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Nramp1 is expressed in neurones and is associated with behavioural and immune responses to stress

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Abstract

The gene *Nramp1* encoding the natural resistance associated macrophage protein (Nramp1) influences susceptibility to intracellular infections and autoimmune diseases, and the humoral response to stress. Nramp1 functions as a proton/divalent cation antiporter in the membranes of late endosomes/lysosomes, regulating cytoplasmic iron levels in macrophages. The *Drosophila* homologue of *Nramp1* is expressed in sensory neurones and macrophages, and influences taste behaviour directly through divalent cation transport. Here we demonstrate that murine Nramp1 is also expressed on neurones as well as microglial cells in the brain and influences the behavioural response to stress, hypothalamus-pituitary-adrenal (HPA) axis activation and mortality following *Toxoplasma gondii* infection in control and pre-stressed mice. We hypothesise that, although differences in HPA activation translate into differences in adrenal enlargement and basal circulating corticosterone levels, the primary influence of Nramp1 is at the level of the neuronal response to stress. These results provide new insight into the possible roles of divalent cation transporters of the *Nramp* gene family in regulating metal ion homeostasis in the brain and its pathological implications.

Keywords

Divalent cation transporter; HPA axis; Toxoplasma; corticotrophin releasing hormone; corticosterone

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INTRODUCTION

Resistance to intracellular infections is influenced by the gene (*Nramp1*, previously termed *Ity/Lsh/Bcg*) mapping to mouse chromosome 1/human 2q35 and encoding the natural resistance associated macrophage protein 1 (Nramp1). The amino-acid sequence of Nramp1 predicts a polytopic integral membrane protein with 10 or 12 putative membrane spanning domains (reviewed [1]). Inbred mice carrying wild type *Nramp1* ($Nramp1^{G169}$) are innately resistant to *Salmonella typhimurium, Leishmania donovani*, and some mycobacterial infections; other inbred strains that have the mutant allele ($Nramp1^{D169}$) are susceptible (reviewed [2]). *Nramp1* also influences susceptibility to *Toxoplasma gondii* infection [3,4]. Allelic associations and/or linkage between human *NRAMP1* and susceptibility to tuberculosis, leprosy, and a range of autoimmune diseases has been demonstrated (reviewed [2]).

The murine Nramp1 protein has been localised to membranes of late endosomes, lysosomes and phagolysosomes of macrophages [5,6]. Nramp1 functions as a proton/divalent cation (Fe^{2+}, Zn^{2+}, Mn^{2+}) antiporter [6A], transporting Fe^{2+} against the H^+ gradient into phagolysosomes where the Fenton reaction produces toxic antimicrobial hydroxyl radicals in macrophages from *Nramp1* wild type mice but not in those from congenic *Nramp1* mutant mice [7]. Endosomal fusion events [8], known to be Zn^{2+} dependant [9,10], and late endosomal acidification [11] are also defective in macrophages from *Nramp1* mutant mice.

The severity of infection in mice and humans is also influenced by the stress response, principally mediated by activation of the hypothalamus-pituitary-adrenal (HPA) axis (reviewed [12]). Stress causes secretion of releasing factors, principally corticotrophin releasing hormone (CRH), from the paraventricular nucleus of the hypothalamus (PVN). These stimulate the pituitary to release adrenocorticotropin hormone, causing the adrenal glands to release glucocorticoids into the blood (predominantly corticosterone in the mouse). The stress response has complex effects upon immunity but these are predominantly suppressive and, in the majority of human and animal experiments, stressors increase susceptibility to infectious diseases. This effect is largely mediated by circulating corticosterone [13].

The murine *Nramp1* gene is implicated in stress-induced immunosuppression. *In vitro*, macrophages from *Nramp1* mutant mice have pleiotropic defects in macrophage activation for killing intracellular pathogens compared with macrophages from *Nramp1* wild type mice (reviewed [14]). Activation of the HPA axis by restraint stress increased the severity of *Mycobacterium avium* infection in *Nramp1* mutant mice but did not affect the ability of congenic *Nramp1* wild type mice to control the mycobacterial infection [15]. This was attributed to differences in the sensitivity of macrophages from *Nramp1* congenic mice to corticosterone because HPA axis activation also caused increased intracellular growth of *M. avium* in macrophages from *Nramp1* mutant, but not wild type mice [16,17]. These *in vivo* and *in vitro* differences were simulated by corticosterone administration and abrogated by surgical or pharmacological adrenalectomy.

To further characterise the role of *Nramp1* in regulating the response to stress, we have examined behavioural, humoral and hypothalamic stress responses following restraint in *Nramp1* congenic mice and have localised the expression of Nramp1 within the brain. We have investigated the relevance of our findings to *Toxoplasma gondii* infection that directly affects the brain.

MATERIALS AND METHODS

Mice

C57BL/10ScSn-(*Lsh*^{s}) (Harlan Olac, Blackthorn, Bicester, England) *Nramp1* mutant and congenic B10.L-*Lsh*^{r} (N20) *Nramp1* wild type [18] mice were bred in-house in a conventional facility. Male mice were studied in 2 experiments that were identical except for mouse age. In experiment 1, mean±SD age was 5.3±0.80, compared with 10.0±2.2 months in experiment 2. Mice were maintained in a 12:12 hour dark:light cycle, housed in cages of 4 to 5 and fed and watered *ad libitum*. All experimental procedures started at 10 a.m., 4 hours after lighting commenced. Blood samples were taken from animals in each experimental group at the same time of day to reduce the effect of circadian rhythms. All of the mice were bred together in adjacent cages and mice in each experimental group were randomly selected from the same cages as controls. In order to prevent auditory or olfactory cues from causing stress responses, mice subjected to or recovering from restraint stress and/or infection were subjected to experimental procedures and housed for the duration of the experiments in rooms sound-insulated and separately ventilated from control animals and animals awaiting experimental procedures. All experimental procedures were carried out under licence from the UK Home Office.

Restraint stress

Mice were placed in 50 ml conical plastic tubes (Becton Dickinson, Oxford, UK) from which a longitudinal slit had been cut to provide ventilation and to prevent hyperthermia. Mice were restrained with their tails emerging from the pointed ends of the closed tubes taped to a steel surface. There was sufficient space for the mice to move but not to alter their orientation within the tubes. *Nramp1* congenic wild type and mutant mice were restrained at the same time to prevent environmental differences from altering experimental conditions for the different genotypes. After a total of 60 minutes restraint, mice were returned to their original cages. All of the mice in each cage were restrained together to prevent stressed and unstressed mice from coming into contact with one another.

Behavioural stress response

Behaviour during the 1 hour of restraint stress was observed. Some mice chewed away areas of the plastic tubes that were used to restrain them and it was noted that this stress-related behaviour differed between the genotypes studied. The borders of the areas that had been bitten away were therefore traced using transparent plastic film. These traces were digitally scanned against length standards and the areas quantified using image analysis software (Openlab version 2, Improvision, Coventry, UK).

Specimens

Thirty minutes after restraint commenced, 200 μl blood was collected into heparinised vessels from the tail veins of 8 mice of each genotype. Plasma was separated by centrifugation at 3,500 g for 5 minutes and frozen at −80°C. Four hours after restraint commenced, these 8 mice were rapidly sacrificed by cervical dislocation and immediate decapitation to allow collection of blood (from the decapitated corpse) without eliciting a second stress response. The brains were then dissected from the skull and dura, snap frozen in liquid nitrogen and stored at −80°C.

As un-stressed controls, an additional 8 mice of each genotype in both experiments were individually killed by cervical dislocation, immediately decapitated and blood collected whilst the brain was excised. The time between picking up each mouse to decapitation was minimised (less than 10 seconds) to reduce the risk of eliciting a stress response.

A cryostat was used to cut 12 μm coronal sections through the mouse brains rostral to the posterior limit of the anterior commisure. Consecutive sections were stained with 0.1% methylene blue (BDH, Lutterworth, UK) and examined under x10 magnification. When the characteristic butterfly shape of the paraventricular nucleus (PVN) of the hypothalamus was reached, every third section was placed onto poly-L-lysine coated ribonuclease free slides (BDH) so that each slide contained 3 to 4 sections distributed throughout the thickness of the PVN. Slides were dried for 5 minutes on a heating block at 37°C and stored at −80°C.

Humoral stress response

Both adrenal glands and the left kidney were excised from each mouse at autopsy and stored in 4% buffered formalin. Surrounding fat was dissected away with the aid of a binocular microscope and the organs were then weighed.

