes. 10 µl-aliquots of SBH dilutions containing 10^{-10}, 10^{-11} or 10^{-12} g of homogenized 263K scrapie brain tissue were mixed in the reaction tubes with 140 µl of NBH. For PMCA samples without 263K prions, 150 µl of NBH were pipetted into the reaction vials. The same set of samples was prepared for control read outs after zero rounds of PMCA. The reaction tubes for PMCA were sealed with parafilm, immersed in 0.2% (w/v) sodium dodecyl sulphate (SDS)/0.3% (w/v) sodium hydroxide (NaOH) for 5 min at room temperature (Lemmer et al. 2008), thoroughly rinsed first with tap water and subsequently with distilled water, and then positioned in a disk-shaped rack on the cup horn of an automatic Q700 sonicator (Misonix Inc., US). One round of PMCA consisted of 24 alternating cycles of incubation for 59 min 20 sec and subsequent ultrasonication (200 W) for 40 sec. The water in the horn chamber ran through a circulation thermostat (CC-304B; Peter Huber Kältemaschinenbau AG, DE). For each new PMCA experiment fresh water was filled into the thermostat, and after termination of the last PMCA round the water was discarded and autoclaved for 90 minutes at 134°C prior to discharge into the wastewater. Using the thermostat's software package "Exclusive" and an external temperature probe (Pt100; PSL Systemtechnik GmbH, DE) the temperature in the horn chamber was set and adjusted to 38.0°C, and recorded every five seconds using an USB stick attached to the thermostat. Furthermore, the flow of water was adjusted to 340±20 ml/min and controlled with a permanently attached flow measurement device (RCT-HBG-Sensor-OMNI; Reichelt Chemietechnik GmbH + Co, DE). After completion of each PMCA round the sealed reaction tubes were once more immersed in 0.2% SDS/0.3% NaOH for 5 min at room temperature, thoroughly rinsed with tap and distilled water, and gently spun down after placing in a 96 well plate in a swing out centrifuge. The parafilm was removed, and, using dedicated pipettes with filter tips, 30 µl aliquots were transferred into 120 µl of fresh NBH in new safe lock tubes for the next PMCA round. Furthermore, 30 µl aliquots from control samples that had been prepared for read out after zero rounds of PMCA, and from reaction samples after one, two, three or four rounds of PMCA were collected and further processed for Western blotting. However, other than previously described (Pritzkow et al. 2011), no phenylmethylsulfonylfluoride (PMSF) was added after mixing with sample loading buffer. Instead, samples were incubated for 10 min in appropriately locked vials in a heating block set to 110°C.

Electrophoresis and Western blotting
SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting for the detection of PrP were performed as described elsewhere (Pritzkow et al. 2011) with the following modifications. SAF 84 (Bertin Pharma SAS, FR) was used as primary antibody. Blots were incubated overnight at 4°C in primary antibody solution (SAF 84, at a concentration of 0.4 µg/ml, in Tris-buffered saline [10 mM Tris HCl, 133 mM NaCl, pH 7.4] containing 3% [w/v] non-fat milk powder and 0.05% [w/v] Tween 20).

Results

Refinement: Prevention of stereotypies in bank voles and enabling of stress-free animal handling for laboratory procedures
After having established and maintained our bank vole colony for some time in Eurostandard Type III cages under the initial animal housing conditions described in the methods section, we observed the occurrence of stereotypical behaviour in a substantial proportion of the animals. As previously described elsewhere for laboratory bank voles (Cooper 2010) the stereotypic behaviour of our animals included gnawing of the wire mash of the cage, jumping, and somersaulting.

In order to alleviate the occurrence of stereotypies in the bank voles we started to house the animals in 2000P cages which have a substantially larger size (612 mm L x 435 mm W x 216 mm H, base area: 2065 cm^2) than the originally used 1290D Eurostandard Type III cages (425 mm L x 276 mm W x 153 mm H, base area: 820 cm^2). Simultaneously, the environment in the cages was enriched by various items that allowed improved bedding, nesting, sheltering, gnawing, hiding and climbing (Figure 1). We were prompted to this approach by the previously reported observation that increased cage sizes and enriched environments are important factors that can help to avoid the occurrence of stereotypies in laboratory bank voles (Ödberg 1987). For inhibiting stereotypies to the greatest possible extent we used cages that were still larger, and enrichment items that were more diverse, than those suggested by Ödberg (1987). Under this set of measures and the stress-reducing handling standards described below, stereotypic behaviour of our bank voles was almost completely absent. If at all, stereotypies only occurred rarely and transiently in a very low number of animals. In this context it is important to note that the housing in large cages with a diverse environmental enrichment should start as early as possible for the bank voles, since established stereotypies are considered to be more difficult to interrupt than developing stereotypies (Ödberg 1987, Cooper 2010). The effectiveness of the adopted refinement measures was independently confirmed during a recent inspection, in November 2017, by the competent animal protection authority of Berlin (LAGeSo) which did not detect any stereotypies in our bank voles.

