Adult vitamin D deficiency exacerbates impairments caused by social stress in BALB/c and C57BL/6 mice

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Abstract

Vitamin D deficiency is prevalent in adults throughout the world. Epidemiological studies have shown significant associations between vitamin D deficiency and an increased risk of various neuropsychiatric and neurodegenerative disorders, such as schizophrenia, depression, Alzheimer's disease and cognitive impairment. However, studies based on observational epidemiology cannot address questions of causality; they cannot determine if vitamin D deficiency is a causal factor leading to the adverse health outcome. The main aim of this study was to determine if AVD deficiency would exacerbate the effects of a secondary exposure, in this case social stress, in BALB/c mice and in the more resilient C57BL/6 mice. Ten-week old male BALB/c and C57BL/6 mice were fed a control or vitamin D deficient diet for 10 weeks, and the mice were further separated into one of two groups for social treatment, either Separated (SEP) or Social Defeat (DEF). SEP mice were placed two per cage with a perforated Plexiglas divider. The DEF mice underwent 10 days of social defeat and during behavioural testing. We found that AVD-deficient mice were more vulnerable to the effects of social stress using a social avoidance test, and this was dependent on strain. These results support the hypothesis that vitamin D deficiency may exacerbate behavioural outcomes in mice vulnerable to stress, a finding that can help guide future studies. Importantly, these discoveries support the epidemiological link between vitamin D deficiency and neuropsychiatric and neurodegenerative disorders; and has provided clues that can guide future studies related to unravelling the mechanisms of action linking adult vitamin D deficiency and adverse brain related outcomes.

KEYWORDS: Vitamin D, social stress, schizophrenia, two-hit exposure; animal model

Introduction

Neuropsychiatric disorders such as schizophrenia have complex aetiology involving both genetic factors and environmental influences (McDonald and Murray, 2000). The idea that multiple factors may contribute to the development of a disorder, led to the creation of the 'two-hit hypothesis' (Maynard et al., 2001). However, recent genome wide association studies show that within the genetics of schizophrenia alone, prior to any environmental exposures, there are thousands of genetic variants of small effect sizes that lead to an increased risk of developing schizophrenia (Wray and Visscher, 2010). Moreover, there are multiple environmental risk factors that can influence the development of disorders. For example, epidemiological studies have identified a number of non-genetic risk factors for schizophrenia including pregnancy and birth complications, maternal infection, immigration, adverse life events, and substance abuse (McDonald and Murray, 2000).

Therefore, it is likely that the accumulation of multiple risk factors leads to the development of complex neuropsychiatric disorders. However, using animal models it is not feasible to look at many 'hits' and therefore a 'two-hit hypothesis' may be a more reasonable approach. For example, a recent study investigated the consequences of combining prenatal immune activation with stress during puberty in mice. They found that the prenatal insult markedly increased the vulnerability of offspring to subsequent stress. Furthermore, the interaction between the two 'hits' led to behavioural alterations relevant to neuropsychiatric disorders, including deficits in sensorimotor gating as assessed by prepulse inhibition (PPI) of the acoustic startle response (ASR) (Giovanoli et al., 2013).

Epidemiological studies show that first generation, dark-skinned migrants to cold climates have increased risk of developing psychosis (Cantor-Graae and Selten, 2005). It was proposed that social stress related to being a member of a minority ethnic group in a foreign country may contribute to this increased risk (Dealberto, 2010). Another hypothesis given was that it may be vitamin D

deficiency, after moving from a country with high sun exposure to one of limited exposure, that increased their risk of developing psychosis (Dealberto, 2010). Developmental vitamin D deficiency has been shown to be a risk factor for the development of schizophrenia later in life (McGrath et al., 2010), however recent animal experiments have suggested that vitamin D deficiency during adulthood also compromises brain function (Groves et al., 2013).

Vitamin D has been shown to be neuroprotective against a range of neuronal insults including excitoxicity and ischemia, both directly and via regulation of NGF and GDNF (Kume et al., 2000; J. Y. Wang et al., 2001; Y. Wang et al., 2002b). 1,25$(OH)_2$D treatment upregulates antioxidant molecules, such as glutathione and iNOS, protecting against free radicals (Garcion et al., 2002; Jain and Micinski, 2013). Furthermore, a recent study in PD has shown that vitamin D supplements prevented a decline in PD-related outcomes compared to those on placebo, suggesting a protective effect of vitamin D against PD.

Vitamin D deficiency has now been shown to reduce glutamine/glutamate levels in BALB/c mice, potentially leaving the mice at risk for increased oxidative stress (Groves et al., 2013). Therefore, the absence of vitamin D could leave an individual more vulnerable to a second 'hit'. For example, it was recently shown in a stroke model, that vitamin D deficient rats had greater infarct volumes compared to controls and this corresponded with greater impairments, post-stroke, in sensorimotor behavioural testing (Balden et al., 2012). Furthermore, the vitamin D deficient animals had significantly lower levels of IGF-1, a neuroprotectant molecule usually elevated after injury, compared to controls (Balden et al., 2012).

Social stress can be assessed in rodents using a number of different approaches, but chronic social defeat has been shown to have long lasting effects on behaviour and brain function (Golden et al., 2011). Using this procedure, mice are exposed to repeated bouts of social defeat by a larger aggressive mouse and then further subjected to continuous psychological stress from sensory interaction through a clear perforated divider in their home cage (Golden et al., 2011). This protocol

has been repeatedly shown to produce social avoidance in a subset of 'susceptible' mice. Susceptible mice show behavioural and physiological changes suggestive of depressive and anxiety symptoms, while 'resilient' mice do not (Golden et al., 2011). For example, a study in rats showed that socially defeated animals displayed weight loss and an enhanced and prolonged response to acoustic startle (Pulliam et al., 2010).

A recent study reported that BALB/c and C57BL/6J mice show a different susceptibility to social defeat stress, with only the BALB/c mice exhibiting long-term social withdrawal (Razzoli et al., 2011). Furthermore, BALB/c mice displayed greater effects of AVD deficiency when compared directly with C57BL/6J mice (Groves et al., 2013). AVD-deficient BALB/c mice also displayed enhanced acoustic startle, suggesting an altered response to stressful stimuli (Groves et al., 2013).

Therefore, the aim of the current study was to examine the effects of a two-hit model combining AVD deficiency and chronic social defeat in both BALB/c and C57BL/6J mice on behaviour, using a social interaction paradigm and PPI of the ASR, two tests relevant to neuropsychiatric disorders and psychosocial stress. It was proposed that AVD deficiency would leave the mice more vulnerable to the second hit of social stress, and that these effects would be greater in the 'susceptible' BALB/c strain

Materials and methods

Animals and housing

Thirty male BALB/c mice, 23 male C57BL/6J mice and 16 CD-1 male mice were used in this study. Ten-week-old BALB/c and C57BL/6J mice (Animal Resources Centre, Canning Vale, WA, Australia) were obtained and housed in groups of 4, in individually ventilated OptiMICE cages (Animal Care Systems, CO, USA). All cages were given bedding (Sanichips, Harlan Laboratories, USA) and nesting material and housed at the Queensland Brain Institute Animal House Facility, University of Queensland. The mice were maintained on a 12-hour light-dark cycle (lights on at

07:00 h) and were housed under incandescent lighting free from UVB radiation. All mice were housed with ad libitum access to food and water. The mice were assigned to either a control diet (Standard AIN93G Rodent diet with 1,500 IU vitamin D3/kg (prior to irradiation with 25 kGy), Specialty Feeds, WA, Australia) or a vitamin D-deficient diet (irradiated with 25 kGy) (Vitamin D Deficient AIN93G Rodent diet, Specialty Feeds, WA, Australia), for 10 weeks prior to the start of behavioural testing; and for the entire duration of the experimental procedures. The 16 CD-1 male mice (Animal Resources Centre, Canning Vale, WA, Australia) were used as the aggressor mice in a social defeat paradigm, and were obtained at 9-10 months old and housed individually in OptiMICE cages (Animal Care Systems, CO, USA). The CD-1 mice were maintained on standard mouse chow (Specialty Feeds, WA, Australia). All experimental work was performed with approval from the University of Queensland Animal Ethics Committee (QBI/202/13/NHMRC), under the guidelines of the National Health and Medical Research Council of Australia.

Experimental design

BALB/c and C57BL/6J mice were tested separately, and after a minimum of 10 weeks on diet the mice were further separated into one of two groups for social treatment, either Social Defeat (DEF) or Separated (SEP) . The DEF mice underwent 10 days of social defeat using the protocol described by (Golden et al., 2011). Sixteen ex-breeder 9-10 month old CD-1 male mice (Animal Resources Centre, Canning Vale, WA, Australia) were used as aggressive residents. All CD-1 mice were screened to confirm that they showed aggressive behaviour during social interaction. DEF mice from both diets were exposed to a different unfamiliar CD-1 resident mouse each day for 10 days, for up to 10 min of interaction. During this exposure all subject mice showed signs of subordination (i.e submissive postures, withdrawal, fleeing, lying on its back, or freezing), assessed visually by the experimenter. After the interaction period, subject mice were separated from the aggressive resident by placing a Plexiglas perforated divider (to allow sensory contact) between them in the resident's home-cage. The mice were housed in this way for the next 24 h, with food and water provided *ab libitum*. To control for the effects of DEF we used identical housing conditions but

without the social defeat; SEP mice were placed two per cage with a perforated Plexiglas divider between them (*n*=7-8 BALB/c, *n*=5-6 C57BL/6J). These mice were moved to a new cage daily for 10 days, so that each day the mice were in a new cage with a new cage mate. At the end of the 10 days, all mice were housed two per cage with a perforated Plexiglas divider between them to the end of behavioural testing, 2 weeks later.

