Stereotactic delivery of M1000 prions to the dorsal CA1 in mice induces acute conditioned fear extinction and spatial memory disturbance

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Introduction

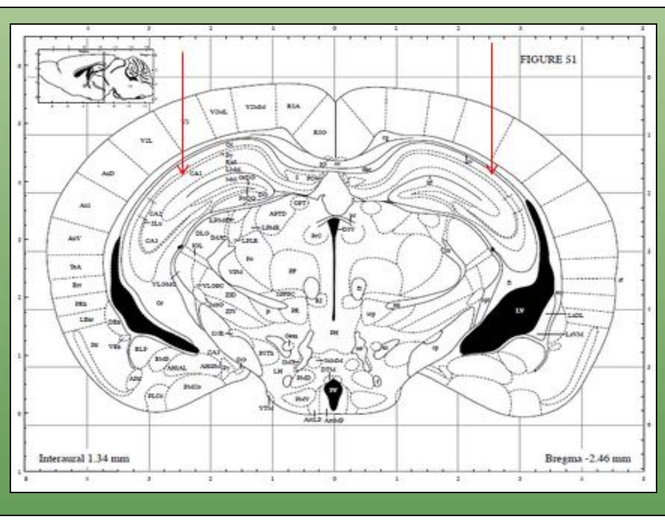
Prion diseases are a group of fatal neurodegenerative diseases with the unique feature of being trans-missible. It has been first described in sheep but identified later also in cattle, deer, elks and other mammals including humans.

The main pathogen responsible for the disease is a proteinaceous infectious unit(pr-i-on) constituted by α-helical cellular PrP (PrP^{c}) misfolded in a β-sheet richer isoform (PrP^{Sc}). With the refolding, PrP^{Sc} acquires the ability to template and refold new endogenous PrP^{c} into further PrP^{Sc} giving rise to accumulation of the abnormal isoform predominantly in the CNS but to a lesser extent at extraneuronal sites and throughout the body, such as within lympho-reticular tissues. Furthermore, PrP^{Sc} acquires new biophysical properties such as insolubility, protein kinase-resistance and resistance to routine 121ºC sterilization.

Recent evidence has shown how it is possible to create synthetic new infectious prions starting from recombinant PrP^{c} refolded in laboratory without the use of DNA or RNA material [1]. Remarkably, although genetic material isn't required, adding nucleotides and lipids to the recombinant prions can enhance the infectivity [2]. In more recent years other proteins responsible for other neurodegenerative diseases have been recognised with some prion-like properties such as α-synuclein fibrils [3], or induction of cerebral Aβ deposition in mice inoculated with synthetic Aβ aggregates [4]. Although our understanding of the spreading and propagation has improved, a detailed understanding of the molecular pathogenesis and mechanisms of neurotoxicity that lead to neuronal death and ultimately to the cognitive symptoms remains elusive. In particular, separating the effect of *de novo* prion propagation from the intrinsic neurotoxic effect of PrP^{Sc} in wild type animals expressing endogenous PrP^{c} is challenging.

Methods & Results

To verify the hypothesis that brain-derived prions harbour intrinsic neurotoxic properties, brain homogenates (10% in PBS) derived from either terminally-sick mice infected with mouse-adapted M1000 prion strain or age-matched controls inoculated with normal brain homogenates (NBH), were injected into 10 week old female WT C57BL/6 mice. The mice were anesthetized with a cocktail of xylazine/ketamine, placed on a stereotactic frame and bilaterally injected over the dorsal CA1 region of the hippocampus (coordinates -2.5mm from bregma, +/- 2.5mm laterally and -1.5mm depth) with 2µL of M1000 or NBH homogenates using a 10µL glass syringe equipped with a 26G needle. Six or seven days following stereotaxic injection, the mice commenced cognitive testing. All testing was performed within 16 days of inoculation to minimise pathogenic contributions from *de-novo* M1000 prion propagations [5] and significant sequestration of local PrP protein as substrate.