So far, in our laboratory, bank voles were primarily used as brain tissue donors for bvPMCA. In order to establish and apply this technique, which potentially provides a powerful in vitro alternative to bank vole bioassays for prions, we needed to breed, keep and eventually euthanise normal bank voles at an age of 55–60 days for dissection of the brain. In order to reduce any stress

FIGURE 2: *Bank vole in a bottle offered for environmental enrichment. Bank voles can be easily lured into such bottles with sunflower seeds and then subjected, in a virtually stress-free state, to laboratory procedures such as weighing or inhalation of anaesthetics.*

of our bank voles as far as possible, the animals were accommodated, from birth, to individual experienced caretakers and veterinarians, who carried out the routine handling and monitoring of the animals or medical procedures, respectively, in a calm routinely manner.

Furthermore, we tentatively resorted from manually grasping or picking up the bank voles to luring them with feed other than the standard feed pellets (e. g. sunflower seeds) into polycarbonate bottles. We noticed that our bank voles frequently entered bottles which were offered as an enrichment item in the cages (Fig. 2), and found that they were even more eager to enter the bottles when these contained sunflower seeds. By luring bank voles with feed into such bottles, the animals could be subjected in a virtually stress-free state to laboratory procedures such as weighing or inhalation of anaesthetics. When bottles with bank voles inside were moved smoothly for handling purposes the animals usually stayed calm, did not show flight behaviour and often continued to eat the sunflower seeds. These refinement effects were achieved with bottles that were at least partially turbid due to repeated reprocessing by washing and autoclaving (see Figs. 1C and 2). Brand-new bottles with clear walls, in contrast, produced a different effect. Here, the bank voles showed a markedly reduced readiness to enter the vessels, possibly due to repellent odours or because they felt less sheltered in colourless clear bottles.

Reduction: Ultra-sensitive bvPMCA with high robustness against 263K scrapie cross-contamination as alternative technology platform to prion bioassays in bank voles

Based on a systematic process analysis we were able to identify bvPMCA conditions that balanced high sensitivity for the detection of 263K scrapie prions with robust absence of false-positive results in non-seeded samples. We found that a tightly controlled reaction temperature of 38°C, a pH of 6.9, a narrow tolerance of the amount of glass beads added to the reaction mixture (20±2 mg), and stringent, yet easily feasible measures against cross-contamination were particularly important factors for successful bvPMCA in our hands.

Figure 3 shows representative findings from a set of three independently performed experiments in each of which unseeded duplicates, and duplicates seeded with 10^{-10} g, 10^{-11} g or 10^{-12} g of 263K scrapie brain tissue were subjected to four rounds of bvPMCA (lanes 1–4 display results from PMCA batches sampled after one, two, three and four PMCA rounds, respectively). No PrP^{TSE} was detected by Western blotting prior to PMCA in reaction batches containing 10^{-10} g/150 ml or lower concentrations of homogenized 263K scrapie brain tissue (lane 0). This is consistent with the previously established sensitivity of our Western blot assay which allows the detection of PrP^{TSE} in the equivalent of 2×10^{-8} g or higher amounts of homogenized brain tissue from terminally ill scrapie hamsters (Thomzig et al. 2003). However, 263K seeding activity could be detected after three rounds of PMCA in six out of six samples spiked with 10^{-10} g, and after four PMCA rounds in six out of six samples spiked with 10^{-11} g of 263K scrapie brain tissue. As exemplified by the uniform PrP staining of samples S1–S3 that had been spiked with 10^{-10} or 10^{-11} g of 263K scrapie brain tissue (Figure 3, first and second row, respectively), PMCA with these amounts of 263K seeds yielded reproducible and consistent amplification results. In contrast, PMCA of samples seeded with 10^{-12} g of 263K scrapie brain tissue produced more varying results, as displayed in the third row of Figure 3. With this seeding dose, only two out of six samples showed detectable amplification of PrP^{TSE} after four rounds of PMCA. Thus, 10^{-12} g of hamster scrapie brain tissue represented a threshold of 263K