Body weight

Body weight was measured on Day 1 prior to the start of the 10-day social stress paradigm and on Day 11 to assess the effects of the stress paradigm on food intake and body weight maintenance.

Serum biochemistry

At the completion of experimental procedures, mice were euthanized by i.p injection of an overdose of Lethabarb at 4 ml/kg body weight and a terminal blood sample was collected via cardiac puncture. The level of 25(OH)D was measured in the serum samples using liquid chromatography-tandem mass spectrometry (Sciex Instruments, ON, Canada) on a 4000 QTrap API AB mass spectrometer (Eyles et al., 2009). Serum corticosterone was determined using DetectX Corticosterone Enzyme Immunoassay kit (KO14-H5, Arbor Assays™, MI, USA) according to the manufacturer's instructions. 5µl of serum per sample was diluted at 1:100, and 50µl of diluted sample was used per well in the assay. Samples were analysed in duplicate in a single assay; limit of detection = 16.9pg/ml; sensitivity = 18.6pg/ml. Corticosterone concentration is expressed in ng/ml. To measure a range of other biochemical parameters, serum samples from 32 mice (*n* = 4/diet/social treatment/strain) were sent to IDEXX Laboratories (Brisbane, Australia). For the full list of biochemical parameters measured, see Table 2.

Behavioural testing

Social interaction test

A modified version of the social interaction test described by (Golden et al., 2011) was used to measure approach/avoidance behaviour toward an unfamiliar target, 24 hours after the final defeat. Social interaction was measured in a 30 x 30 cm arena with a metal wire cage (12 x 5 cm x 8 cm high) at one end (Figure 1). In the first phase of the test, subjects were individually placed in the arena opposite the metal wire cage while it was empty and their movement was recorded for 150 s (habituation phase) using a USB digital camera and recording software. They were returned to their home-cage for 30 s. In the second phase of the test, an unfamiliar aggressive CD-1 mouse was confined within the metal wire cage and the subject was placed back in the arena for a further 150 s and their movement was again recorded (interaction phase). The test was performed under red light conditions.

The duration of the subject's presence within the interaction zone (defined as a 5 cm wide area surrounding the wire cage) and in the avoidance zone (defined as the far corners opposite the metal cage, 7.5 cm square) (Figure 1) as well as the average distance of the test mouse to the centre point of the metal cage and the total distance travelled (cm) were obtained using Ethovision v9 software (Noldus Information Technology, The Netherlands).

Prepulse inhibition of the acoustic startle response

PPI of the ASR was used to measure sensorimotor gating (Harms et al., 2008) using startle chambers (SR-Lab, San Diego Instruments, CA, USA), which consisted of a Plexiglass cylinder (5 cm diameter x 12 cm long) mounted on an elevated Plexiglass base within a dark chamber. A speaker situated 24 cm above the cylinder was used to provide background noise within the chamber set to 70 dB as well as the acoustic pulses of white noise throughout the testing.

Testing began with an acclimatisation period of 300 s of 70 dB background noise. The mice then underwent a total of 130 trials (26 different blocks of 5 trials). To assess within-session habituation (WSH), startling pulses of 110 dB were presented at the start (post acclimatisation) and end of the testing. The mice were exposed to a range of pulse intensities (80, 90, 100, 110 and 120 dB) to

measure ASR and a range of prepulse to pulse intervals (8, 16, 32, 64, 128 and 256 ms) before a 120 dB pulse to measure PPI. The median values for each block of 5 trials were used for analysis, with PPI being calculated with the formula: %PPI = [(startle amplitude of ASR trial - startle amplitude on prepulse trial)/startle amplitude of ASR trial] x 100 (Harms et al., 2008).

Statistical analysis

Results were analysed for statistical significance using SPSS (version 20.0) software. All data were analysed for the main effects of Diet (control or AVD-deficient), Social Treatment (SEP or DEF), and Strain (C57BL/6J or BALB/c) using ANOVA or, where appropriate, repeated measures ANOVA. Significant differences ($p < 0.05$) were followed up with post-hoc student t-tests.

Results

Body Weight

Starting body weights were not different for Social Treatment ($F_{1,43} = 0.34$, $p = 0.562$) and Diet ($F_{1,43} = 0.40$, $p = 0.530$) groups, but were significantly different between Strains ($F_{1,43} = 42.91$, $p < 0.001$) with C57BL/6J mice weighing more than BALB/c mice. At the end of the Social Treatment, the change in bodyweight was significantly different for Social Treatment ($F_{1,43} = 5.99$, $p = 0.019$) but not for Strain ($F_{1,43} = 0.93$, $p = 0.339$) or Diet ($F_{1,43} = 2.38$, $p = 0.130$) groups, with DEF mice losing weight compared to SEP mice (Table 1), providing conformation that the social defeat protocol worked.

Serum biochemistry

There was a significant main effect of Strain ($F_{1,43} = 152.41$, $p < 0.001$) and Diet ($F_{1,43} = 937.07$, $p < 0.001$) on 25(OH)D levels, with higher levels seen in C57BL/6J mice compared to BALB/c mice and higher levels in Controls compared to AVD-deficient mice, see Table 2 for details. As expected, the AVD-deficient mice had levels of 25(OH)D at the lower level of detection.

There was no significant main effects of Strain ($F_{1,46} = 0.47$, $p = 0.496$), Diet ($F_{1,46} = 1.65$, $p = 0.206$) or Social Treatment ($F_{1,46} = 1.49$, $p = 0.229$) on serum corticosterone levels. However, when strains were analysed separately, there was a significant effect of Diet ($F_{1,13} = 4.93$, $p = 0.045$) and Social Treatment ($F_{1,13} = 14.46$, $p = 0.002$) in C57BL/6J mice. DEF mice had higher levels of serum corticosterone when compared to SEP mice. Furthermore, the AVD-deficient mice had increased serum corticosterone levels when compared to the control mice (Figure 2b). In the BALB/c strain, there was no significant effect of Diet ($F_{1,33} = 0.46$, $p = 0.504$) or Social Treatment ($F_{1,33} = 0.13$, $p = 0.726$) on serum corticosterone levels (Figure 2a).

There was a significant main effect of Strain ($F_{1,17} > 4.38$, $p < 0.047$) on the level of a number of other biochemical parameters including sodium, potassium, glucose, calcium, total protein, albumin, bilirubin, aspartate aminotransferase, and creatine kinase.

There was a significant main effect of Social Treatment ($F_{1,17} = 5.49$, $p = 0.032$) on the level of total serum protein, with a Strain x Diet interaction ($F_{1,17} = 4.51$, $p = 0.049$). When analysing the strains separately, there was a significant effect of Social Treatment ($F_{1,11} = 6.27$, $p = 0.029$) on total serum protein levels in C57BL/6J mice, but no effect of Diet ($F_{1,11} = 4.26$, $p = 0.063$), with reduced levels in the Social Defeat mice. There were no significant effects in BALB/c mice.

Analysing the strains separately, there was only a significant effect of Social Treatment ($F_{1,12} = 4.86$, $p = 0.048$) on sodium levels in BALB/c mice, with lower levels in DEF mice compared to SEP mice. There were no significant effects of Diet on any measure when strains were analysed separately.

Social defeat duration

In the socially defeated mice, there was no significant effect of Diet ($F_{1,22} = 1.52$, $p = 0.230$) on the duration of the daily defeats. There was a significant effect of Strain ($F_{1,22} = 198.83$, $p < 0.001$) on the duration of the daily defeats. Defeats were terminated by the experimenter early in BALB/c mice due to the intensity of the defeats (multiple attacks within a 60 sec time period, despite

defensive behaviour shown by the BALB/c mice), however all C57BL/6J mice completed the full ten min defeat each day for the 10 days (Table 1).

Social interaction test

Habituation phase

During the habituation phase of the social interaction test there was no significant main effect of Diet or Social Treatment on any measure, there was however a significant main effect of Strain on the average distance to the metal cage ($F_{1,44} = 13.88$, $p = 0.001$), with the C57BL/6J mice having a shorter average distance compared to the BALB/c mice (Table 1).

Interaction phase

During the interaction phase there were four main measures analysed; the average distance to the CD-1, distance travelled, time in the interaction zone and time in the avoidance zone.