Duplicate plasma samples were diluted 1:100 in buffer and total plasma corticosterone was measured using antiserum provided by G. Makara (Institute of Experimental Medicine, Budapest, Hungary) and ^{125}I-corticosterone tracer (specific activity 2 to 3 mCi/μg, ICN Biomedicals, CA, USA). The limit of detection of the assay was 11 ng/ml. The intra-assay co-efficient of variation was 11%.

Hypothalamic stress response

Quantitative *in situ* hybridisation was used to measure CRH mRNA in the PVN sections as previously described [19,20]. All sections were processed and quantified together: triplicate sections from groups of 8 wild type and mutant, control and stressed mice from both experiments. The probes used were 48-mer oligonucleotides complementary to exonic CRH mRNA (Perkin-Elmer, London, UK). Terminal deoxytransferase was used to label the 3' end of the probe with ^{35}S-deoxy-ATP (1,000 Ci/mmol) to a specific activity of 5.5×10^8 dpm/mol. The specificity of the probes has been determined [19] and representative images have been published previously [21,22]. The autoradiograph images of PVN CRH mRNA were quantified using a digital image analysis system (Image 1.22, NIH, Bethesda, MD, USA). In order to compensate for the non-linear response of the film to radioactivity, the standard curve was derived from ^{35}S-labelled standards exposed with the PVN sections. The autoradiograph image from each characteristically shaped PVN was outlined and exposure within that area was quantified compared with the background exposure from the surrounding brain.

Preparation of whole brain embryonic cell cultures

Pregnant N20 wild type mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation 1 to 2 days before expected delivery and their embryos were immediately excised. Three embryos with a mean crown-rump length of 24 mm, equivalent to a gestational age of approximately 20 days (E20), were dissected in ice cold phosphate buffered saline (PBS, pH 7.4) containing 0.6% glucose (w/v) under a ×20 magnification. All reagents were supplied by Sigma, Gillingham, UK, unless otherwise stated. Whole brains were removed, the meningeal tissue was carefully excised and neural tissue was sectioned into 1 mm^3 portions which were pooled. These were incubated in trypsin (207 U/ml in DNase I) for 20 minutes at 37°C, and then washed 3 times in DNase I (240 U/ml in 0.6% glucose/PBS). Tissue was then mechanically triturated into a single cell suspension using a flame-polished Pasteur pipette. Cell viability was assessed using the trypan blue exclusion method [23]. Cells were plated directly onto poly-L-lysine (0.01%) coated, 13 mm glass coverslips in 24 well NUNC (Denmark) tissue culture plates at a density of 50,000 live cells per coverslip. They were allowed to differentiate in sterile culture medium (1.0 ml per well of 70% DMEM, 30% HAMS, Gibco, Paisley, Scotland) supplemented with foetal calf serum (1% v/v), B27 nutritional supplement (2% v/v; Gibco) containing 100 units penicillin

G, 100 μg streptomycin and 0.25 μg amphotericin B per ml (Gibco). Cultures were maintained in a humidified incubator (95% air, 5% CO_2) at 37°C. Half of the medium in the culture wells was replenished every third day. On day 7, phase contrast microscopy revealed extensive differentiation of the embryonic cells into inter-digitating cells with the morphology of neurones, astrocytes, microglia and oligodendrocytes. Coverslips were then fixed in paraformaldehyde (4% v/v in PBS; pH 7.4) and stored at 4°C in PBS (pH 7.4 containing 0.01% sodium azide) for immunochemical analysis.

Immunochemistry

Brain sections were warmed to room temperature, fixed in acetone for 10 minutes, post-fixed in ice cold methanol for 5 minutes and then washed in PBS. Sections and cultured embryonic brain cells were stained by indirect fluorescence immunochemistry as described [24].

Blocking—Non-specific staining was reduced by incubation for 1 hour with 3% fatty-acid-free bovine serum albumin (BSA) in Triton-Tris buffered saline (TTBS, 154 mM NaCl, 10 mM Tris, 0.01% sodium azide, 0.1% Triton x100, pH 7.4). Mouse brain sections that were to be labelled with mouse anti-NeuN primary antibodies were then incubated in affinity purified Fab-immunoglobulin fragment goat anti-mouse IgG (Jackson Immuno Research laboratories, West Grove, PA, USA) in 3% BSA in TTBS for 24 hours at 37°C. This prevented secondary anti-mouse IgG antibodies from directly labelling IgG within blood vessels.

Primary anti-Nramp1 antibodies—The blocking solution was replaced with fresh dilutions of 2 pooled primary antibodies in 3% BSA in TTBS and these were co-incubated at 4°C in a humidified chamber for 16 hours. Rabbit polyclonal anti-N-terminal Nramp1 serum was used at 1:250 dilution. This antibody epitope maps to the Nramp1-specific amino acid sequence PSADQGTF at position 43 to 50 [6]. The sequence recognised has no protein homology with published Nramp2 sequence. Rabbit polyclonal anti-C-terminal Nramp1 serum [25] was used at 1:500 dilution. Monoclonal rat anti-N-terminal Nramp1 antibody 3N4B3 (1:50 dilution, 38 μg IgG/ml) was from the same fusion as anti-N-terminal Nramp1 monoclonals previously described [6], but has not been epitope mapped. This antibody was harvested from hybridoma hollow-fibre culture in RPMI 1640 (Gibco), affinity purified with protein G, eluted in pH 2.2 glycine and dialysed against PBS/azide (Cymbus Biotechnology Ltd, Chandlers Ford, UK).

Primary cell marker antibodies—The neuronal cell markers NeuN (Chemicon, Harrow, UK) and β-tubulin III (Sigma, both 1:500 dilution = 2 μg IgG/ml) were used to stain adult brain and embryonic cell cultures, respectively. Cells of the macrophage/monocyte lineage, including microglia, were stained with the rat anti-mouse F4/80 antibody (Serotec, Oxford, UK; 1:10 dilution = 100 μg IgG/ml). Astrocytes were stained with rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Dako, Glostrup, Denmark, 1:750 dilution = 5.5 μg protein/ml). The oligodendrocyte cell marker Gal-C (Boehringer, 1:200 dilution) was also used. Rat anti-mouse macrosialin IgG2a (1:500 dilution) was kindly provided by Dr Thierry Lang (Institut Pasteur, Paris).

Secondary antibodies—After washing in PBS, pairs of highly specific fluoroscein (FITC) or Texas red (TRITC) labelled secondary anti mouse (Vecta, Peterborough, UK) rat (Jackson) or rabbit (Sigma) IgG antibodies were co-incubated for 5 hours at room temperature. The secondary antibodies used had been raised against IgG from the species of the primary antibodies used in that particular experiment and were both diluted 1:50 in 3% BSA in TTBS together with 1 ng/ml Hoescht (Sigma) nuclear marker.

Controls—In each experiment, 4 additional tissue sections or cell preparations were stained as controls with anti-Nramp1 antibody omitted, cell marker antibody omitted, with both primary antibodies omitted and with all primary and secondary antibodies omitted to test for the possibility of antibody cross-reactivity between animal species. For polyclonal rabbit anti-Nramp1 antibody controls, appropriate dilutions of rabbit pre-immune serum were applied.

Analysis—Sections were then washed in 3 changes of PBS, rinsed in distilled water and mounted in Citifluor AF1 (Agar Scientific, Kent, UK). Staining was viewed with an Olympus Provis AX70 fluorescent microscope (London, UK), mounted with a Hamamatsu C47-42-95 digital camera (Photonics KK, Japan) and images captured with Openlab software. Exposures for FITC and TRITC channels varied between 75 and 400 ms. The microscope, camera and printer settings were standardised within experiments for stained and control sections, all of which had been incubated in parallel.

Functional immune challenge: *Toxoplasma gondii* infection

In parallel with the restraint procedure for animals that were sacrificed for tissue samples, additional *Nramp1* congenic mice were restrained for 1 hour and then infected. Groups of 20 mice were restrained prior to infection in experiment 1 and groups of 10 mice in experiment 2. Twenty-four hours after restraint stress, these mice were infected by gavage with 10 oocysts of the Beverley strain of *T. gondii*, as described [4]. In both experiments, larger groups of age-matched, unstressed mice were infected as controls. Survival was noted daily at 10 a.m. for 85 to 100 days. In experiment 1, 4 unstressed infected mice of each genotype were sacrificed 10 days post infection and tissues collected as described above for immunochemical investigation.

