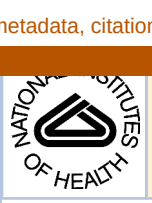

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Caloric restriction alleviates abnormal locomotor activity and dopamine levels in the brain of the methionine sulfoxide reductase A knockout mouse

Derek B. Oien[a], **Gregory L. Osterhaus**[b], **Brandi L. Lundquist**[a], **Stephen C. Fowler**[a,c], and **Jackob Moskovitz**[a,*]

[a]Department of Pharmacology & Toxicology, University of Kansas, Lawrence, KS USA

[b]Department of Chemistry, University of Kansas, Lawrence, KS USA

[c]Life Span Institute, University of Kansas, Lawrence, KS USA

Abstract

Oxidative stress is associated with the aging process, a risk factor for neurodegenerative diseases, and decreased by reduced energy intake. Oxidative modifications can affect protein function; the sulfur-containing amino acids, including methionine, are particularly susceptible to oxidation. A methionine sulfoxide can be enzymatically reduced by the methionine sulfoxide reductase (Msr) system. Previously, we have shown that *MsrA*$^{-/-}$ mice exhibit altered locomotor activity and brain dopamine levels as function of age. Previous studies have demonstrated that a caloric restriction enhances antioxidant defense and reduces the action of reactive oxygen species. Here we examine locomotor behavior and dopamine levels of *MsrA*$^{-/-}$ mice after caloric restriction starting at 8 months of age and ending at 17 months. The *MsrA*$^{-/-}$ mice did not have any significant difference in spontaneous distance traveled when compared to controls at 17 months of age. In contrast, our previous report showed decreased locomotor activity in the *MsrA*$^{-/-}$ mice at 12 months of age and older when fed *ad-libitum*. After completion of the caloric restriction diet, dopamine levels were comparable to control mice. This differs from the abnormal dopamine levels previously observed in *MsrA*$^{-/-}$ mice fed *ad-libitum*. Thus, caloric restriction had a neutralization effect on MsrA ablation. In summary, it is suggested that caloric restriction alleviates abnormal locomotor activity and dopamine levels in the brain of the methionine sulfoxide reductase A knockout mouse.

Keywords

Oxidative stress; Protein oxidation; Caloric restriction; Locomotor activity; Dopamine; Aging

INTRODUCTION

Oxidative stress is associated with the aging process, is a risk factor for several neurodegenerative diseases, and is decreased by reduced energy intake. Oxidative damage to

*Corresponding author: Jackob Moskovitz, 1251 Wescoe Hall Drive, 5064 Malott Hall, Lawrence, KS 66045, moskovij@ku.edu, Phone: (785)864-3536, Fax: (785)864-5219

cells and tissues results from reactive oxygen species production. The production of these species is often a byproduct of metabolism that can affect lipids, nucleic acids, and proteins. The sulfur-containing side chains of methionine and cysteine are particularly susceptible to oxidation. Oxidation of a cysteine residue can be reduced by various antioxidants, but methionine sulfoxide is unique in that it is reduced by the methionine sulfoxide reductase (Msr) system. The Msr enzymes include MsrA and MsrB, which reduce the *S* and *R* sulfoxide enantiomers, respectively [15]. MsrA is considered the major Msr because it positively regulates MsrB expression [14,18]. Lack of MsrA has been demonstrated in several organisms to cause increased hypersensitivity to oxidative stress [22,25] and premature death [16,19]. Recent evidence has found oxidized methionine residues associated with age-related neurodegenerative conditions [2,5]. However, the role of methionine oxidation in the pathogenesis of neurodegenerative diseases is still unknown [22].

Reduced energy intake is known to decrease free radical production and oxidative damage in mammals, extend life-span, and slow age-related health problems in various species [12]. An ongoing study with rhesus monkeys supports the latter concept [3]. Furthermore, caloric restriction (CR) is neuroprotective [11] and has benefits that oppose brain aging, including oxidative stress pathways and related pathological states [4]. In rodents, feeding conditions have been found to affect locomotor activity [9,13], and restrictive diets can increase dopamine (DA) signaling [1,8].