There were significant main effects of Diet ($F_{1,44} = 4.64$, $p = 0.037$), Strain ($F_{1,44} = 19.19$, $p < 0.001$) and Social Treatment ($F_{1,44} = 30.48$, $p < 0.001$) on the average distance to CD-1 with a Strain x Social Treatment interaction ($F_{1,44} = 15.31$, $p < 0.001$). Analysing the strains separately, there was a significant effect of Social Treatment in C57BL/6J ($F_{1,19} = 4.69$, $p = 0.043$) and in BALB/c mice ($F_{1,25} = 31.88$, $p < 0.001$), with DEF mice having a greater average distance to CD-1 compared to SEP mice. There was also a significant effect of Diet ($F_{1,25} = 4.70$, $p = 0.040$) in the BALB/c strain only, with AVD-deficient mice having a greater distance to CD-1 compared to control mice (Figure 2a and b).

There was a significant main effect of Social Treatment ($F_{1,44} = 21.05$, $p < 0.001$) and Strain ($F_{1,44} = 13.80$, $p = 0.001$) but not of Diet ($F_{1,44} = 0.01$, $p = 0.935$) and no interaction. C57BL/6J mice travelled further than BALB/c mice and SEP mice travelled further than DEF mice (Table 1).

There was a significant main effect of Strain ($F_{1,44} = 33.48$, $p < 0.001$) and Social Treatment ($F_{1,44} = 15.49$, $p < 0.001$), but not of Diet ($F_{1,44} = 0.11$, $p = 741$) on the time spent in the interaction zone. The DEF mice spent less time in the interaction zone compared to SEP mice and BALB/c mice spent less time in this zone compared to C57BL/6J mice (Figure 2c and d).

There were significant main effects of Strain ($F_{1,44} = 10.11$, $p = 0.003$), Social Treatment ($F_{1,44} = 6.34$, $p = 0.016$) and Diet ($F_{1,44} = 4.86$, $p = 0.033$) on the time spent in the avoidance zone. When analysing the strains separately, there was no significant effect of Social Treatment or Diet in the C57BL/6J mice. In the BALB/c mice, there was a significant effect of Social Treatment ($F_{1,25} = 6.32$, $p = 0.019$) and Diet ($F_{1,25} = 4.75$, $p = 0.039$) on the time spent in the avoidance zone. DEF mice spent more time in the avoidance zone compared to SEP mice and AVD-deficient mice spent more time in the avoidance zone compared to control mice (Figure 2e and f).

Prepulse inhibition of the acoustic startle response

There was a significant Strain x Diet x Social Treatment interaction ($F_{1,43} = 12.54$, $p = 0.001$) with significance found in the main effects of Strain, Social Treatment and Diet in the ASR. In the BALB/c strain (Figure 3a and c), there was a Diet x Social Treatment interaction ($F_{1,24} = 4.38$, $p = 0.047$). Post-hoc t-tests showed a significant difference between DEF controls and SEP control mice, with DEF leading to a blunted ASR but no significant difference between Social Treatments in AVD-deficient mice. In C57BL/6J mice (Figure 3b and d), there was a main effect of Diet ($F_{1,19} = 15.79$, $p = 0.001$) but not of Social Treatment ($F_{1,19} = 1.67$, $p = 0.212$), with a Diet x Social Treatment interaction ($F_{1,19} = 7.52$, $p = 0.013$). In the DEF mice, AVD deficiency led to a blunted ASR compared to controls and compared to SEP mice.

There was a significant main effect of Strain ($F_{1,43} = 6.16$, $p = 0.017$) and Diet ($F_{1,43} = 9.31$, $p = 0.004$) on the response to the initial 110 dB pulse given at the start of the session, with AVD-deficient mice reacting less than controls and BALB/c mice reacting less than C57BL/6J mice. Due to the effect of Diet on the response to the initial pulse, AVD-deficient mice showed no WSH ($F_{1,19}$

= 0.01, $p = 0.933$), however, control mice did show a significant WSH ($F_{1,24} = 6.58$, $p = 0.017$) (Figure 4). There was no effect of Social Treatment on the initial pulse ($F_{1,43} = 1.61$, $p = 0.212$) or on WSH ($F_{1,43} = 0.03$, $p = 0.876$).

There were no significant differences in PPI scores for Strain ($F_{1,43} = 0.70$, $p = 0.407$), Diet ($F_{1,43} = 0.23$, $p = 0.633$) or Social Treatment ($F_{1,43} = 0.18$, $p = 0.671$) (Figure 5). When strains were analysed separately, there were still no significant differences seen.

Discussion

The main finding from this study was that AVD deficiency exacerbated impairments caused by social stress and the degree of impairment was dependent on strain. In BALB/c mice but not C57BL/6J mice, AVD deficiency exacerbated the social deficit seen following social stress compared to controls. Corticosterone levels increased in response to both social stress and vitamin D deficiency in C57BL/6J mice but interestingly in BALB/c mice no change in corticosterone was observed following social stress regardless of dietary manipulation. In the ASR, the main difference was seen in the C57BL/6J mice; when AVD deficiency was combined with social defeat, it led to a blunted ASR, compared to either treatment on their own. In addition, AVD deficiency altered WSH across both strains due to a blunted initial response. Therefore we can accept the hypothesis that combining two-hits leads to greater detrimental outcomes compared to either treatment alone.

Biochemical parameters were measured in a subset of mice from each group and although there were strain differences for a number of parameters, there was no significant difference between AVD-deficient mice and control mice on any measure other than vitamin D levels. Importantly, calcium and phosphate levels, which are known to be regulated by vitamin D (Holick, 2007) were not altered in the AVD-deficient mice. Therefore, we can conclude that the model of AVD deficiency used in the current study has limited confounding factors and we can be more confident that alterations reported using this model have occurred due to the effects of vitamin D deficiency.

Social avoidance is a negative symptom of schizophrenia (Hansen et al., 2009), a symptom of autism (Richer, 1976) and it is also a symptom of depression and anxiety (Gorman, 1996). A previous study in group-housed BALB/c and C57BL/6J mice showed no social interaction impairments with AVD deficiency (Groves et al., 2013). Although the current study showed that chronic social defeat led to a mild social deficit in C57BL/6J mice, AVD deficiency did not exacerbate the mild social deficit caused by the chronic social defeat. However in BALB/c mice, AVD deficiency did exacerbate the social deficits following chronic social defeat and also increased the deficit following the separated housing condition. The two strains used in this study differ on a range of physiological parameters and behavioural responses, such as the non-emotional, high locomotor C57Bl/6 strain compared with the highly emotional, neophobic BALB/c strain. A previous study reported significant differences between BALB/c and C57Bl/6 mice in response to AVD deficiency. For example, BALB/c mice showed significant effects of AVD deficiency across a range of behaviours while C57BL/6 mice had a subtle behavioural phenotype (Groves et al., 2013). With respect to brain neurochemistry, BALB/c AVD-deficient mice exhibited an imbalance between excitatory and inhibitory neurotransmitters, with reductions in glutamate and glutamine levels and elevated levels of GABA, whereas C57BL/6J mice had increased levels of the dopamine (DA) metabolite, homovanillic acid (HVA), and 5HIAA, a metabolite of 5-hydroxytryptamine (5-HT). (Groves et al., 2013). The differential effects of AVD deficiency on BALB/c (glutamate/GABA) and C57BL/6J (DA/5HT) mice may contribute to the different behavioural responses observed in the current study.

Impairments in PPI of the ASR are relevant to schizophrenia (Moriwaki et al., 2009), however ASR on its own is generally not considered important as a behavioural readout for schizophrenia. It has been suggested that PPI is difficult to assess when the ASR itself has been modulated by a specific treatment, as we have seen in these experiments. However, we did not see any significant difference in PPI. The % PPI values in the C57BL/6J mice are comparable to previously published data using this strain; with the same protocol and equipment that was used in the current study (Harms et al., 2008). Although, results using the BALB/c strain have not previously been published using this

protocol, there was no significant difference in % PPI between the two strains in this study. PPI values do vary depending on the particular protocol used, as well as the facility and batch of mice. For example, a study by (van den Buuse, 2013) showed higher rates of % PPI and ASR in the BALB/c strain compared to the C57BL/6J strain and compared to the BALB/c mice in this study.

The relevance of altered ASR is not as easily defined, although changes in fear and anxiety can alter ASR (Davis et al., 1997), as can changes in stress response pathways (X. Wang et al., 2002a). Furthermore, enhanced response to acoustic startle has been previously proposed to reflect aspects of traumatic psychosocial stress (Pulliam et al., 2010).

AVD deficiency in group-housed BALB/c mice has previously been shown to increase the ASR compared to controls (Groves et al., 2013). In this experiment there was no significant main effect of diet on the ASR when analysing BALB/c mice separately. It is possible that the combination of social stress and AVD deficiency has blunted the ASR back to levels comparable to controls. However, there were differences in housing between the current study and the previous study (Groves et al., 2013) and the different housing conditions may have impacted on the ASR response. The level of ASR in this experiment was comparable to the controls in the previous study (Groves et al., 2013). In C57BL/6J mice, there was a significant effect on ASR when AVD deficiency was combined with chronic social defeat leading to a blunted ASR. Studies in humans have shown blunted startle in unipolar and bipolar depression (Forbes et al., 2005; Dichter and Tomarken, 2008), and in Parkinson's disease patients (Bowers et al., 2006), suggesting that it may be indicative of a depressive-like phenotype.