Statistical analysis

All processing and measurements were done blind on coded samples. The continuous variables were approximately normally distributed and are expressed and plotted as mean + standard error of the mean (SEM). Because the sample sizes were too small to prove normality, the non-parametric Mann-Whitney U test was used to analyse differences between groups. Mortality curves were analysed with the Log Rank test and Cox regression models. The latter were also used to test for possible effects of mouse age on mortality within each experiment. Statview statistical software (version 5, SAS Institute Inc., NC, USA) was used.

RESULTS

Behavioural stress response

Nramp1 wild type mice struggled persistently, attempting to escape from restraint and to bite the experimenter handling them. In contrast, *Nramp1* mutant mice were noted to be relatively docile and exhibited little aggressive behaviour throughout the experiment. Consistent with these empirical observations, the areas of plastic tube bitten away by restrained mice (Figure 1a) were significantly greater for *Nramp1* wild type than mutant mice (experiment 1 $p<0.0002$; experiment 2 $p<0.04$, Figure 1b). In experiment 2, 3 *Nramp1* resistant mice died immediately after restraint. Autopsy revealed no macroscopic abnormality.

Humoral stress response

Figure 2a shows adrenal gland and kidney mass in *Nramp1* congenic mice. *Nramp1* mutant mice had significantly heavier adrenal glands than wild type mice (experiment 1 $p<0.0001$,

experiment 2 $p<0.02$). Kidney mass was measured to control for the size of the mice and did not differ between wild type and mutant mice in either experiment.

Figure 2b shows plasma corticosterone concentrations in control, un-stressed *Nramp1* congenic mice and in mice during and 4 hours after restraint stress. Before restraint stress, *Nramp1* mutant mice had significantly higher corticosterone concentrations than wild type mice (14 fold greater in experiment 1, $p<0.004$; 2.1 fold greater in experiment 2, $p<0.04$). During restraint stress, corticosterone concentrations were significantly elevated compared with pre-restraint controls ($p<0.0001$ for all groups). The absolute corticosterone concentrations during restraint did not differ significantly between wild type and mutant mice in either experiment. Four hours after restraint stress had commenced, corticosterone concentrations had fallen to levels similar to those in control mice prior to restraint and there was a non-significant trend towards *Nramp1* mutant mice again having higher concentrations than wild type mice (1.6 fold greater in both experiments).

Figure 2c shows the relative increase in corticosterone comparing during stress/pre-stress levels for each group. This ratio may be physiologically relevantand was significantly greater for *Nramp1* wild type than mutant mice ($p<0.005$ in both experiments).

Hypothalamic stress response

Figure 3 shows the results of quantitative *in situ* hybridisation for CRH mRNA in the PVN of the hypothalamus. Before restraint stress, the wild type and mutant mice had similar levels of CRH mRNA in both experiments. In experiment1, mice recovering from restraint had significantly elevated CRH mRNA compared with controls (wild type and mutant $p<0.02$) and levels were higher in wild type than mutant mice ($p<0.04$). The results were similar in experiment 2, but the death of 3 wild type mice and the failure of 1 hybridisation slide halved the size of this experimental group and the difference between wild type and mutant mice did not reach statistical significance ($0.05<p<0.08$).

Immunochemistry

Figure 4 shows selected results of immunochemical staining of brain sections and embryonic brain cell cultures. Polyclonal anti-N-terminal (Figure 4a and b) and anti-C-terminal (not shown) Nramp1 antibodies gave similar results. Both antibodies stained cytoplasmic vesicles within the same cells as specific mouse anti-neuronal cell markers in brain sections (NeuN which stains the nuclei and peri-nuclear cytoplasm [26,27]; Figure 4a) and in brain cell cultures (β-tubulin III [28]; Figure 4b). The identification of these cells as neurones was supported by their nuclear morphology and, for cultured cells, their characteristic inter-digitating processes. Monoclonal rat anti-N-terminal Nramp1 antibody 3N4B3 (Figure 4c and f) similarly stained cytoplasmic vesicles within the same cells as the neuronal cell marker β-tubulin III in brain cell cultures (Figure 4c). The polyclonal and monoclonal anti-Nramp1 antibodies exactly co-localised with one another in brain cell cultures (not shown).

Polyclonal anti-N-terminal (Figure 4d and e) and anti-C-terminal (not shown) Nramp1 antibodies also stained cytoplasmic vesicles within a second, sparse population of cells in brain sections (Figure 4d) and brain cell cultures (Figure 4e). These were identified as cells of the macrophage/monocyte/microglial lineage by co-staining with the pan-macrophage marker F4/80 [29] and their morphology was that of microglial cells.

Within brain cell cultures, rabbit polyclonal anti-N-terminal Nramp1 antibody partially co-localised with rat anti-macrosialin antibody, a marker for late endosomes [30] (not shown). Astrocytes had highly characteristic morphology, were positive for the rabbit polyclonal

anti-GFAP antibody [31] and were negative for rat monoclonal anti-Nramp1 antibodies (Figure 4f).

Cultured brain cells with the characteristic morphology of oligodendrocytes were rarely seen and anti-Nramp1 antibodies did not label cells with this morphology. However, oligodendrocytes only stained with the specific cell marker Gal-C under conditions that prevented Nramp1 staining (no permeabilisation) and these methodological limitations prevented definitive proof that Nramp1 was not expressed on oligodendrocytes.

Anti-Nramp1 antibody staining was positive in brain sections from wild type and mutant *Nramp1* congenic mice throughout the brain sections, including neurones within the PVN. Neuronal and macrophage/microglial staining seemed brighter in wild type than mutant mice and appeared to be increased 10 days post-infection in wild type but not in mutant mice. Objective quantification to confirm these subjective impressions of protein expression was beyond the resolution of the immunochemical technique used.

Control brain sections and cultured brain cell preparations incubated without either anti-Nramp1 or cell marker primary antibodies confirmed the specificity of the secondary antibodies, resulting in no visible staining at the microscope, camera and printer settings used to produce the images in figure 4.

Functional immune challenge: *Toxoplasma gondii* infection

Figure 5 shows Kaplan-Meier cumulative survival plots following *T. gondii* infection in *Nramp1* congenic mice. In unstressed mice in experiment 1 (Figure 5a), mutant mice had greater mortality than wild type mice (Log Rank test $p<0.0001$; Cox regression $p<0.001$) and restraint stress prior to infection had no significant effect on mortality.

In experiment 2 (Figure 5b), *Nramp1* genotype did not significantly affect survival in the absence of stress. In wild type mice, restraint stress did not significantly affect mortality, but in mutant animals, restraint stress prior to infection increased mortality compared with unstressed mutant mice (Log Rank test $p<0.03$; Cox regression $p<0.02$). Stressed mutant mice also had significantly increased mortality compared with stressed wild type mice (Log Rank test $p<0.007$; Cox regression $p<0.002$). Cox regression analysis confirmed that minor age variations within each of the experiments did not significantly influence survival. There were no deaths in uninfected wild type and mutant mice over these periods.

DISCUSSION

Results presented here demonstrate that *Nramp1* genotype is associated with behaviour and the neural immune response to stress in addition to infectious disease susceptibility. These effects might reasonably have been interpreted as differences in the ability of cells of the macrophage/microglial cell lineage to respond to corticosterone triggered by activation of the HPA axis [16,17]. However, our finding that Nramp1 is expressed in neurones, including those in the PVN, suggests a more direct influence of Nramp1 in HPA axis activation. This is supported by our observation that stress by restraint directly induces mRNA for CRH in the brains of *Nramp1* wild type to a greater extent than mutant mice.

Our pilot experiments concerning the behavioural response to restraint stress strongly suggest that *Nramp1* mutant mice exhibited attenuated escape-behaviour. Studies in other mouse strains have shown that some behavioural effects of restraint stress may be simulated by administration of CRH [32,33], even after hypophysectomy [32], and are partially prevented by a CRH antagonist [33,34]. The deficient CRH stress responses that we observed in congenic *Nramp1* mutant mice may therefore directly account for their

abnormal behavioural stress responses compared with wild type mice. Our study is not the first to identify a role for a *Nramp* gene family member in regulating behavioural responses. A mutation in the *malvolio* gene, the *Drosophila* homologue for mouse and human *Nramp1*/*NRAMP1*, causes abnormal taste behaviour [35]. Interestingly, malvolio is expressed in both macrophages and sensory neurones [35]. The *malvolio* mutation can be complemented with human *NRAMP1* [36], and the abnormal taste behaviour is suppressed when mutant flies are reared in the presence of, or given a 2 hour exposure to, $MnCl_2$ or $FeCl_2$ [37]. $ZnCl_2$ inhibits the effect of $MnCl_2$ but does not itself restore taste behaviour. Hence, these authors conclude that *malvolio* functions as a $Mn^{2+}/Fe^{2+}/Zn^{2+}$ transporter, with Mn^{2+} and/or Fe^{2+} being functionally involved in transduction of taste perception in *Drosophila*.