As a model to study the methionine oxidation *in vivo*, the methionine sulfoxide reductase A knockout ($MsrA^{-/-}$) mouse was created [16]. This mouse was described as having elevated levels of brain pathologies [25] and an abnormal walking pattern [16]. Moreover, a genomic profile of $MsrA^{-/-}$ mice showed an up- and down-regulation of several genes associated with oxidative stress, signal transduction, and neuroregulation [20]. In a recent study, it was shown that $MsrA^{-/-}$ mice exhibit lower locomotor activity with age [23]. The $MsrA^{-/-}$ brains contained significantly higher levels of DA up to 12 months of age relative to wild-type (WT) control mice, while lower levels of DA were observed at 16 months of age. Moreover, $MsrA^{-/-}$ mice were less responsive to amphetamine, the release of DA was increased in the striatal regions, and the expression pattern of tyrosine hydroxylase activating protein correlated with age-dependent DA levels. Thus, it was suggested that DA regulation and signaling pathways are impaired in $MsrA^{-/-}$ mice, which may contribute to their abnormal behavior. Here we describe $MsrA^{-/-}$ mice on CR conditions and compare the results to our recent experiments showing abnormal locomotor behavior and dopamine levels in brain [23]. The $MsrA^{-/-}$ mice were placed on feeding restrictions starting at eight months of age, and these restrictions were continued until up to the age of 17 months. The mice were analyzed for locomotor behavior and brain DA levels.

MATERIALS AND METHODS

The $MsrA^{-/-}$ and WT mice were previously described [16,23] and all applied procedures were approved by the KU Institutional Animal Care and Use Committee.

Caloric restriction

The $MsrA^{-/-}$ and WT control mice were both fed on an *ad-libitum* (AL) diet until they reached eight months of age. Initial weights prior to caloric restriction were calculated by taking the average weight over three days for each mouse. Each mouse was then fed daily an amount of chow equal to 10% body weight until the weight of the mouse was reduced by 15%. All mice achieved this reduction within the first two weeks of restriction. Each mouse was maintained at this body weight for the duration of the experiment by adjusting food availability. The average weight losses within each mouse type and between the mouse groups did not

significantly differ ($P<0.05$; data not shown). Likewise, previous report indicated similar weights in *MsrA*$^{-/-}$ and WT mice as function of age on an AL diet [16].

Total distance traveled

Locomotor activity was assessed with four concurrently operative force-plate actometers [7]. The actometer and associated peripherals, including the methods of data collection and analysis, were used as previously described [7]. In this study, the total distance traveled for each *MsrA*$^{-/-}$ and WT control mouse on CR was measured for 30 minutes. Measurements were taken before CR at the age of eight months and each subsequent month while mice were on CR. Measurements were taken at the same time of day each month. Data presented are the mean ±standard deviation. The distance traveled was analyzed with a repeated-measures two-way ANOVA with genotype as the between-groups factor and month as the repeated-measures factor. Significance was further determined by the polynomial trend order 2 post-hoc test.

Dopamine concentrations in brain

The concentration of DA in the brain tissue was analyzed when mice on CR were 12 months old and 17 months old. The DA concentrations in *MsrA*$^{-/-}$ and WT control mice were measured immediately after the respective analysis of distance traveled by methods described previously [23]. Briefly, postmortem brain cerebrum was homogenized in a solution containing 0.2 M perchloric acid and dihydrobenzylamine as the internal standard. Insoluble material was removed by centrifugation and supernatants were injected into a C18 reverse-phase HR-80 catecholamine column (ESA Inc., Bedford, MA). The mobile phase consisted of 94% 50 mM sodium phosphate/0.2 mM EDTA/1.2 mM heptanesulfonic acid (pH 3.2) solution and 6% methanol. Peaks were detected by an ESA 8 Channel CoulArray system and analyzed by CoulArray data analysis. Statistical analysis was carried out using *Student's t-test* to determine if the observed changes between the two mouse types had a significant effect ($P<0.05$).

Detection of protein-methionine sulfoxide in brain

Post-mortem cerebra from both mouse types at 12 months of age, fed either CR (four months) or AL diets, were homogenized in PBS buffer, centrifuged, and the corresponding supernatants were collected. Equal protein amounts (50 μg) from each brain extract were separated by gel-electrophoresis in 4–20% SDS-gel followed by western blot analysis using anti-methionine sulfoxide antibodies or anti-tyrosine hydroxylase antibodies, as previously described [21].