The duration of the social defeats were often terminated early by the experimenter (average of six and a half min, in lieu of the standard 10 min duration), due to the aggressive nature of the social defeats for the BALB/c strain. Due to the intensity of the protocol in the BALB/c mice, there may have been ceiling effects that would prevent all differential effects of diet from being seen. Interestingly, we noted effects of AVD deficiency in the separated mice in the BALB/c strain and

propose that being separated from cage mates during this period was enough to produce a mild social stress in this strain. Therefore, a better control such as group-housed mice should be used in any future experiments. Furthermore, a milder form of social stress such as social isolation or restraint stress may be enough of a second hit in this strain.

By contrast, the C57BL/6J strain was a more robust, larger sized, aggressive strain compared to the BALB/c strain and was more resilient to the social defeat paradigm. All bouts of defeat lasted the full 10 min, however all mice were submissive towards the CD-1 mice and there was a significant difference in behaviour between the separated C57BL/6J mice and the social defeat C57BL/6J mice. However, overall the C57BL/6J mice were more resilient to social defeat compared to the BALB/c mice.

Corticosterone levels are usually elevated in response to stress as part of the natural hypothalamic-pituitary-adrenal (HPA) axis (Smith and Vale, 2006). Furthermore, chronic stress can lead to long-term elevation of corticosterone due to a hyperactive HPA response (Hollis and Kabbaj, 2014). In the more resilient C57BL/6 mice, they showed a significant increase in serum corticosterone levels in response to chronic social defeat. However, in the vulnerable BALB/c strain no significant increase in corticosterone was observed following chronic social stress suggesting that they were unable to mount an appropriate stress response. One study examining the differences between susceptible and unsusceptible mice to chronic SD showed that in the unsusceptible mice serum corticosterone was still elevated four weeks later but in susceptible mice it was reduced (Krishnan et al., 2007).

Previous studies have shown differential corticosterone responses from the two strains which is highly dependent on type of stress and also the period of time between the stress and blood draw (Palumbo et al., 2010; Browne et al., 2011; Savignac et al., 2011; Benedetti et al., 2012). For example, (Browne et al., 2011) showed that BALB/c had increased corticosterone 1h following chronic restraint stress but not following acute stress, whereas, C57BL/6J mice had increased

corticosterone levels 1h after acute restraint stress but no change following chronic restraint stress. Another study showed no change in corticosterone levels in BALB/c mice following chronic social defeat, similar to our finding, but decreased corticosterone levels in C57BL/6J mice (Savignac et al., 2011). There is some evidence to suggest that vitamin D regulates the production of glucocorticoids in both humans and mice (Lundqvist et al., 2010; Lundqvist et al., 2012) and that vitamin D deficiency may modulate corticosterone (Tesic et al., 2015) which may explain why there is an effect of vitamin D deficiency in C57BL/6J on corticosterone levels.

Higher levels of BDNF in the hippocampus are both necessary and sufficient for resilience to chronic stress (Bergstrom et al., 2008) and reductions in BDNF induce depressive-like behaviours and anhedonia (Taliaz et al., 2011). Furthermore, other neurotrophic factors are also reduced in rats susceptible to chronic stress (Bergstrom et al., 2008). Vitamin D has been shown to stimulate a range of neurotrophic factors as reviewed by (Garcion et al., 2002) and therefore, vitamin D deficiency may lead to reductions in neurotrophic factors important for neuroprotection against chronic stress. Furthermore, chronic stress has been shown to induce elevated levels of oxidative stress (Fontella et al., 2005; Patki et al., 2013). Altered glutamate synaptic transmission, in the form of reduced vesicular glutamate transporter 1 (VGLUT1) enhances vulnerability to stress-induced social avoidance and enhanced anxiety and depressive-like behaviours (Tordera et al., 2007; Venzala et al., 2012). VGLUT1 heterozygous knockout mice also have impairments in long-term memory (Tordera et al., 2007). A wide range of components involved in glutamatergic neurotransmission including receptors and transporters and pre- and post-synaptic components have been associated with susceptibility to chronic stress as reviewed by (Franklin et al., 2012).

AVD-deficient mice showed greater susceptibility to chronic social stress compared to control mice and although the socially defeated mice were not tested for depressive-like behaviours other than social avoidance, it is plausible to suggest, based on previous research (Venzala et al., 2012), that AVD-deficient mice might be more vulnerable to the induction of a depressive-like phenotype following social stress. However, AVD deficiency on its own may be insufficient to induce a

depression-like phenotype because previous studies using the forced swim test as a measure of behavioural despair have shown that group-housed AVD-deficient mice showed similar levels of immobility to controls (Groves et al., 2013).

This experiment is the first to analyse the effects of combining the two adult environmental exposures of vitamin D deficiency and social stress. Future studies should extend the behavioural tests to address a more comprehensive screen of domains relevant to neuropsychiatric disorders, such as tests of anxiety, psychomimetic-induced locomotion and cognitive impairments. Other important experiments would be to look at changes in brain neurochemistry. Perhaps in future experiments, alternatives to social stress as the 'second hit' could be used, for example cannabis use is a risk factor for the development of schizophrenia and could be combined with AVD deficiency.

There is also growing evidence of an association between vitamin D deficiency and a substantially increased risk of all-cause dementia and AD (Littlejohns et al., 2014). Future experiments could test the additional effect of vitamin D deficiency on already well-characterized animal models of Alzheimer's disease. It is possible that a lack of neuroprotection caused by vitamin D deficiency may exacerbate the symptom severity of diseases such as Alzheimer's disease.

Conclusion

We found that AVD deficiency exacerbated impairments caused by social stress and the degree of impairment was strain dependent. These results extend our previous work related to developmental vitamin D exposures, and indicate that AVD deficiency may leave the brain more vulnerable to stress-related second hits. Furthermore, these findings could provide a mechanism of action underpinning the increased risk of psychosis in dark skinned migrants to cold climates; or the increased risk of AD, or PD severity in vitamin D deficient individuals.

References

Balden R, Selvamani A, Sohrabji F (2012) Vitamin D deficiency exacerbates experimental stroke injury and dysregulates ischemia-induced inflammation in adult rats. Endocrinology 153:2420-2435.

Benedetti M, Merino R, Kusuda R, Ravanelli MI, Cadetti F, dos Santos P, Zanon S, Lucas G (2012) Plasma corticosterone levels in mouse models of pain. European journal of pain 16:803-815.

Bergstrom A, Jayatissa MN, Mork A, Wiborg O (2008) Stress sensitivity and resilience in the chronic mild stress rat model of depression; an in situ hybridization study. Brain Res 1196:41-52.

Bowers D, Miller K, Mikos A, Kirsch-Darrow L, Springer U, Fernandez H, Foote K, Okun M (2006) Startling facts about emotion in Parkinson's disease: blunted reactivity to aversive stimuli. Brain 129:3356-3365.

Browne CA, Clarke G, Dinan TG, Cryan JF (2011) Differential stress-induced alterations in tryptophan hydroxylase activity and serotonin turnover in two inbred mouse strains. Neuropharmacology 60:683-691.

Cantor-Graae E, Selten JP (2005) Schizophrenia and migration: a meta-analysis and review. Am J Psychiatry 162:12-24.

Davis M, Walker DL, Lee Y (1997) Amygdala and bed nucleus of the stria terminalis: differential roles in fear and anxiety measured with the acoustic startle reflex. Philos Trans R Soc Lond B Biol Sci 352:1675-1687.

Dealberto MJ (2010) Ethnic origin and increased risk for schizophrenia in immigrants to countries of recent and longstanding immigration. Acta Psychiatr Scand.

Dichter GS, Tomarken AJ (2008) The chronometry of affective startle modulation in unipolar depression. J Abnorm Psychol 117:1-15.

Eyles D, Anderson C, Ko P, Jones A, Thomas A, Burne T, Mortensen PB, Norgaard-Pedersen B, Hougaard DM, McGrath J (2009) A sensitive LC/MS/MS assay of 25OH vitamin D3 and 25OH vitamin D2 in dried blood spots. Clin Chim Acta 403:145-151.

Fontella FU, Siqueira IR, Vasconcellos AP, Tabajara AS, Netto CA, Dalmaz C (2005) Repeated restraint stress induces oxidative damage in rat hippocampus. Neurochem Res 30:105-111.

Forbes EE, Miller A, Cohn JF, Fox NA, Kovacs M (2005) Affect-modulated startle in adults with childhood-onset depression: relations to bipolar course and number of lifetime depressive episodes. Psychiatry Res 134:11-25.

Franklin TB, Saab BJ, Mansuy IM (2012) Neural mechanisms of stress resilience and vulnerability. Neuron 75:747-761.

Garcion E, Wion-Barbot N, Montero-Menei CN, Berger F, Wion D (2002) New clues about vitamin D functions in the nervous system. Trends Endocrinol Metab 13:100-105.

Giovanoli S, Engler H, Engler A, Richetto J, Voget M, Willi R, Winter C, Riva MA, Mortensen PB, Feldon J, Schedlowski M, Meyer U (2013) Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice. Science 339:1095-1099.