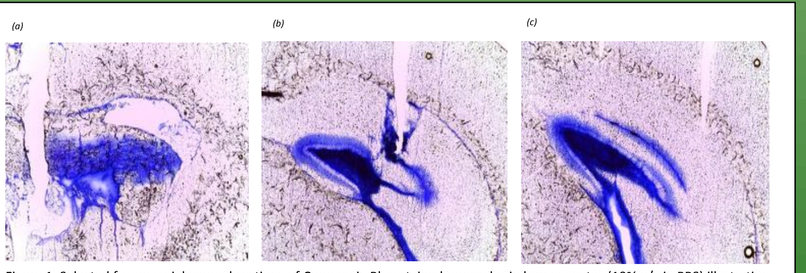
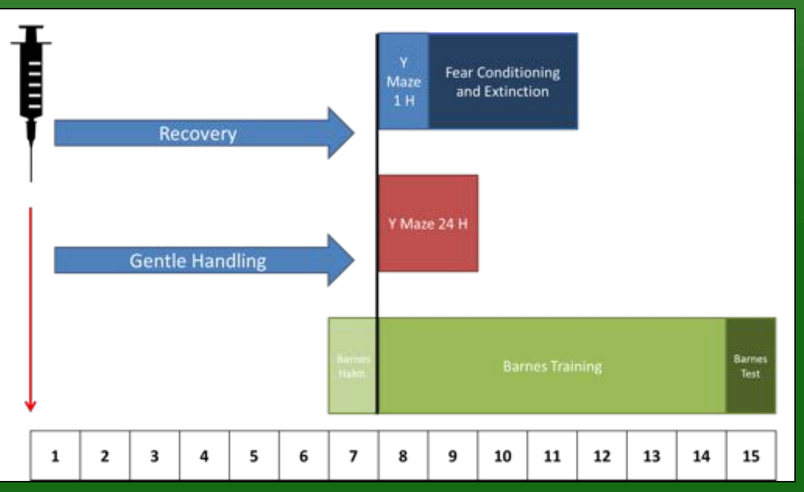


Figure 1. Selected frozen serial coronal sections of Coomassie Blue-stained mouse brain homogenates (10%w/v in PBS) illustrating the anatomical extent of a 2µl injection into the CA1 region using the bregma coordinates -2.5mm, ±2.5mm lateral and depth 1.5mm. Each figure show the distribution of the inocula at ~-1mm (*a*), injection site -2.5mm (*b*) and ~-2.9mm from bregma (*c*).