Evidence for effects of dietary exposure to divalent cations on behaviour also exists in vertebrates. In rats, a period of severe early iron deficiency causes long-term behavioural effects [38,39] and elevated basal plasma corticosterone concentrations [40]. The corticosteroid stress response is also impaired in humans deficient in iron [41] and in brown trout living in water contaminated by the cations cadmium and zinc [42]. Such effects may be regulated by dietary uptake of divalent cations through the action of Nramp2 in the gut [43,44], and/or through cellular regulation of divalent cations in macrophages/microglial cells and neurones. In macrophages, Nramp2 is involved in the cellular uptake of divalent cations and co-localises with transferrin receptor in the early endosomal compartment [45]. Nramp2 acts as a proton/ divalent cation symporter [44], transporting divalent cations from the low pH of the early endosome into the cytoplasm [45]. Nramp1 localises to the late endosomal/lysosomal compartment [5,6] and acts as a proton/divalent cation antiporter [6A] to transport divalent cations from the cytoplasm to the low pH of these vesicles [7]. Both molecules therefore regulate cytoplasmic divalent cation concentrations. In the *Nramp1* mutant we have studied here, the ability to transport divalent cations from the cytoplasm to the late endosomal/lysosomal compartment is thought to be impaired. One of the direct effects of this is failure to deliver Fe^{2+} to these vesicles to participate in the Fenton reaction and generate antimicrobial hydroxyl radicals [7]. This is clearly important in resistance to infection. An important bi-product of this defect is to alter cytoplasmic iron levels [7,46]. Hence, *Nramp1* mutant macrophages have higher levels of cytoplasmic iron [7] that directly influences mRNA stability for a range of macrophage activation markers [47]. We hypothesise that such a mechanism could account for the differences in mRNA levels for CRH observed here between *Nramp1* wild type and mutant mice.

Nramp1 may influence the HPA axis through a direct effect on neuronal function in response to stress by restraint, but it is clear that there are baseline differences in HPA axis activation leading to differences in adrenal size and function in *Nramp1* congenic mouse strains. Animals used in these experiments were housed in a conventional animal facility, but have never screened positive for any intra-macrophage pathogens. Hence, it seems unlikely that these baseline differences are due to chronic sub-clinical infections to which *Nramp1* mutant mice are more susceptible than wild type mice are. Differences in the immunosuppressive response to environmental stress may contribute. Paradoxically, although *Nramp1* wild type mice made a stronger response to restraint stress in terms of enhanced CRH mRNA and HPA axis activation, it is mutant mice which showed signs (larger adrenals, higher basal plasma corticosterone) of chronic HPA axis activation. This may reflect failure to respond appropriately to negative feedback regulation by corticosterone, leading to sustained high basal levels of corticosterone and immunosuppression contributing to generalised susceptibility to macrophage infection [15-17].

There are parallels between our finding that infection-susceptible mutant mice have attenuated HPA stress responses and studies in inbred rat strains. Experiments with the

immature, female Lewis rat have suggested that a defect in the HPA axis response to acute stress can predispose these rats to autoimmune and inflammatory phenomena. This defect has been located to the hypothalamic PVN as the Lewis strain is unable to increase CRH mRNA in response to stress. In contrast, the histocompatible Fisher strain of rat has a robust stress response and is resistant to these conditions [48-50]. The Lewis and Fisher strains also demonstrate differences in stress behaviour. Interestingly, transplantation of Fisher embryonic neuronal tissue into Lewis rats reduces this predisposition to inflammatory disorders [51] and attenuates the behavioural differences [52]. Similarly in the present study, we have noted that the *Nramp1* mutant mice had reduced CRH mRNA associated with infectious disease susceptibility and behavioural stress responses. Although we do not know whether the responses in rats come under *Nramp1* control, these data provide further evidence for the important protective role of the HPA axis in response to infection and disease and support accumulating evidence of the role of CRH in mediating these effects [53-57].

In the case of our experiments with *T. gondii* infection, it was interesting that 5 month old mice in experiment 1, mutant mice were significantly more susceptible to infection without stress, and that mortality occurred slowly over 10 to 60 days post infection consistent with chronic suppression of the immune response. In contrast, the mortality observed in stressed 10 month old mutant mice occurred rather dramatically between days 10 to 20 post infection, at a time when *Toxoplasma* is known to begin to cause inflammation within the brain [58]. Failure to suppress the inflammatory response to infection in the brain leads to toxoplasmic encephalitis and death [59], similar to that seen with direct delivery of lipopolysaccharide to the brain side of the blood brain barrier [60]. *Toxoplasma* is a potent inducer of macrophage inflammatory responses [61]. It would therefore be of interest to determine whether Nramp1 expression directly influences microglial cell and/or neuronal responses to *Toxoplasma*, a factor that may be important during congenital infection and development of associated clinical sequelae.

We have demonstrated here for the first time that Nramp1 is expressed in neurones and is of direct significance in activation of the HPA axis. Defects in Nramp1/Nramp2 functions may also contribute to the inappropriate accumulation of metal ions in affected neurones in the substantia nigra in patients with Parkinson's [62-64] and other [65,66] neurodegenerative diseases. Our observation that Nramp1 is expressed in the vesicular compartment of neurones therefore opens up a new area of interest in metal ion homeostasis and its pathological implications.

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REFERENCES

1. Cellier M, Belouchi A, Gros P. Resistance to intracellular infections: Comparative genomic analysis of *Nramp*. Trends Genet. 1996; 12:201–204. [PubMed: 8928221]
2. Blackwell JM, Searle S. Genetic regulation of macrophage activation: understanding the function of Nramp1 (=Ity/Lsh/Bcg). Immunol.Lett. 1999; 65:73–80. [PubMed: 10065630]
3. McLeod R, Skamene E, Brown CR, Eisenhauer PB, Mack DG. Genetic regulation of early survival and cyst number after peroral *Toxoplasma gondii* infection of A × B/B × A recombinant inbred and B10 congenic mice. J Immunol. 1989; 143:3031–3034. [PubMed: 2809214]
4. Blackwell JM, Roberts CW, Roach TIA, Alexander J. Influence of macrophage resistance gene *Lsh/Ity/Bcg* (candidate *Nramp*) on *Toxoplasma gondii* infection in mice. Clin Exp Immunol. 1994; 97:107–112. [PubMed: 8033407]