Golden SA, Covington HE, 3rd, Berton O, Russo SJ (2011) A standardized protocol for repeated social defeat stress in mice. Nat Protoc 6:1183-1191.

Gorman JM (1996) Comorbid depression and anxiety spectrum disorders. Depress Anxiety 4:160-168.

Groves NJ, Kesby JP, Eyles DW, McGrath JJ, Mackay-Sim A, Burne TH (2013) Adult vitamin D deficiency leads to behavioural and brain neurochemical alterations in C57BL/6J and BALB/c mice. Behav Brain Res 241:120-131.

Hansen CF, Torgalsboen AK, Melle I, Bell MD (2009) Passive/apathetic social withdrawal and active social avoidance in schizophrenia: difference in underlying psychological processes. J Nerv Ment Dis 197:274-277.

Harms LR, Eyles DW, McGrath JJ, Mackay-Sim A, Burne TH (2008) Developmental vitamin D deficiency alters adult behaviour in 129/SvJ and C57BL/6J mice. Behav Brain Res 187:343-350.

Holick MF (2007) Vitamin D Deficiency Medical Progress:. The New Eng J of Med 357:266.
Hollis F, Kabbaj M (2014) Social defeat as an animal model for depression. ILAR J 55:221-232.
Jain SK, Micinski D (2013) Vitamin D upregulates glutamate cysteine ligase and glutathione reductase, and GSH formation, and decreases ROS and MCP-1 and IL-8 secretion in high-glucose exposed U937 monocytes. Biochem Biophys Res Commun 437:7-11.
Krishnan V et al. (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell 131:391-404.
Kume T, Nishikawa H, Tomioka H, Katsuki H, Akaike A, Kaneko S, Maeda T, Kihara T, Shimohama S (2000) p75-mediated neuroprotection by NGF against glutamate cytotoxicity in cortical cultures. Brain Res 852:279-289.
Littlejohns TJ, Henley WE, Lang IA, Annweiler C, Beauchet O, Chaves PH, Fried L, Kestenbaum BR, Kuller LH, Langa KM, Lopez OL, Kos K, Soni M, Llewellyn DJ (2014) Vitamin D and the risk of dementia and Alzheimer disease. Neurology 83:920-928.
Lundqvist J, Norlin M, Wikvall K (2010) 1alpha,25-Dihydroxyvitamin D3 affects hormone production and expression of steroidogenic enzymes in human adrenocortical NCI-H295R cells. Biochimica et biophysica acta 1801:1056-1062.
Lundqvist J, Wikvall K, Norlin M (2012) Vitamin D-mediated regulation of CYP21A2 transcription - a novel mechanism for vitamin D action. Biochimica et biophysica acta 1820:1553-1559.
Maynard TM, Sikich L, Lieberman JA, LaMantia AS (2001) Neural development, cell-cell signaling, and the "two-hit" hypothesis of schizophrenia. Schizophr Bull 27:457-476.
McDonald C, Murray RM (2000) Early and late environmental risk factors for schizophrenia. Brain Res Brain Res Rev 31:130-137.
McGrath JJ, Burne TH, Feron F, Mackay-Sim A, Eyles DW (2010) Developmental vitamin D deficiency and risk of schizophrenia: a 10-year update. Schizophr Bull 36:1073-1078.
Moriwaki M, Kishi T, Takahashi H, Hashimoto R, Kawashima K, Okochi T, Kitajima T, Furukawa O, Fujita K, Takeda M, Iwata N (2009) Prepulse inhibition of the startle response with chronic schizophrenia: a replication study. Neurosci Res 65:259-262.
Palumbo ML, Canzobre MC, Pascuan CG, Rios H, Wald M, Genaro AM (2010) Stress induced cognitive deficit is differentially modulated in BALB/c and C57Bl/6 mice: correlation with Th1/Th2 balance after stress exposure. Journal of neuroimmunology 218:12-20.
Patki G, Solanki N, Atrooz F, Allam F, Salim S (2013) Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress. Brain Res 1539:73-86.
Pulliam JV, Dawaghreh AM, Alema-Mensah E, Plotsky PM (2010) Social defeat stress produces prolonged alterations in acoustic startle and body weight gain in male Long Evans rats. J Psychiatr Res 44:106-111.
Razzoli M, Carboni L, Andreoli M, Ballottari A, Arban R (2011) Different susceptibility to social defeat stress of BalbC and C57BL6/J mice. Behav Brain Res 216:100-108.
Richer J (1976) The social-avoidance behaviour of autistic children. Anim Behav 24:898-906.
Savignac HM, Finger BC, Pizzo RC, O'Leary OF, Dinan TG, Cryan JF (2011) Increased sensitivity to the effects of chronic social defeat stress in an innately anxious mouse strain. Neuroscience 192:524-536.
Smith SM, Vale WW (2006) The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues in clinical neuroscience 8:383-395.
Taliaz D, Loya A, Gersner R, Haramati S, Chen A, Zangen A (2011) Resilience to chronic stress is mediated by hippocampal brain-derived neurotrophic factor. J Neurosci 31:4475-4483.
Tesic D, Hawes JE, Zosky GR, Wyrwoll CS (2015) Vitamin D Deficiency in BALB/c Mouse Pregnancy Increases Placental Transfer of Glucocorticoids. Endocrinology 156:3673-3679.
Tordera RM, Totterdell S, Wojcik SM, Brose N, Elizalde N, Lasheras B, Del Rio J (2007) Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). Eur J Neurosci 25:281-290.

van den Buuse M (2013) Exploring the role of 5-HT1A receptors in the regulation of prepulse inhibition in mice: implications for cross-species comparisons. ACS Chem Neurosci 4:149-160.

Venzala E, Garcia-Garcia AL, Elizalde N, Delagrange P, Tordera RM (2012) Chronic social defeat stress model: behavioral features, antidepressant action, and interaction with biological risk factors. Psychopharmacology (Berl) 224:313-325.

Wang JY, Wu JN, Cherng TL, Hoffer BJ, Chen HH, Borlongan CV, Wang Y (2001) Vitamin D(3) attenuates 6-hydroxydopamine-induced neurotoxicity in rats. Brain Res 904:67-75.

Wang X, Su H, Copenhagen LD, Vaishnav S, Pieri F, Shope CD, Brownell WE, De Biasi M, Paylor R, Bradley A (2002a) Urocortin-deficient mice display normal stress-induced anxiety behavior and autonomic control but an impaired acoustic startle response. Mol Cell Biol 22:6605-6610.

Wang Y, Chang CF, Morales M, Chiang YH, Hoffer J (2002b) Protective effects of glial cell line-derived neurotrophic factor in ischemic brain injury. Ann N Y Acad Sci 962:423-437.

Wray NR, Visscher PM (2010) Narrowing the boundaries of the genetic architecture of schizophrenia. Schizophr Bull 36:14-23.

Figure Legends

Figure 1 Social interaction arena layout

Figure 2 Effects of chronic social defeat on plasma corticosterone levels

There was no significant effect of Diet or Social Treatment in BALB/c mice on corticosterone levels (a). However, there were significant main effects for both Diet and Social Treatment in C57BL/6J mice (b); AVD-Deficient mice had higher corticosterone levels than Control mice, and DEF mice had higher corticosterone levels than SEP mice. Mean ± SEM (* main effect, $p < 0.05$)

Figure 3 Results of the social interaction test

The results of the social interaction test in BALB/c mice **(a, c and e)** and in C57BL/6J mice **(b, d and f)**. The average distance to the CD-1 mouse was significantly further in DEF mice compared to SEP mice in both strains **(a and c)**, however in the BALB/c strain **(a)**, there was also a significant increase in the average distance to the CD-1 mouse in AVD-deficient SEP mice compared to control SEP mice. The time spent in the interaction zone was significantly less for DEF mice compared to SEP mice in both strains **(b and d)**, with no significant differences between AVD-deficient and control mice in either strain. The time spent in the avoidance zone was significantly greater in DEF mice compared to SEP mice in BALB/c mice only **(e)**, furthermore AVD-deficient mice spent significantly (# main effect of Diet, $p < 0.05$) more time in the avoidance zone compared to controls **(e)**. Mean ± SEM (*$p < 0.05$)