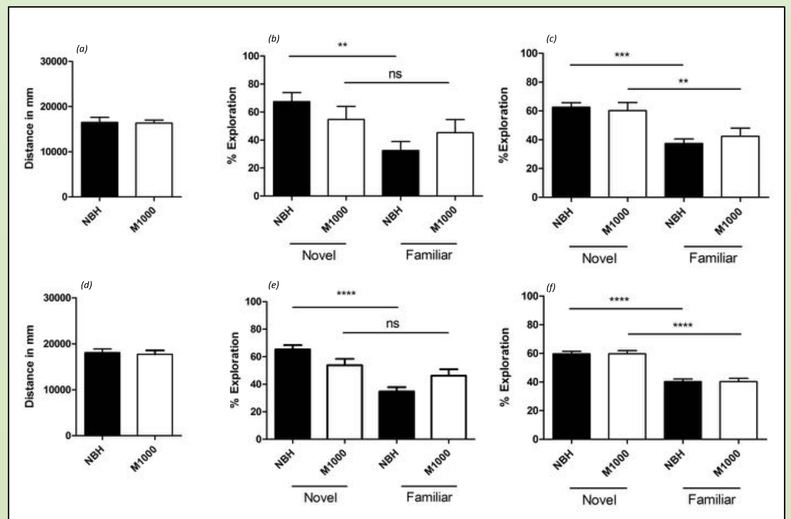


Figure 2. Means (+/-SEM) of Y-maze at 1 hour (*a,b,c*) and 24 hours (*d,e,f*). At both time points, there is no difference in exploration distance over the total test-phase (5 minutes). (*b,e*) While the control group (NBH) is able to discriminate the new arm from the familiar arm (1 hour test phase t=3.827, df=18, p = 0.0012; 24 hours test phase t=6.858, df=18, p < 0.0001) during the first 30 seconds, the M1000 group is unable to discriminate between the arms (1 hour test phase t=0.7157, df=18, p = 0.4845; 24 hours test phase t=1.143, df=18, p = 0.2681). (*c,f*) The inter-group difference observed disappears during exploration in the remaining 4.5 minutes of the test-phase (NBH 1 hour test phase t=4.290, df=18, p = 0.0004; NBH 24 hours test phase t=7.624, df=18, p < 0.0001; M1000 1 hour test phase M1000 t=3.585, df=18, p = 0.0025; M1000 24 hours test phase t=5.893, df=18, p < 0.0001) (N=10 each group).

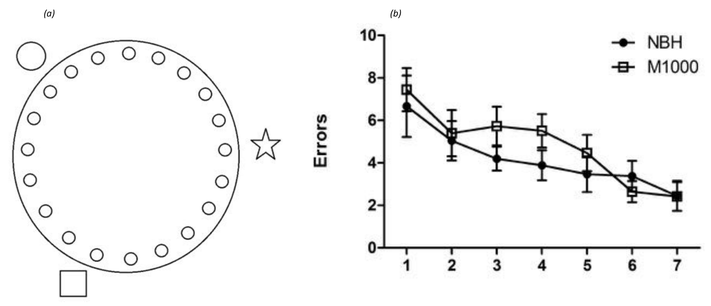


Figure 3. (*a*) Schematic representation of the Barnes maze arena with visual cues to facilitate spatial navigation of the maze; for each mouse, the escape box is randomly placed under one of the escape hole and the position is not changed for the entire acquisition phase (21 trials). (*b*) Mean (+/-SEM) of errors committed before successfully exploring the escape hole. The average errors are represented in blocks of 3 trials per day from day 1 (8 days after injection) until day 7 (14 days after injection). 2W RM ANOVA shows a significant effect of the 21 learning trials grouped in blocks of 3 ("Block" effect, $F_{6,168}$= 7.84, p <0.0001, N=15 each group).