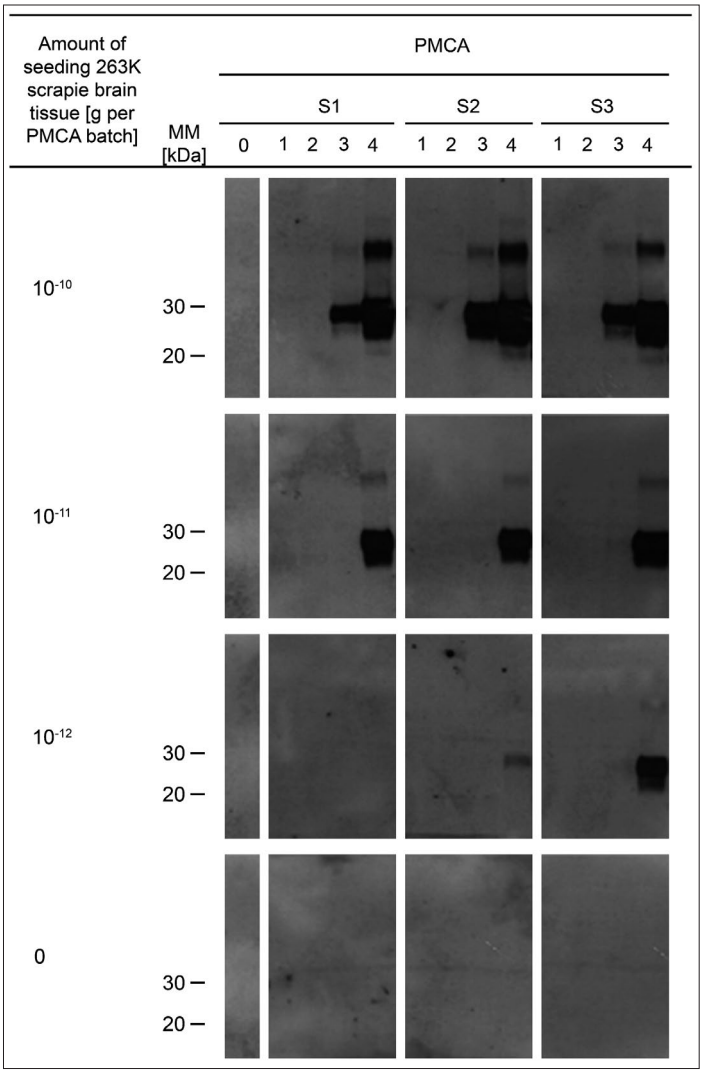

FIGURE 3: *Sensitivity and specificity of PMCA optimized for the amplification of 263K scrapie-associated PrP^{TSE} in bank vole reaction substrate. Western blot detection of PrPres, the proteinase K-resistant core of scrapie-associated PrP, after PMCA seeded with the indicated amounts of 263K scrapie brain tissue. Exemplary results are shown for three samples each (S1–S3). Lane MM, indicators of the typical molecular mass of PrPres in the range of ~30 to ~20 kDa. Lane 0 represents 3.75 µl from reaction mixtures prior to PMCA. Lanes 1–4 represent 3.75 µl from reaction mixtures after one, two, three and four rounds of amplification.*

seeding activity that could be detected only in a fraction of our bvPMCAs. Detectable cross-contamination of unseeded controls with 263K prions did neither occur in six out of six samples from this set of three independently performed PMCA experiments (for representative findings see Figure 3, bottom row), nor in further 10 negative controls similarly subjected to bvPMCA (not shown). This demonstrates that our bvPMCA was able to consistently detect 263K-associated seeding activity still in samples spiked with 10^{-11} g of 263K scrapie brain tissue, which corresponded to the sensitivity previously established for bank vole-adapted v586 prions (Cosseddu et al. 2011). At the same time, cross-contaminations of bvPMCA samples with 263K seeding activity higher than that level could be reliably excluded, although our laboratory setting is intensely exposed to the handling of 263K scrapie prions.

The results with our bvPMCA obtained so far suggest a consistent quantitative correlation between the amount of 263K seeding material, the number of PMCA rounds, and the staining intensity of detected PrPres for samples containing ≥ 10^{-11} g of 263K scrapie brain tissue. If further confirmed for 263K and other prion strains, such correlation could be prospectively used for a quantitative measurement of prion-associated seeding activities in bank vole substrate as previously described for hamster-based PMCA (Pritzkow et al. 2011).