Figure 4 Results for the acoustic startle response

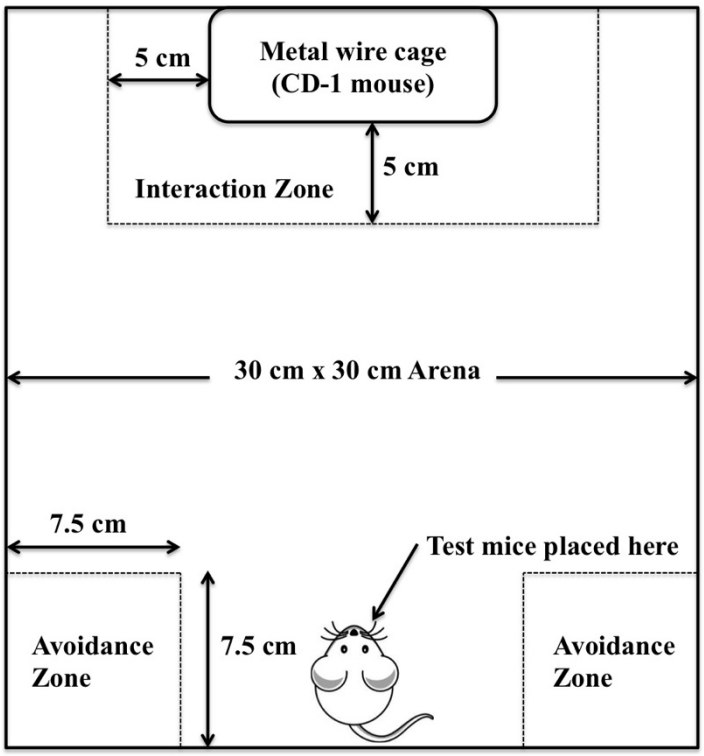
ASR in BALB/c **(a and c)** and C57BL/6J **(b and d)** mice. There was a significant Diet x Social Treatment interaction in BALB/c mice. Post-hoc t-tests showed a significant difference between DEF controls and SEP control mice, with DEF leading to a blunted ASR but no significant difference between Social Treatments in AVD-deficient mice. In C57BL/6J mice, AVD deficiency led to a blunted acoustic startle response in DEF mice **(d)**. Mean ± SEM (*$p < 0.05$)

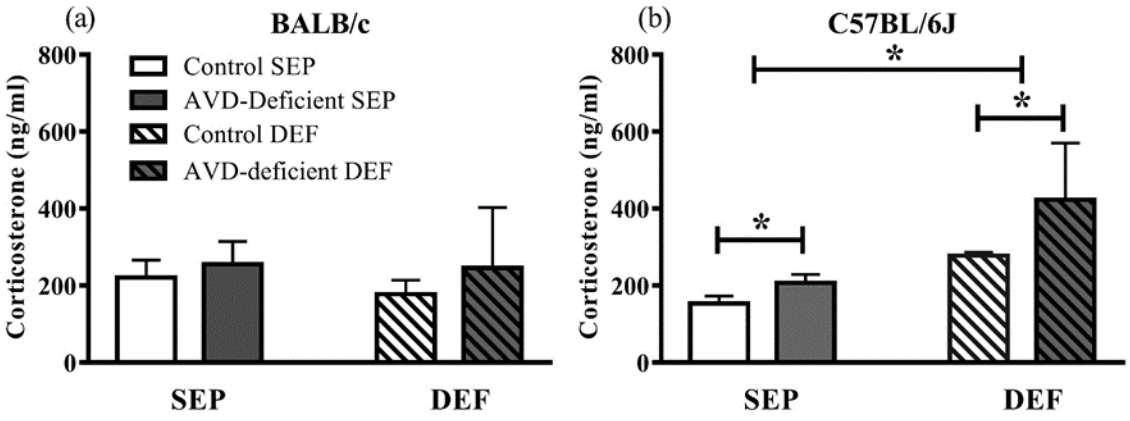
Figure 5 Results for within-session habituation

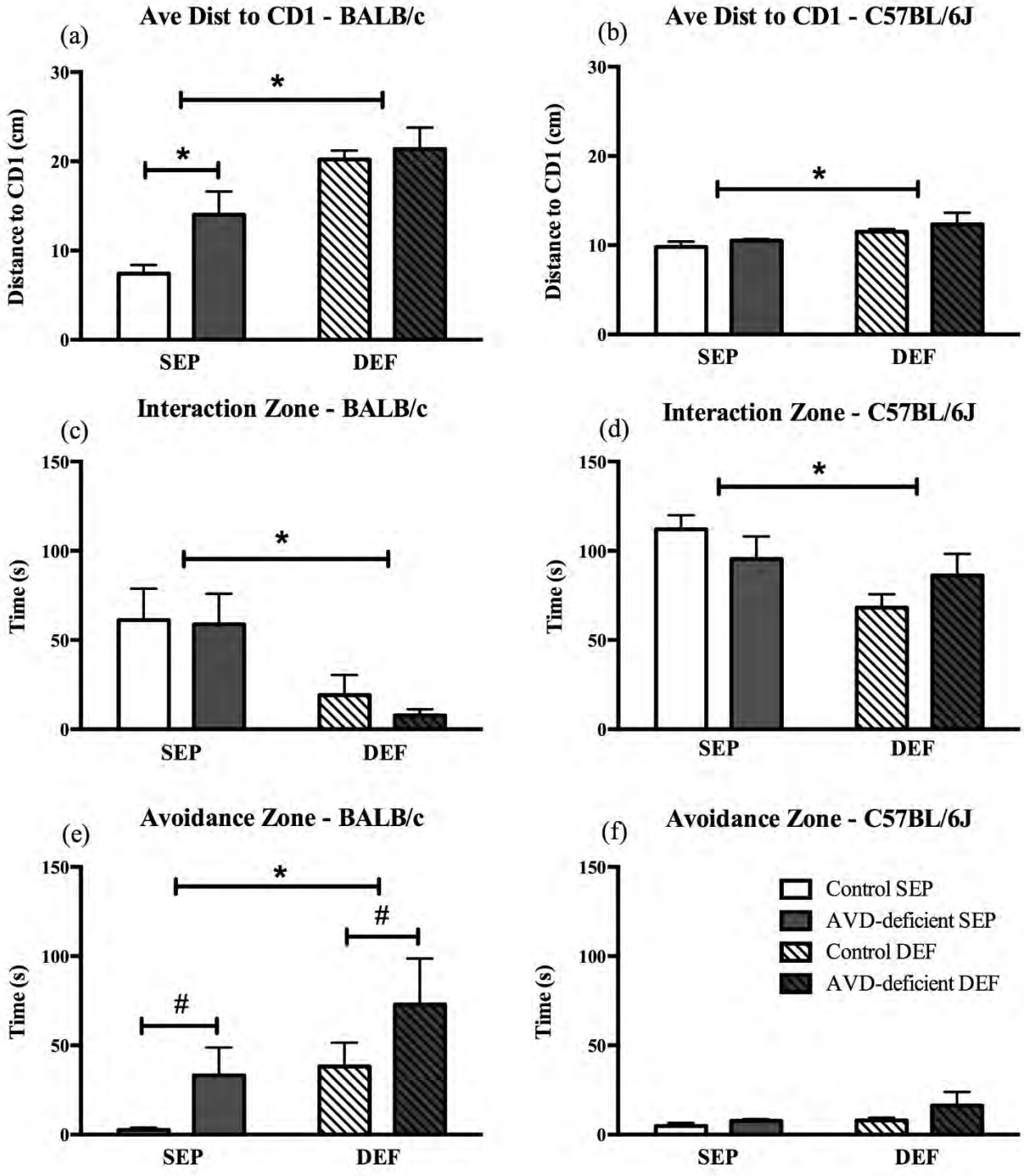
WSH in BALB/c strain **(a and c)** and in C57BL/6J strain **(b and d),** SEP mice shown in **a** and **b**, with DEF mice shown in **c** and **d**. Overall, AVD deficiency led to a significantly reduced response to the initial 110 dB pulse. Due to this reduced initial response, AVD-deficient mice had no WSH, although controls did show WSH. Mean ± SEM (*$p < 0.05$)

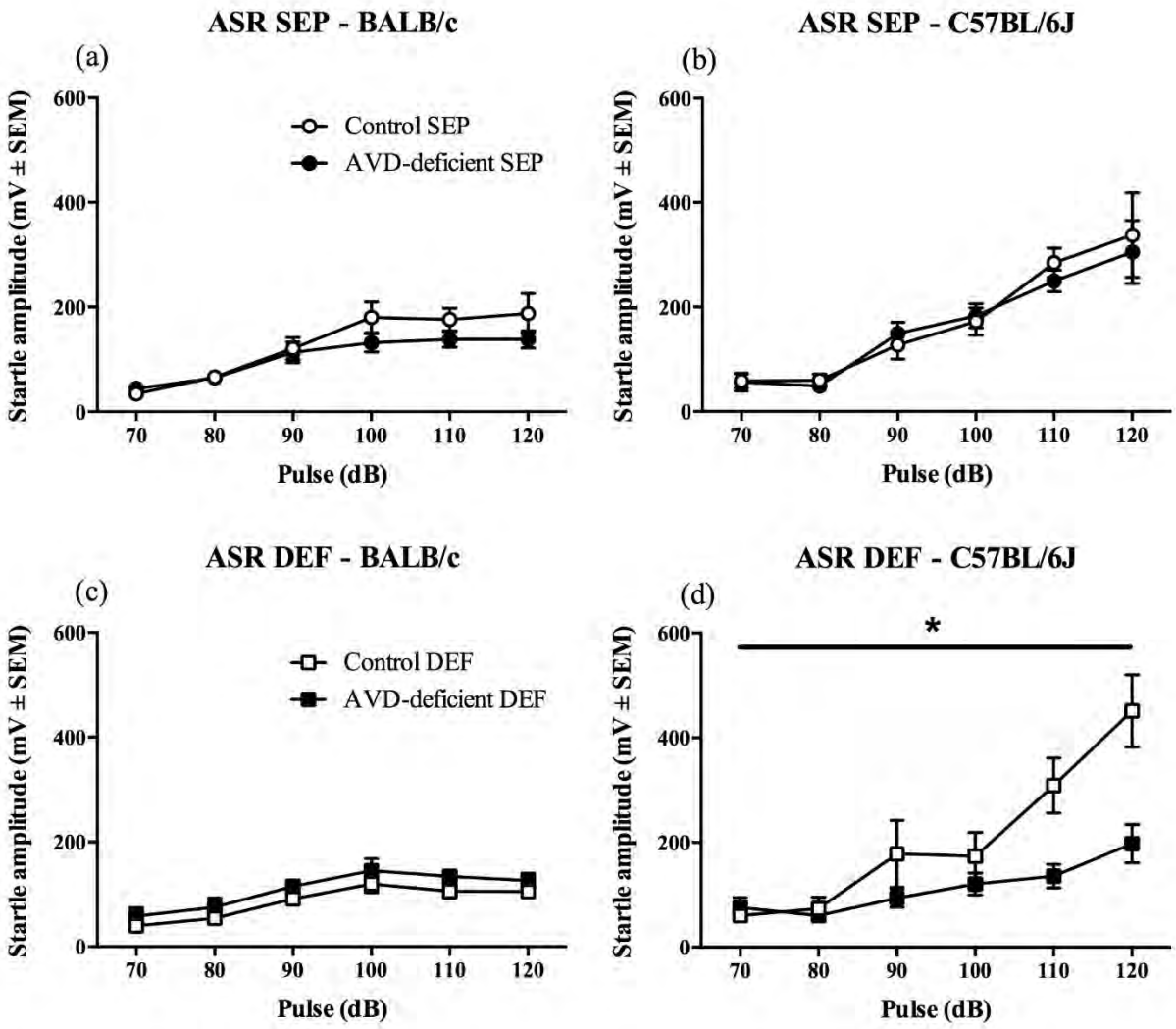
Figure 6 Results for prepulse inhibition of the acoustic startle response