5. Gruenheid S, Pinner E, Desjardins M, Gros P. Natural resistance to infections with intracellular pathogens: The *Nramp1* protein is recruited to the membrane of the phagosome. J Exp Med. 1997; 185:717–730. [PubMed: 9034150]
6. Searle S, Bright NA, Roach TIA, Atkinson PGP, Barton CH, Meloen RH, Blackwell JM. Localisation of *Nramp1* in macrophages: modulation with activation and infection. J Cell Sci. 1998; 111:2855–2866. [PubMed: 9730978]
6A. Goswami T, Bhattacharjee A, Babal P, Searle S, Moore E, Li M, Blackwell JM. Nramp1 is a H^+/ divalent cation antiporter. Biochem J. 2001 in press.
7. Zwilling BS, Kuhn DE, Wikoff L, Brown D, LaFuse W. The role of iron in *Nramp1* mediated inhibition of mycobacterial growth. Infect Immun. 1999; 67:1386–92. [PubMed: 10024586]
8. de Chastellier C, Frehel C, Offredo C, Skamene E. Implication of phagosome-lysosome fusion in restriction of *Mycobacterium avium* growth in bone marrow macrophages from genetically resistant mice. Infect Immun. 1993; 61:3775–3784. [PubMed: 8359899]
9. Aballay A, Sarrouf MN, Colombo MI, Stahl PD, Mayorga LS. Zn2+ depletion blocks endosome fusion. Biochem J. 1995; 312:919–923. [PubMed: 8554539]
10. Aballay A, Arenas NG, Quest AF, Mayorga LS. A factor with a zinc- and phorbol ester-binding domain is necessary for endosome fusion. Exp Cell Res. 1997; 235:28–34. [PubMed: 9281349]
11. Hackam DJ, Rotstein OD, Zhang W, Gruenheid S, Gros P, Grinstein S. Host resistance to intracellular infection: mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification. J Exp Med. 1998; 188:351–364. [PubMed: 9670047]
12. Chrousos GP. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med. 1995; 332:1351–62. [PubMed: 7715646]
13. Sternberg EM. Neural-immune interactions in health and disease. J Clin Invest. 1997; 100:2641–7. [PubMed: 9389725]
14. Blackwell JM. Structure and function of the natural-resistance-associated macrophage protein (*Nramp1*), a candidate protein for infectious and autoimmune disease susceptibility. Mol Med Today. 1996; 2:205–211. [PubMed: 8796889]
15. Brown DH, Sheridan J, Pearl D, Zwilling BS. Regulation of mycobacterial growth by the hypothalamus-pituitary-adrenal axis: differential responses of *Mycobacterium bovis* BCG-resistant and -susceptible mice. Infect Immun. 1993; 61:4793–4800. [PubMed: 8406880]
16. Brown DH, LaFuse W, Zwilling BS. Cytokine-mediated activation of macrophages from *Mycobacterium bovis* BCG-resistant and -susceptible mice: Differential effects of corticosterone on antimycobacterial activity and expression of the *Bcg* gene (candidate *Nramp*). Infect Immun. 1995; 63:2983–2988. [PubMed: 7622220]
17. Brown DH, Zwilling BS. Activation of the hypothalamic-pituitary-adrenal axis differentially affects the anti-mycobacterial activity of macrophages from BCG-resistant and susceptible mice. J Neuroimmunol. 1994; 53:181–187. [PubMed: 8071432]
18. Blackwell JM, Toole S, King M, Dawda P, Roach TIA, Cooper A. Analysis of *Lsh* gene expression in congenic B10.L-*Lsh*^{r} mice. Curr Top Microbiol Immunol. 1988; 137:301–309. [PubMed: 3138075]
19. Young WS III, Mezey E, Siegel RE. Quantitative *in situ* hybridization histochemistry reveals increased levels of corticotropin-releasing factor mRNA after adrenalectomy in rats. Neurosci Lett. 1986; 70:198–203. [PubMed: 3490635]
20. Harbuz MS, Lightman SL. Responses of hypothalamic and pituitary mRNA to physical and psychological stress in the rat. J Endocrinol. 1989; 122:705–11. [PubMed: 2809478]
21. Harbuz M, Russell JA, Sumner BE, Kawata M, Lightman SL. Rapid changes in the content of proenkephalin A and corticotrophin releasing hormone mRNAs in the paraventricular nucleus during morphine withdrawal in urethane-anaesthetized rats. Brain Res Mol Brain Res. 1991; 9:285–91. [PubMed: 1645431]
22. Lightman SL, Young WS III. Corticotrophin-releasing factor, vasopressin and pro-opiomelanocortin mRNA responses to stress and opiates in the rat. J Physiol-London. 1988; 403:511–23. [PubMed: 3267021]
23. Rani BU, Singh NI, Ray A, Rao KS. Procedure for isolation of neuron- and astrocyte-enriched fractions from chick brain of different ages. J Neurosci Res. 1983; 10:101–5. [PubMed: 6887279]

24. Mosedale DE, Metcalfe JC, Grainger DJ. Optimization of immunofluorescence methods by quantitative image analysis. J Histochem Cytochem. 1996; 44:1043–50. [PubMed: 8773570]
25. Atkinson PGP, Blackwell JM, Barton CH. *Nramp1* locus encodes a 65 kDa interferon-g inducible protein in murine macrophages. Biochem J. 1997; 325:779–786. [PubMed: 9271100]
26. Yakovlev AG, Knoblach SM, Fan L, Fox GB, Goodnight R, Faden AI. Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. J Neurosci. 1997; 17:7415–24. [PubMed: 9295387]
27. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci. 1997; 17:5820–9. [PubMed: 9221780]
28. Banerjee A, Roach MC, Trcka P, Luduena RF. Increased microtubule assembly in bovine brain tubulin lacking the type III isotype of beta-tubulin. J Biol Chem. 1990; 265:1794–9. [PubMed: 2404018]
29. Lawson LJ, Perry VH, Gordon S. Turnover of resident microglia in the normal adult mouse brain. Neuroscience. 1992; 48:405–15. [PubMed: 1603325]
30. Smith MJ, Koch GL. Differential expression of murine macrophage surface glycoprotein antigens in intracellular membranes. J Cell Sci. 1987; 87:113–9. [PubMed: 3312248]
31. Laping NJ, Teter B, Nichols NR, Rozovsky I, Finch CE. Glial fibrillary acidic protein: regulation by hormones, cytokines, and growth factors. Brain Pathol. 1994; 4:259–75. [PubMed: 7952267]
32. Berridge CW, Dunn AJ. CRH and restraint-stress decrease exploratory behavior in hypophysectomized mice. Pharmacol Biochem Behav. 1989; 34:517–9. [PubMed: 2623010]
33. Krahn DD, Gosnell BA, Grace M, Levine AS. CRH antagonist partially reverses CRH- and stress-induced effects on feeding. Brain Res Bull. 1986; 17:285–9. [PubMed: 3490298]
34. Berridge CW, Dunn AJ. A corticotropin-releasing factor antagonist reverses the stress-induced changes of exploratory behavior in mice. Horm Behav. 1987; 21:393–401. [PubMed: 3499373]
35. Rodrigues V, Cheah VPY, Ray K, Chia W. *malvolio*, the *Drosophila* homologue of mouse *Nramp1* (*Bcg*), is expressed in macrophages and in the nervous system and is required for normal taste behaviour. EMBO J. 1995; 14:3007–3020. [PubMed: 7621816]
36. Orgad S, Nelson H, Segal D, Nelson N. Metal ions suppress the abnormal taste behavior of the Drosophila mutant malvolio. J Exp Biol. 1998; 201:115–120. [PubMed: 9390942]
37. D'Souza J, Cheah PY, Gros P, Chia W, Rodrigues V. Functional complementation of the *malvolio* mutation in the taste pathway of *Drosophila melanogaster* by the human natural resistance-associated macrophage protein 1 (Nramp-1). J Exp Biol. 1999; 202:1909–15. [PubMed: 10377272]
38. Weinberg J, Levine S, Dallman PR. Long-term consequences of early iron deficiency in the rat. Pharmacol Biochem Behav. 1979; 11:631–8. [PubMed: 538052]
39. Weinberg J, Brett LP, Levine S, Dallman PR. Long-term effects of early iron deficiency on consummatory behavior in the rat. Pharmacol Biochem Behav. 1981; 14:447–53. [PubMed: 7232471]
40. Coleman R, Tanne Z, Nahir M, Shomrat D, Miller-Lotan R, Youdim M. Ultrastructural changes in mitochondria of the adrenal cortex of iron- deficient rats. Acta Anat. 1995; 152:33–40. [PubMed: 7604676]
41. Saad MJ, Morais SL, Saad ST. Reduced cortisol secretion in patients with iron deficiency. Ann Nutr Metab. 1991; 35:111–5. [PubMed: 1651678]
42. Norris DO, Donahue S, Dores RM, Lee JK, Maldonado TA, Ruth T, Woodling JD. Impaired adrenocortical response to stress by brown trout, *Salmo trutta*, living in metal-contaminated waters of the Eagle River, Colorado. Gen Comp Endocrinol. 1999; 113:1–8. [PubMed: 9882538]
43. Canonne-Hergaux F, Gruenheid S, Ponka P, Gros P. Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. Blood. 1999; 93:4406–4417. [PubMed: 10361139]
44. Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA. Cloning and characterization of a mammalian proton- coupled metal-ion transporter. Nature. 1997; 388:482–488. [PubMed: 9242408]