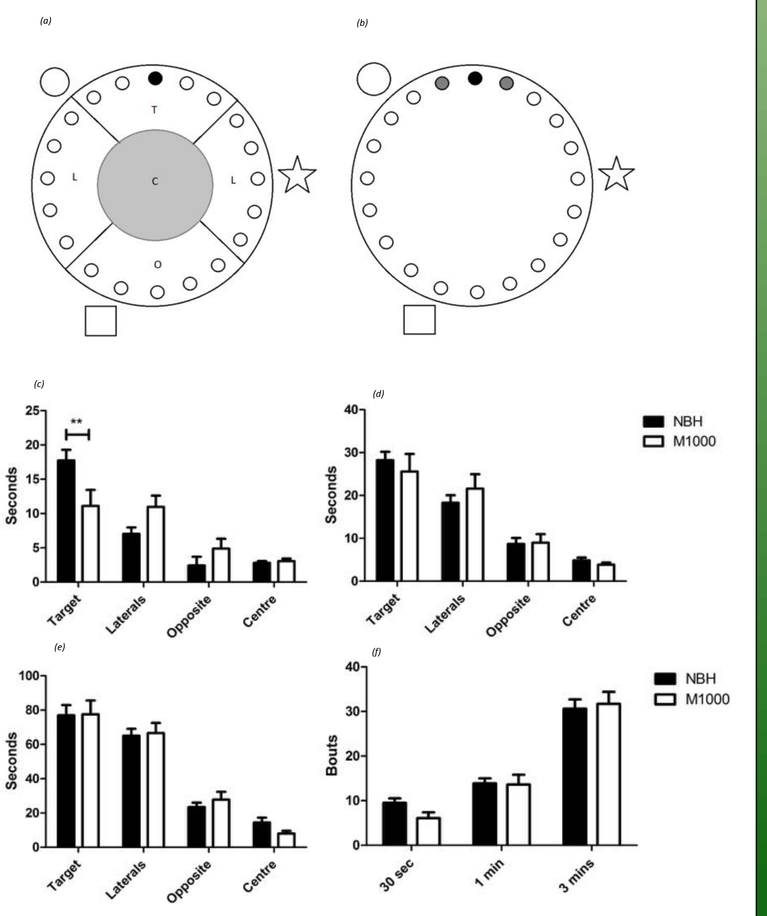


Figure 4. (*a*) Schematic representations of the areas of the Barnes maze used to assess spatial memory during the test-phase. Barnes maze divided into quadrants: the target quadrant (T) previously containing the escape hole (located in black), lateral quadrants (L), the opposite quadrant to the target (O) and the inner circle where the mouse starts the test (C). (*b*) Barnes maze representation for the scoring of the number of visits (bouts) during the test-phase: the escape hole where the box was placed during training (in black) and the adjacent holes (in grey). Results from the test-phase are given as quadrant exploration and as number of bouts during the first 30 seconds, first 1 minute and after 3 minutes. Although both groups explore preferentially the target quadrant after 1 minute ($F_{3,72}$= 40.50, p < 0.0001) and 3 minutes ($F_{3,72}$= 84.02, p < 0.0001)(*d,e*), there is a significant interaction of "Inoculum and Quadrant" ($F_{3,72}$= 5.67, p = 0.0015, Bonferroni post-hoc test t = 3.381, p < 0.01) during the first 30 seconds of the test phase (*c*). No difference in total bouts at 30 seconds, 1 minute or 3 minutes (*f*)(N=10 each group).

Group	Conditioning	p value	Tone Extinction 24 hours	p value	Tone Extinction 48 hours	p value
NBH	0.0920 ± 0.0920	0.33	17.56 ± 10.56	0.02	13.98 ± 10.33	0.37
M1000	0.00		6.592 ± 10.03		10.57 ± 7.066	

Table 1. Mean ± SEM % freezing baseline for the different phases of fear conditioning and tone extinction