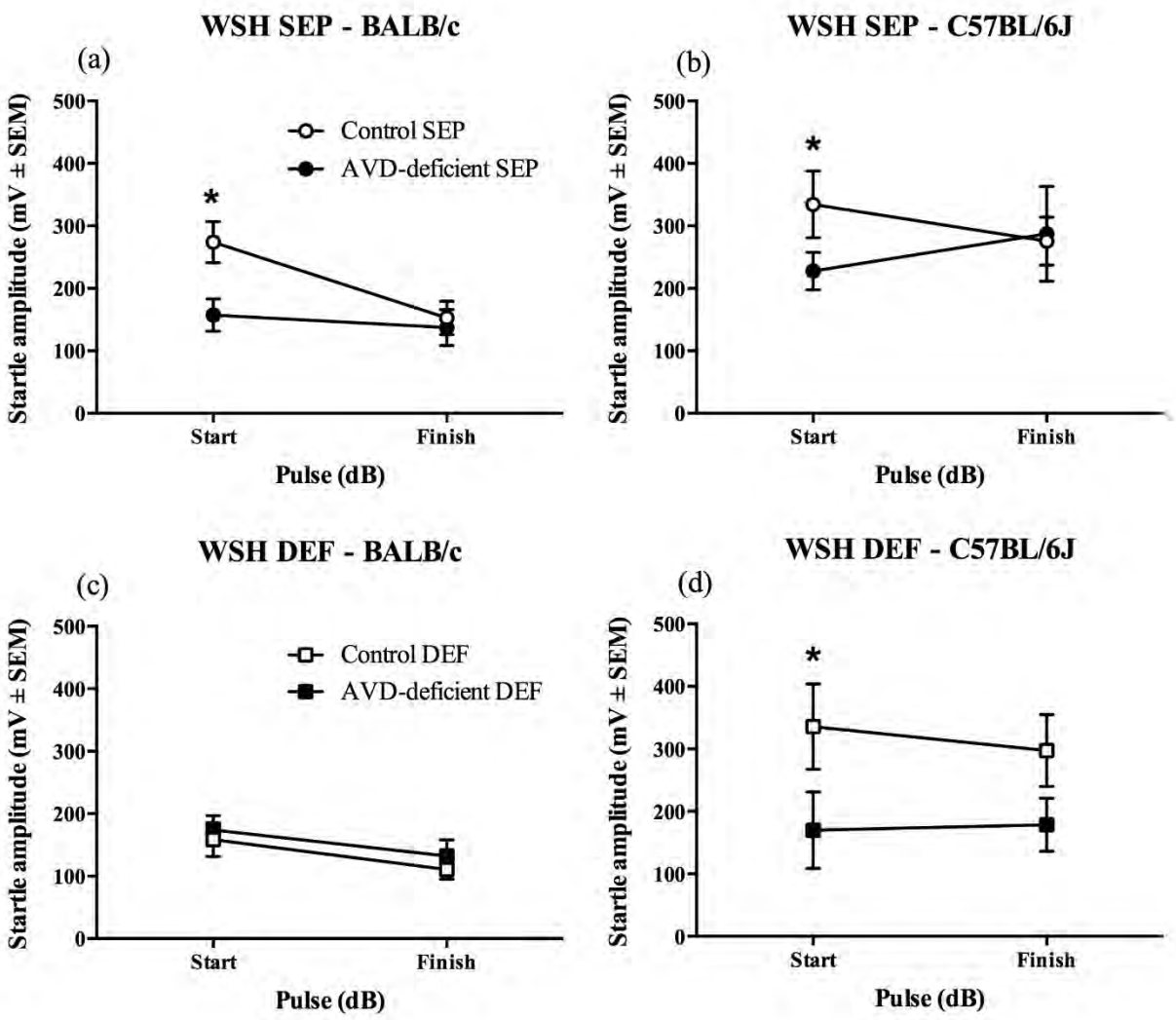
There were no significant differences on PPI scores. Mean ± SEM


5 cm
Metal wire cage
(CD-1 mouse)
5 cm
Interaction Zone
30 cm x 30 cm Arena
7.5 cm
Test mice placed here
Avoidance
Zone
7.5 cm
Avoidance
Zone


(a)
BALB/c
Control SEP
AVD-Deficient SEP
Control DEF
AVD-deficient DEF
Corticosterone (ng/ml)
800
600
400
200
0
SEP
DEF
(b)
C57BL/6J
*
*
*
Corticosterone (ng/ml)
800
600
400
200
0
SEP
DEF


(a)
Ave Dist to CD1 - BALB/c
Distance to CD1 (cm)
30
20
10
0
*
*
SEP
DEF
(b)
Ave Dist to CD1 - C57BL/6J
Distance to CD1 (cm)
30
20
10
0
*
SEP
DEF
(c)
Interaction Zone - BALB/c
Time (s)
150
100
50
0
*
SEP
DEF
(d)
Interaction Zone - C57BL/6J
Time (s)
150
100
50
0
*
SEP
DEF
(e)
Avoidance Zone - BALB/c
Time (s)
150
100
50
0
*
#
#
SEP
DEF
(f)
Avoidance Zone - C57BL/6J
Time (s)
150
100
50
0
Control SEP
AVD-deficient SEP
Control DEF
AVD-deficient DEF
SEP
DEF


ASR SEP - BALB/c
(a)
Control SEP
AVD-deficient SEP
Startle amplitude (mV ± SEM)
600
400
200
0
70
80
90
100
110
120
Pulse (dB)
ASR SEP - C57BL/6J
(b)
Startle amplitude (mV ± SEM)
Pulse (dB)
ASR DEF - BALB/c
(c)
Control DEF
AVD-deficient DEF
Startle amplitude (mV ± SEM)
Pulse (dB)
ASR DEF - C57BL/6J
(d)
*
Startle amplitude (mV ± SEM)
Pulse (dB)


WSH SEP - BALB/c
(a)
Control SEP
AVD-deficient SEP
Startle amplitude (mV ± SEM)
500
400
300
200
100
0
*
Start
Finish
Pulse (dB)
WSH SEP - C57BL/6J
(b)
Startle amplitude (mV ± SEM)
500
400
300
200
100
0
*
Start
Finish
Pulse (dB)
WSH DEF - BALB/c
(c)
Control DEF
AVD-deficient DEF
Startle amplitude (mV ± SEM)
500
400
300
200
100
0
Start
Finish
Pulse (dB)
WSH DEF - C57BL/6J
(d)
Startle amplitude (mV ± SEM)
500
400
300
200
100
0
*
Start
Finish
Pulse (dB)