45. Gruenheid S, Canonne-Hergaux F, Gauthier S, Hackam DJ, Grinstein S, Gros P. The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. J Exp Med. 1999; 189:831–841. [PubMed: 10049947]
46. Atkinson PGP, Barton CH. Ectopic expression of *Nramp1* in COS-1 cells modulates iron accumulation. FEBS Letters. 1998; 425:239–242. [PubMed: 9559656]
47. Brown DH, LaFuse WP, Zwilling BS. Stabilized expression of mRNA is associated with mycobacterial resistance controlled by *Nramp1*. Infect Immun. 1997; 65:597–603. [PubMed: 9009318]
48. Sternberg EM, Young WS III, Bernardini R, Calogero AE, Chrousos GP, Gold PW, Wilder RL. A central nervous system defect in biosynthesis of corticotropin-releasing hormone is associated with susceptibility to streptococcal cell wall-induced arthritis in Lewis rats. Proc Natl Acad Sci U S A. 1989; 86:4771–5. [PubMed: 2786636]
49. Mason D. Genetic variation in the stress response: susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. Immunol Today. 1991; 12:57–60. [PubMed: 2059305]
50. Sternberg EM, Hill JM, Chrousos GP, Kamilaris T, Listwak SJ, Gold PW, Wilder RL. Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis- susceptible Lewis rats. Proc Natl Acad Sci U S A. 1989; 86:2374–8. [PubMed: 2538840]
51. Misiewicz B, Poltorak M, Raybourne RB, Gomez M, Listwak S, Sternberg EM. Intracerebroventricular transplantation of embryonic neuronal tissue from inflammatory resistant into inflammatory susceptible rats suppresses specific components of inflammation. Exp Neurol. 1997; 146:305–14. [PubMed: 9270039]
52. Misiewicz B, Poltorak M, Gomez M, Glowa JR, Gold PW, Sternberg EM. Intracerebroventricularly transplanted embryonic neuronal tissue from inflammatory-resistant F344/N rats decreases acoustic startle responses in inflammatory-susceptible LEW/N rats. Cell Transplant. 1996; 5:287–91. [PubMed: 8689039]
53. Harbuz MS, Jessop DS, Chowdrey HS, Blackwell JM, Larsen PJ, Lightman SL. Evidence for altered control of hypothalamic CRH in immune- mediated diseases. Ann NY Acad Sci. 1995; 771:449–458. [PubMed: 8597421]
54. Harbuz MS, Leonard JP, Lightman SL, Cuzner ML. Changes in hypothalamic corticotrophin-releasing factor and anterior pituitary pro-opiomelanocortin mRNA during the course of experimental allergic encephalomyelitis. J Neuroimmunol. 1993; 45:127–32. [PubMed: 8331157]
55. Brady LS, Page SW, Thomas FS, Rader JL, Lynn AB, Misiewicz-Poltorak B, Zelazowski E, Crofford LJ, Zelazowski P, Smith C, et al. 1,1′-Ethylidenebis[L-tryptophan], a contaminant implicated in L- tryptophan eosinophilia myalgia syndrome, suppresses mRNA expression of hypothalamic corticotropin-releasing hormone in Lewis (LEW/N) rat brain. Neuroimmunomodulation. 1994; 1:59–65. [PubMed: 8528886]
56. Shanks N, Moore PM, Perks P, Lightman SL. Endocrine correlates of murine systemic lupus erythematosus in the MRL lpr/lpr model. Ann N Y Acad Sci. 1997; 823:252–5. [PubMed: 9292051]
57. Sakic B, Laflamme N, Crnic LS, Szechtman H, Denburg JA, Rivest S. Reduced corticotropin-releasing factor and enhanced vasopressin gene expression in brains of mice with autoimmunity-induced behavioral dysfunction. J Neuroimmunol. 1999; 96:80–91. [PubMed: 10227427]
58. Ferguson DJ, Graham DI, Hutchison WM. Pathological changes in the brains of mice infected with *Toxoplasma gondii*: a histological, immunocytochemical and ultrastructural study. Int J Exp Pathol. 1991; 72:463–74. [PubMed: 1883744]
59. Freund YR, Sgarlato G, Jacob CO, Suzuki Y, Remington JS. Polymorphisms in the tumor necrosis factor alpha (TNF-alpha) gene correlate with murine resistance to development of toxoplasmic encephalitis and with levels of TNF-alpha mRNA in infected brain tissue. J Exp Med. 1992; 175:683–8. [PubMed: 1740661]
60. Perry VH, Andersson PB. The inflammatory response in the CNS. Neuropathol Appl Neurobiol. 1992; 18:454–9. [PubMed: 1454134]

61. McLeod R, Remington JS. Influence of infection with Toxoplasma on macrophage function, and role of macrophages in resistance to Toxoplasma. Am J Trop Med Hyg. 1977; 26:170–86. [PubMed: 339761]

62. Jellinger KA, Kienzl E, Rumpelmaier G, Paulus W, Riederer P, Stachelberger H, Youdim MB, Ben-Shachar D. Iron and ferritin in substantia nigra in Parkinson's disease. Adv Neurol. 1993; 60:267–72. [PubMed: 8420142]

63. Gerlach M, Ben-Shachar D, Riederer P, Youdim MB. Altered brain metabolism of iron as a cause of neurodegenerative diseases? J Neurochem. 1994; 63:793–807. [PubMed: 7519659]

64. Hirsch EC, Faucheux BA. Iron metabolism and Parkinson's disease. Mov Disord. 1998; 13:39–45. [PubMed: 9613717]

65. Gerlach M, Riederer P, Youdim MB. Molecular mechanisms for neurodegeneration. Synergism between reactive oxygen species, calcium, and excitotoxic amino acids. Adv Neurol. 1996; 69:177–94. [PubMed: 8615127]

66. Multhaup G. Amyloid precursor protein, copper and Alzheimer's disease. Biomed Pharmacother. 1997; 51:105–11. [PubMed: 9181045]

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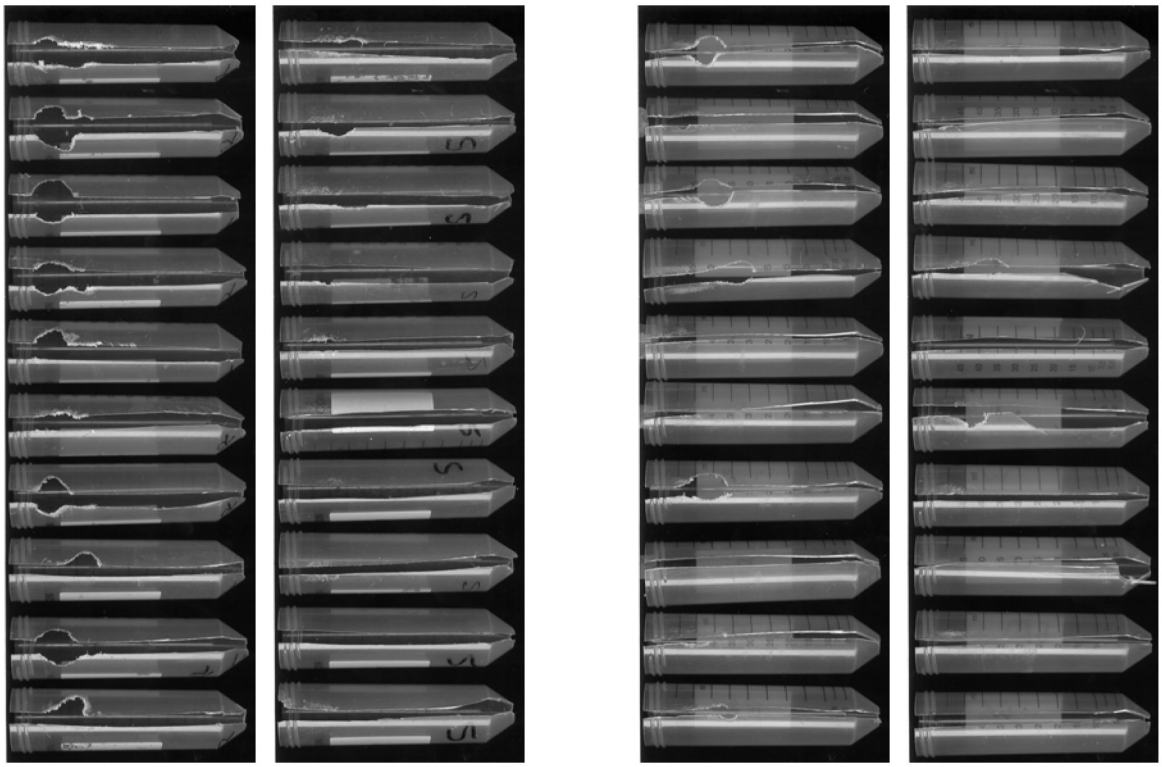