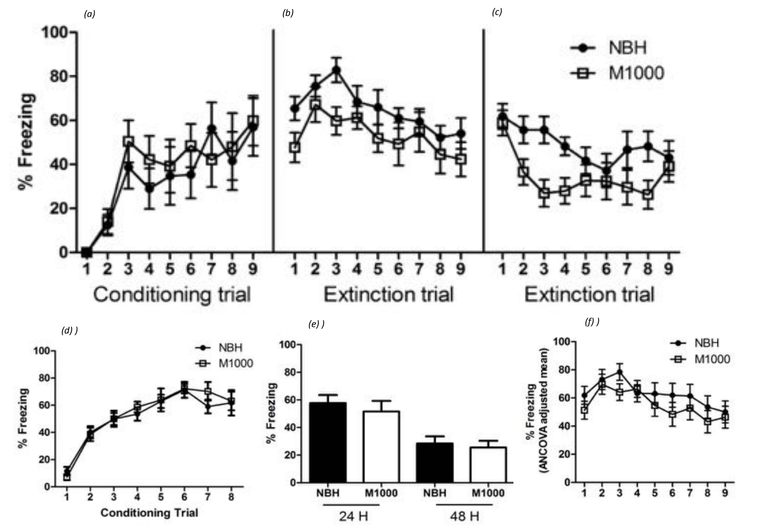


Figure 5. Mean (±SEM) of % of time freezing observed during each conditioning trial for CS (*a*) and CS extinction testing grouped in blocks of 5 stimuli at 24 (*b*) and 48 (*c*) hours. Mean (±SEM) of % time freezing observed during each ITI between CS-US pairings (*d*). Average (±SEM) of % time freezing observed during 3 minutes of context extinction test at 24 hours and 48 hours (*e*). A 2W ANOVA RM during the tone conditioning reveals a main effect of trials ($F_{8,144}$=9.22, p <0.0001)(*a*). 2W ANCOVA RM does not show a trial Block or Inoculum effect during 24 hours CS extinction (*f*). At 48 hours CS extinction instead, 2W ANOVA RM shows a significant effect of trial Block ($F_{8,144}$=4.55, p <0.0001) and a main Inoculum effect ($F_{1,18}$=4.68, p =0.0442). An analysis of the ITI-elicited freezing shows a main effect of the Trials ($F_{7,126}$=42.21, p =0.0001) during conditioning (*d*) and no difference in context-elicited freezing at 24 or 48 hours (*e*)(N=10 each group).

Discussions

The results derived from the Y-Maze, indicate a discrimination memory deficit of the new *versus* the old arm in the experimental group during the first 30 seconds, when the novelty component of the test is maximal. The effect observable at 1 hour is shown during the 24 hour test-phase as well suggesting that the effect is not limited to short-term memory but potentially to a more general effect on the hippocampal circuitry involved in the retrieval of memories. Interestingly, by the end of the test both experimental M1000 and control NBH groups explore the new arm preferentially suggesting a similarly efficient memory consolidation at short and long term.

Similar results are confirmed through the Barnes test where despite similar learning curves over 7 days with an interesting progression over days 3 & 4 of training, the M1000 group explores less efficiently on day 8 the Target Quadrant, where the escape box was located throughout the training. Congruently with the Y-maze data, this effect is observable only during the first 30 seconds of the test-phase when memory requirements are arguably the greatest due to the imminent environmental stress of the buzzer alarm. Although not statistically significant, a trend in less bouts to the escape box and the immediately adjacent holes is observable for the M1000 group correlating with more time spent outside the Target Quadrant. This again suggests a proper execution of the spatial navigation task with similar learning curves but a less efficient recall of the spatial memory. A similar dissociation between learning and retrieval has been reported also in rats following partial lesioning of the hippocampus with Morris Water maze [6].

Following the interesting results on spatial memory tasks, we wanted to investigate also if bilateral hippocampal M1000 prion injections could affect non-declarative associative learning and memory such as fear conditioning and tone/context extinction. As expected, the pairing of the CS with the US is similar in both groups: the association of the two cues is highly reliant of amydgala integrity and in our acute model the inocula spreading doesn't expand beyond hippocampal region. During the CS tone extinction, the analysis shows a significantly different extinction rate at 48 hours after training. Context extinction on the other hand, is similar in both groups at 24 and 48 hours. This second result could potentially suggest that the entity of hippocampal damage derived from single M1000 injection in not sufficient to affect the task or, due to the different ethological relevance of the environment during the task, the memory of the context in fear conditioning is qualitatively different from declarative memory.

Taken together, the observations show for the first time through a robust behavioural model that brain-derived prions harbour intrinsic neurotoxic properties in the setting of minimising *de novo* prion propagation and the dorsal hippocampus is a region susceptible to such toxicity. The degree of change in behaviour is subtle but reproducible and observable across various behavioural testing paradigm. Furthermore, as published literature has shown, the acute toxicity might not be correlated to an increased inflammatory response or direct neuronal loss in the hippocampus. Further analysis of brains harvested at different time points after inoculation might elucidate molecular mechanisms underlying the behavioural changes observed such as a selective astrocytic insult, transient decrease in LTP efficiency or synaptic pruning.

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