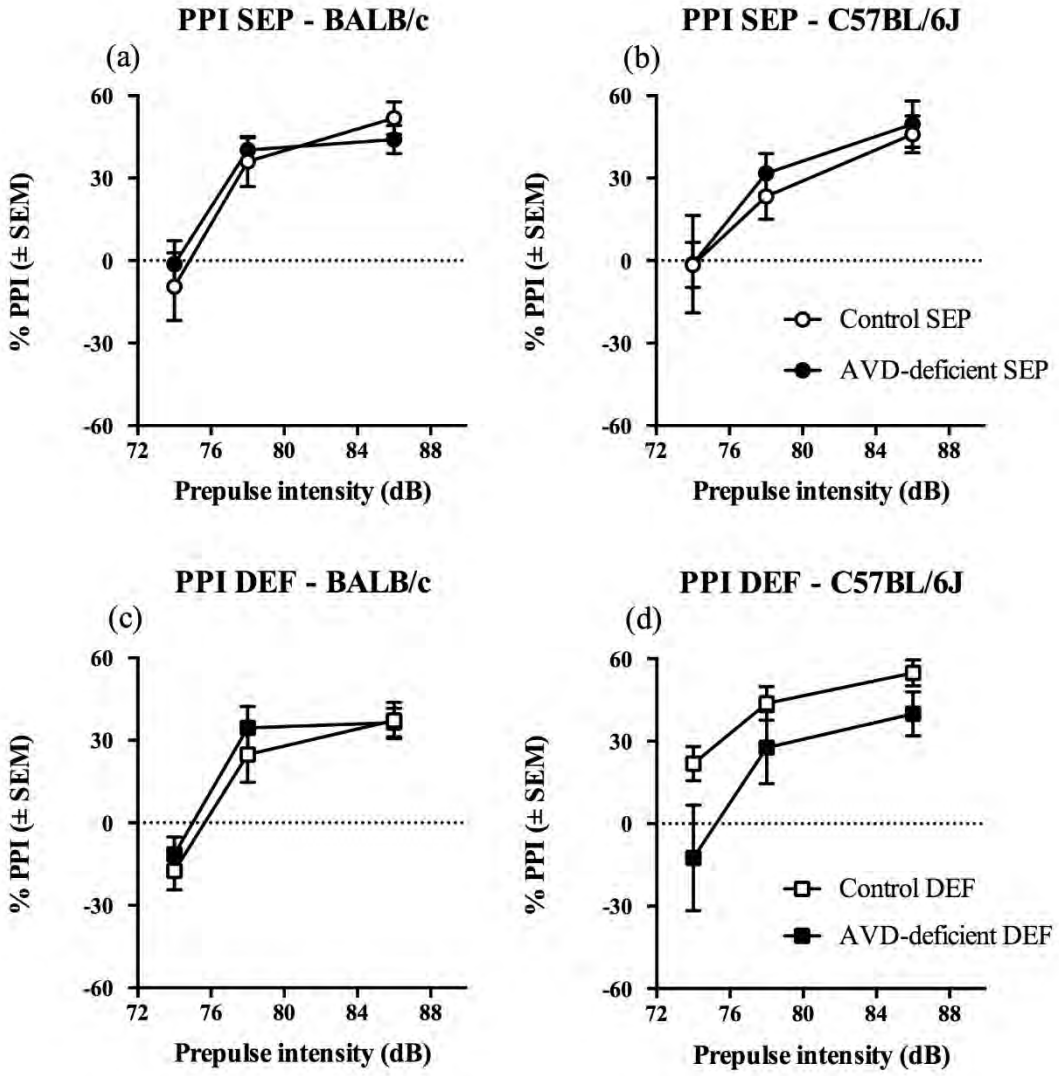

PPI SEP - BALB/c
(a)
% PPI (± SEM)
60
30
0
-30
-60
72
76
80
84
88
Prepulse intensity (dB)
PPI SEP - C57BL/6J
(b)
Control SEP
AVD-deficient SEP
PPI DEF - BALB/c
(c)
PPI DEF - C57BL/6J
(d)
Control DEF
AVD-deficient DEF

Table 1 Summary of results for social defeat and social interaction not included in Figure 2

Mean ± SEM

Social Defeat and Interaction	BALB/c Control SEP	BALB/c AVD SEP	BALB/c Control DEF	BALB/c AVD DEF	C57BL/6J Control SEP	C57BL/6J AVD SEP	C57BL/6J Control DEF	C57BL/6J AVD DEF
Starting Body Weight (g) *	27.4 ± 0.6	26.9 ± 0.8	25.6 ± 1.0	26.8 ± 0.4	32.3 ± 1.9	32.2 ± 0.8	33.0 ± 1.7	30.9 ± 1.4
Finishing Body Weight (g) *	26.9 ± 0.6	27.1 ± 0.8	23.5 ± 0.8	25.3 ± 0.8	31.5 ± 1.4	31.7 ± 0.9	27.9 ± 1.1	29.4 ± 0.7
Social Defeat - Ave Duration (s) *	—	—	417.5 ± 13.2	382.5 ± 24.9	—	—	600.0 ± 0	600.0 ± 0
Habituation * - Ave Dist to CD-1 (cm)	13.4 ± 1.9	14.0 ± 1.1	13.6 ± 1.9	15.1 ± 2.4	9.0 ± 1.5	10.5 ± 0.4	9.3 ± 0.8	10.5 ± 0.2
Habituation - Dist travelled (cm)	599.9 ± 63.6	722.2 ± 71.5	636.6 ± 69.5	805.3 ± 98.5	774.0 ± 165.0	822.7 ± 44.0	713.9 ± 67.3	725.0 ± 43.5
Habituation - Interaction Zone (s)	80.58 ± 14.03	64.16 ± 4.78	44.93 ± 10.97	50.43 ± 12.92	93.45 ± 18.14	87.02 ± 19.40	91.02 ±10.47	105.87 ± 7.20
Habituation - Avoidance Zone (s)	16.88 ± 11.99	19.10 ± 8.16	6.69 ± 2.11	27.35 ± 13.77	6.06 ± 1.99	6.91 ± 1.00	5.98 ±1.85	6.96 ±1.25
Interaction * - Dist travelled (cm)	475.6 ± 63.0	474.8 ± 89.3	284.9 ± 34.0	175.9 ± 31.7	599.8 ± 62.1	582.4 ± 56.3	380.9 ± 75.7	493.7 ± 41.7

(*) Denotes a significant main effect of strain ($p < 0.05$)

Table 2 Summary of results for biochemical parameters

Mean ± SEM

Biochemistry (*n* = 4/group)	BALB/c Control SEP	BALB/c AVD SEP	BALB/c Control DEF	BALB/c AVD DEF	C57BL/6J Control SEP	C57BL/6J AVD SEP	C57BL/6J Control DEF	C57BL/6J AVD DEF
25-OHD$_3$ (*n* = 5-8)*	26.85 ± 0.56	2.21 ± 1.11	28.57 ± 2.06	2.99 ± 1.23	62.15 ± 4.11	1.90 ± 0.36	66.54 ± 2.89	1.02 ± 0.30
Sodium*	161 ± 1	161 ± 1	159 ± 1	159 ± 1	156 ± 1	156 ± 1	158 ± 4	157 ± 1
Potassium*	6 ± 0	6 ± 0	6 ± 0	6 ± 0	10 ± 2	8 ± 1	8 ± 0	7 ± 1
Chloride	113 ± 1	111 ± 0	110 ± 1	112 ± 1	108 ± 1	108 ± 1	114 ± 3	110 ± 1
Glucose*	8 ± 1	9 ± 1	7 ± 2	15 ± 5	28 ± 8	27 ± 2	18 ± 7	20 ± 4
Urea	8 ± 0	8 ± 0	9 ± 1	9 ± 0	8 ± 1	8 ± 1	8 ± 1	10 ± 3
Calcium*	2.1 ± 0.1	2.1 ± 0.0	1.7 ± 0.4	2.0 ± 0.0	2.4 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.3 ± 0.3
Phosphate	3 ± 0	3 ± 0	3 ± 1	3 ± 0	4 ± 1	4 ± 0	4 ± 1	4 ± 0
Total Protein *	50 ± 1	50 ± 1	40 ± 8	50 ± 1	56 ± 2	54 ± 2	53 ± 0	43 ± 5
Albumin*	24 ± 1	24 ± 0	20 ± 4	25 ± 0	27 ± 1	29 ± 1	28 ± 1	29 ± 5
Globulin	25 ± 0	25 ± 1	20 ± 4	26 ± 1	28 ± 3	25 ± 1	25 ± 0	15 ± 9
A:G Ration	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	0 ± 1
Total Bilirubin*	1 ± 0	1 ± 0	0 ± 0	1 ± 0	3 ± 1	2 ± 0	3 ± 1	1 ± 1
Alkaline Phosphatase	78 ± 4	93 ± 8	69 ± 15	81 ± 6	87 ± 18	69 ± 9	63 ± 14	51 ± 9
Aspartate Aminotransferase*	273 ± 57	178 ± 11	138 ± 5	172 ± 44	476 ± 143	374 ± 129	315 ± 84	247 ± 121
Alanine Aminotransferase	46 ± 7	30 ± 2	25 ± 2	28 ± 7	410 ± 244	217 ± 96	92 ± 31	78 ± 48
Creatine Kinase*	42 ± 11	58 ± 8	39 ± 11	41 ± 10	39 ± 8	33 ± 8	25 ± 2	31 ± 8
Cholesterol	3 ± 0	3 ± 0	2 ± 0	3 ± 0	4 ± 0	4 ± 0	3 ± 1	3 ± 0
Triglyceride	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0

(*) Denotes a significant main effect of strain ($p < 0.05$)

Conflict of Interest

The authors declare no conflicts of interest.

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