Wild type Mutant Wild type Mutant

Experiment 1 Experiment 2

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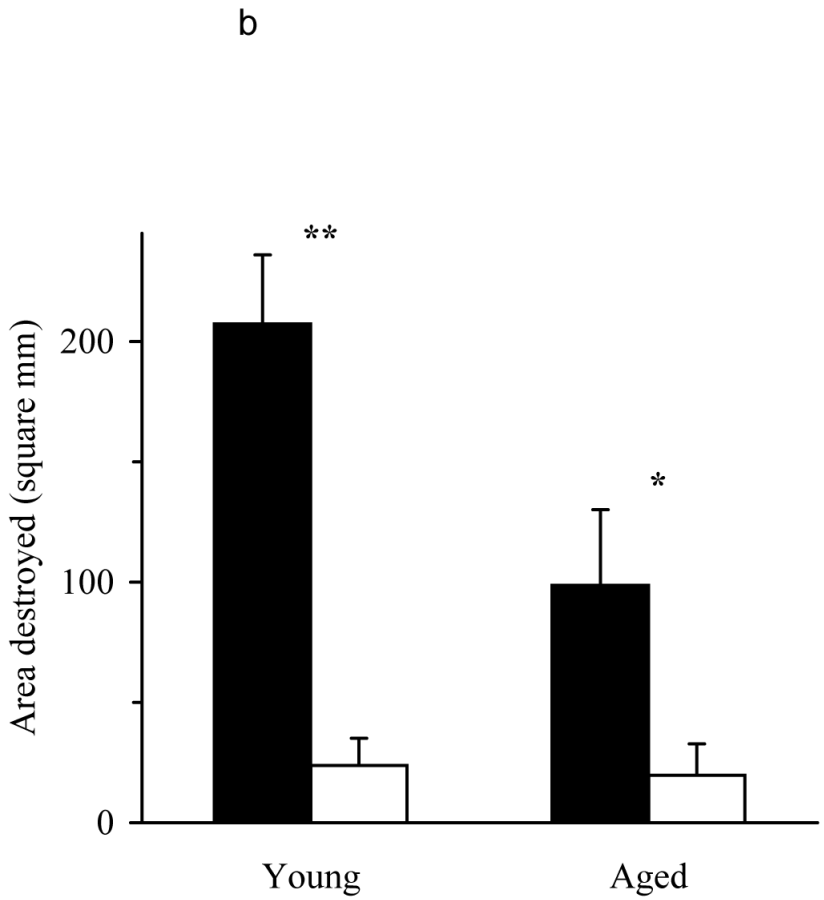

Figure 1.

Behavioural stress responses in *Nramp1* congenic mice. (**a**) Photographs of the 50 ml plastic tubes, some of which were partially destroyed by restrained mice in duplicate experiments. (**b**) Quantification of the areas of the plastic tubes shown in **a** that were destroyed during restraint of *Nramp1* wild type (filled bars) and mutant (open bars) mice. Results are mean + SEM. Mann-Whitney U test: wild type vs. mutant **p<0.005; *p<0.05

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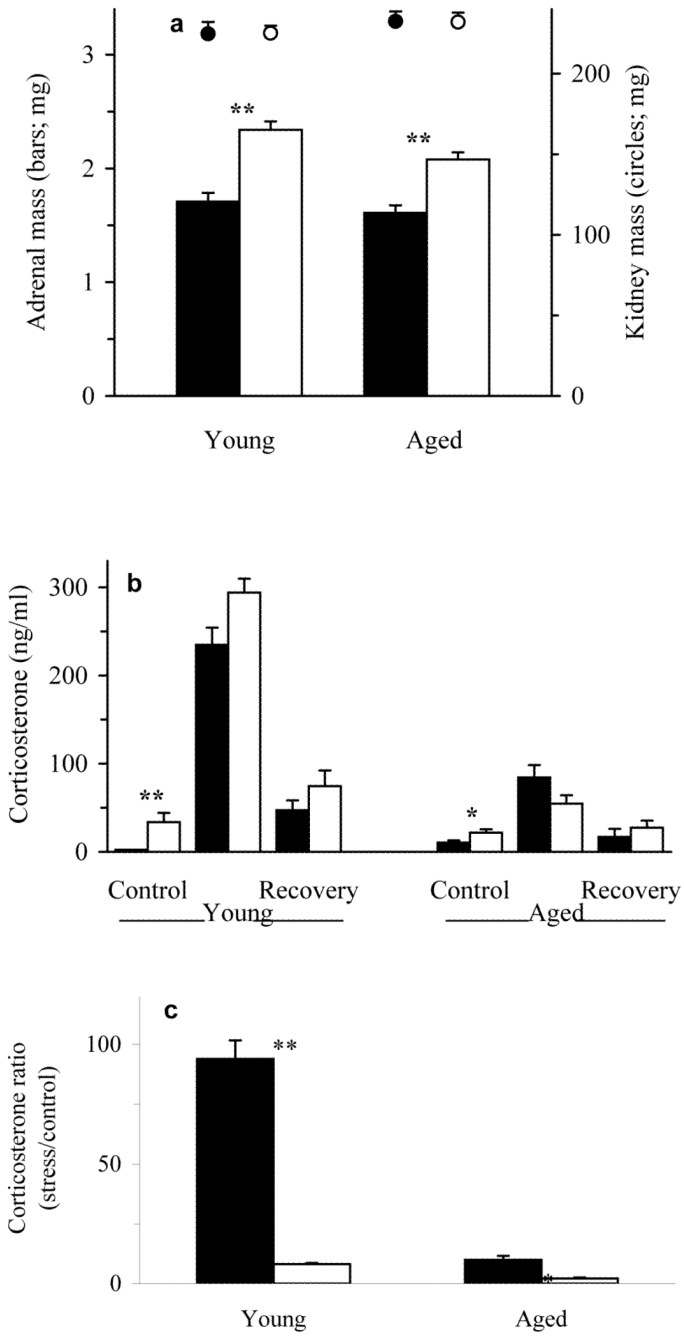

Figure 2.

Humoral stress response in *Nramp1* congenic mice. (**a**) Adrenal mass (bars) differed between genotypes whereas kidney mass (circles), a measure of animal size, did not. (**b**) Plasma corticosterone concentrations prior to, during and after 4 hours recovery following restraint stress. (**c**) Plasma corticosterone stress response calculated as the ratio of corticosterone concentrations during/prior to stress for each genotype and experiment. Results are mean + SEM for *Nramp1* wild type (closed symbols) and mutant (open symbols) mice. Mann-Whitney U test: wild type vs. mutant **p<0.005 *p<0.05

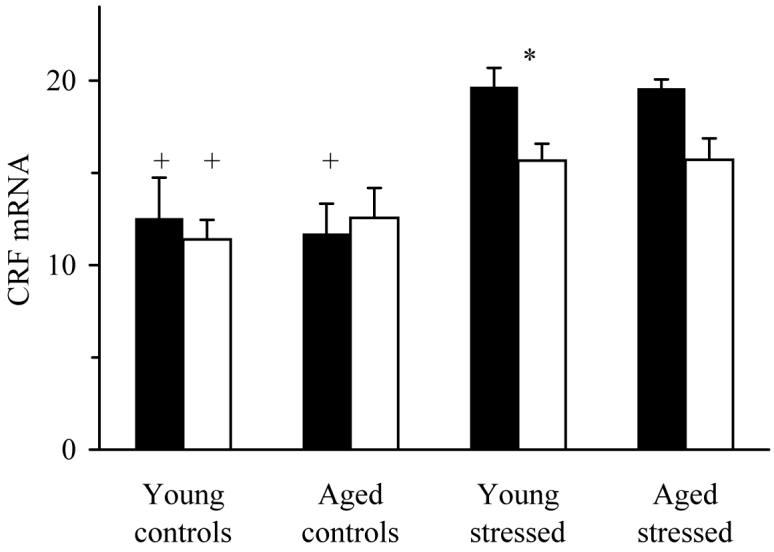

Figure 3.

Hypothalamic stress response in *Nramp1* congenic mice. Corticotrophin releasing hormone (CRH) mRNA quantitation by *in situ* hybridisation in the paraventricular nucleus of the hypothalamus in un-stressed controls and in mice stressed by restraint. Results are mean + SEM (arbitrary units) for *Nramp1* wild type (closed bars) and mutant (open bars) mice. Mann-Whitney U test: wild type vs. mutant *$p<0.05$; control vs. stress +$p<0.05$.