Discussion

Refinement

According to Ödberg (1987) stereotypies are generally characterised by a "relatively invariant pattern, regular repletion and apparent uselessness", and "all situations eliciting stereotypies involve a chronic conflict and bear a common element, which is frustration". Furthermore, stereotypies can cause injuries, e. g. broken tails or facial lesions due to collisions with cage elements (Cooper 2010). Therefore, the occurrence of stereotypies in laboratory bank voles severely impairs animal welfare and calls for alleviating measures. Prompted by previous findings by Ödberg (1987) we addressed the problem of stereotypies in our bank vole colony by housing the animals in quite large cages with environmental enrichment. However, while enrichment elements for the housing of bank voles traditionally often consisted of merely hay or twigs, we used a broader, very diverse range of enrichment items to offer the animals improved conditions for bedding, nesting, sheltering, hiding and climbing. With these practical measures and the stress-reducing handling standards described in the following paragraph we have been able to avoid the occurrence of stereotypies in our animals almost completely.

For stress reduction, bank voles were accommodated from birth to individual and well-trained caretakers and veterinarians. In our animal facility, the bank voles were predominantly in contact with female personnel. This was expected to prevent distress by olfactory exposure to males, as previously observed in male and female mice (Sorge et al. 2014). In addition, we established a simple handling method using bottles by which bank voles can be subjected without stress to laboratory procedures such as weighing, inhalation anaesthesia, or humane euthanasia with isoflurane. This approach resembled the previously reported use of tunnels for the handling of mice, which was found to led the animals to voluntary approach, low anxiety and acceptance of physical restraint (Hurst and West 2010). Bank voles are more vivid and excitable than many strains of mice, though. While this emphasized the need for gentle handling procedures, it also cast some uncertainty whether a stress-free handling of bank voles by using bottles would be feasible. However, our animals did not show any aversion or anxiety when they voluntarily entered bottles lured by sunflower seeds, and the use of bottles led them to accept physical restrain without visible stress. Although these refinements on stereotypies and stress-free handling were achieved in a breeding colony of bank voles used for the generation of brain tissue samples, they are likely to be similarly effective when performing proper animal experimentation (e. g. infection assays) with bank voles in prion research and beyond.

Reduction

Highly-sensitive bvPMCA will be only established to a broader extent as a viable in vitro alternative to prion bioassays in bank voles when its basic problem of potential cross-contamination with prevalent prion strains such as 263K scrapie in TSE laboratories can be reliably prevented. Stringent conditions that excluded cross-contamination with bank vole-adapted v586 prions have been identified in a seminal study by Cosseddu et al. (2011). They included i) the use of multiply safecup vials (article number 72.733.200; Sarstedt AG + Co KG, DE), ii) wearing, of all personnel, of disposable coats, and iii) changing the water of the sonicator circuit and decontaminating its plastic holder and horn cap by immersion in bleach after each PMCA round. Our protocol eases these rigorous demands in that it does not require the last two safety procedures, and also allows to use more conventional 0.5 ml reaction vials without screw caps. This is also of particular importance with respect to PMCA formats that use reaction volumes larger than 100 µl, since multiply safecup vials (which are currently being offered by Sarstedt only as a "non core item" that is not listed in their 2018/2019 catalogue) have a volume limit of this size. Our study revealed a practical approach of how cross-contamination with 263K scrapie prions, potentially a prime disruptive factor for bvPMCA, can be reliably controlled despite ultra-efficient PrP^{TSE} amplification. This provides a basic technology platform that can be prospectively used as a versatile in vitro alternative to prion bioassays in bank voles. To this end, the procedure would be particularly helpful if it were adapted to prions from sporadic and genetic forms of human TSEs, for which other sensitive in vitro assays are still lacking. Although bvPMCA probably cannot completely replace bank vole bioassays for prions, it may substantially help to reduce them.

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Conflict of interest

The authors confirm that no conflicts of interest are associated with this publication, and that no financial support was given which could have influenced the outcome of this study.

Ethical approval

The breeding of bank voles has been approved by the competent animal protection authority in Berlin (Landesamt für Gesundheit und Soziales [LAGeSo], Berlin, Germany; current approval number: IC 114 – ZH 22). Although not mandatory, we reported euthanasia of normal bank voles from our colony to the competent animal protection authority in Berlin (LAGeSo; Registration Numbers T 0286/09, T 0300/15).

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Authors contribution

LP: Contributed to study conception, performed data acquisition, analysed and interpreted data, critically revised the manuscript. PK: Advised on experimental study design, performed data acquisition, analysed and interpreted data, critically revised the manuscript.. CT-R: Advised on experimental study design, analysed and interpreted data, critically revised the manuscript. AT: Contributed to study conception, analysed and interpreted data, critically revised the manuscript. MB: Contributed to study conception, analysed and interpreted data, drafted and finalized the manuscript.

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