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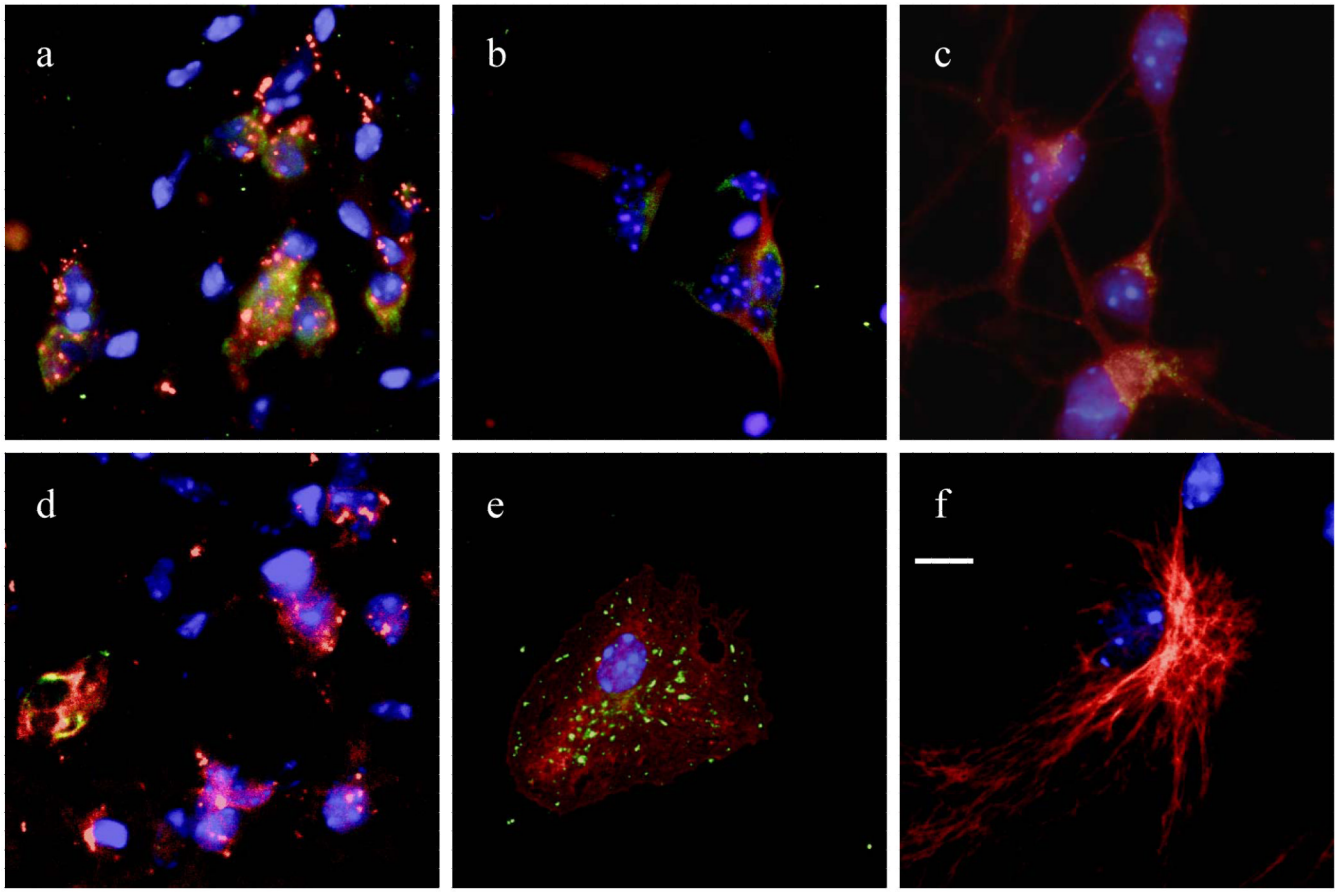

Figure 4. Immunochemistry

Indirect fluorescent immunochemical triple co-staining of frozen mouse brain sections in **a** and **d** and cultured embryonic mouse brain cells in **b**, **c**, **e** and **f**, all viewed at x400 magnification. Nuclei are stained with Hoescht and appear blue. In (**a**), (**b**) and (**c**), green fluorescence identifies vesicular cytoplasmic Nramp1 expression within neurones that are identified by their morphology and red fluorescence for neuronal marker antibodies NeuN in **a** and β-tubulin III in **b** and **c**. In (**d**), Nramp1 expression (red) is also seen in macrophages/microglia labelled (green) for a pan-macrophage marker (F4/80). In (**e**), green fluorescence identifies vesicular cytoplasmic Nramp1 expression within a cultured embryonic microglial cell identified by its morphology and red fluorescence for F4/80. In (**f**), Nramp1 expression (green) is not seen in GFAP (red) immuno-reactive astrocytes. Polyclonal rabbit anti-Nramp1 antibodies were used in **a**, **b**, **d** and **e**; monoclonal rat anti-Nramp1 antibodies in **c** and **f**. The bar in **f** represents 10 μm.

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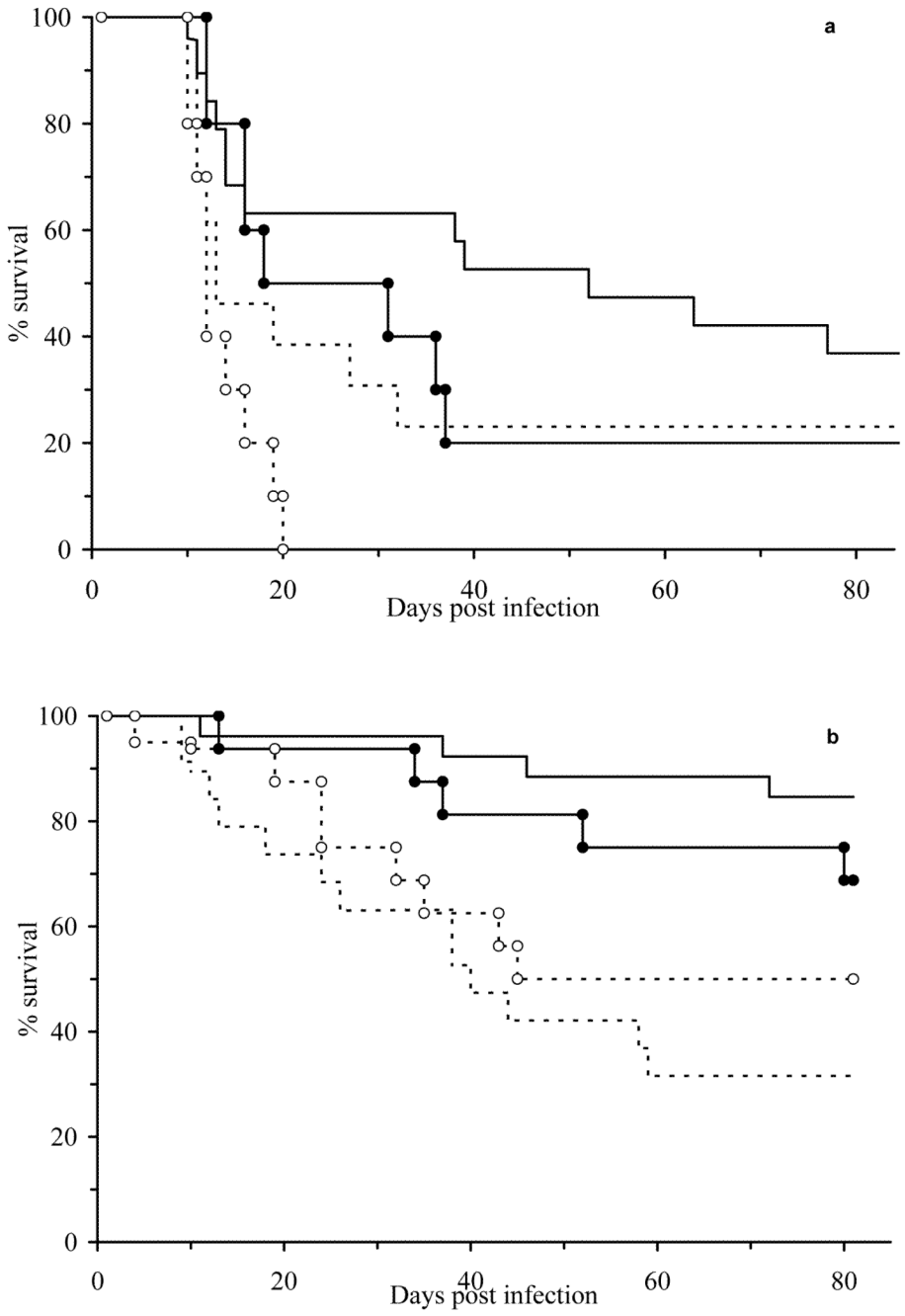

Figure 5.

Kaplan-Meier cumulative survival plots for *Nramp1* congenic mice following oral infection with *T. gondii* cysts. (**a**) 5 month old mice. (**b**) 10 month old mice. Solid lines with/without circles denote *Nramp1* wild type mice; dashed lines with/without circles denote mutant mice. Lines marked with circles denote groups of mice that were subjected to 1 hour of restraint stress a day before infection.