RESULTS

Total distance traveled of MsrA$^{-/-}$ and wild-type mice on caloric restriction

Changes in spontaneous locomotor activity can be indicative of neuronal dysfunction and neurodegenerative diseases [10]. In a previous study, the *MsrA*$^{-/-}$ mice showed significantly less locomotor activity when compared to WT mice, including the spontaneous distance traveled. There was no significant difference between *MsrA*$^{-/-}$ and WT mice for distance traveled in the overall experiment (Fig. 1; $P=0.661$, main effect). Also, there were no significant differences found between distances traveled among the genotypes during both the beginning and the end months of the CR diet (Fig. 1). The *MsrA*$^{-/-}$ mice traveled significantly more only in the intermediate months as judged by the polynomial trend order 2 post-hoc test ($P=0.001$, month-by-type interaction effect). The lack of difference before the CR diet (when mice were fed AL at 8 months of age; Fig. 1) is comparable to the distances traveled by *MsrA*$^{-/-}$ and WT mice at 6 months of age that were also fed on an AL diet [23]. The increase and later non-significant difference of *MsrA*$^{-/-}$ mice on a CR diet is a contrast to the ~50% decrease in distance traveled by *MsrA*$^{-/-}$ mice when on an AL diet at the age of 12 months [23].

Dopamine concentrations in brains of mice fed caloric restriction diet

Dopamine levels from the nigrostriatal pathway can influence the direct and indirect movement pathways. In a previous study, abnormal DA levels in the brain tissue of *MsrA*$^{-/-}$ mice were found, relative to WT mice [22]. The concentration of DA in the brain tissue of *MsrA*$^{-/-}$ and WT mice did not significantly differ at the ages of 12 months and 17 months (Fig. 2) when on CR. There was a small, non-significant relative decline in DA levels of *MsrA*$^{-/-}$ mice at 17 months of age, which may be similar to the significant decrease between *MsrA*$^{-/-}$ and WT mice when fed on an AL diet (Table 1) [23]. This latter observation suggests that the positive effect of the CR diet on *MsrA*$^{-/-}$ DA levels may be limited in advancing age. Furthermore, the increased DA concentration of 16-month-old WT control mice on an AL diet [23] was not detected in WT mice fed on a CR diet at 17 months of age. This observation suggests that the CR diet reduced brain oxidative stress below levels that normally prompt DA elevation [23].

Methionine oxidation of 15 kDa protein in mouse brain

We have applied our novel anti-methionine sulfoxide antibodies [21] to identify methionine sulfoxide proteins in brain extracts of both mouse types under AL and CR diets. There was an enhanced methionine oxidation in a ~15 kDa brain protein from *MsrA*$^{-/-}$ mice under AL diet, and to lesser extent in WT mice (Fig. 3). Interestingly, under CR diet, the levels of methionine oxidation in this protein were reduced in both mouse types. Other methionine sulfoxide-containing proteins were detected in the western blot analysis, but their band intensities were not sufficient to determine the effect of CR diet due to the assay sensitivity limits. Further purification and enrichment of this protein will be performed to facilitate the identification of this protein by mass spectrometry analysis. Also, there was no change in tyrosine hydroxlase expression in all cases, which serves both as an internal control for protein loading and evidence for lack of obvious neurodegeneration in both mouse types brains.

DISCUSSION

The *MsrA*$^{-/-}$ mice are hypersensitive to oxidative stress and exhibit accelerated age-related pathologies [17,23,25]. We have previously reported that the *MsrA*$^{-/-}$ mice exhibit decreased locomotor activity and abnormal DA levels in the brain tissue. Here, it is demonstrated that reduced availability of food can alleviate abnormal locomotor activity and DA levels in the brain of *MsrA*$^{-/-}$ mice. However, there is a lack of solid evidence for mechanisms involved in alteration of methionine sulfoxide content and DA signaling via methionine oxidation. Thus, it is still difficult to predict if a decrease in reactive oxygen species or compensatory response prompted by the CR diet, or both, are responsible for the observed changes.

The reduced availability of food and corresponding loss of body weight has been shown to correspond to a reduction of oxidative stress and reactive oxygen species [12]. In this study, the mice had reduced access to the same food that was given when fed on an AL diet. No differences in the feeding habits of *MsrA*$^{-/-}$ and WT mice were observed, and this is further supported by a previous report demonstrating that the weights of *MsrA*$^{-/-}$ and WT mice are not different on an AL diet or with age [16]. Furthermore, a 20% reduction in body weight has been shown to improve DA receptor signaling in rodents [1]. The *MsrA*$^{-/-}$ mice have been previously reported to exhibit decreased locomotor activity and abnormal DA levels in the brain tissue relative to WT mice [23]. However, in the current study there was no significant difference in locomotor activity between the two mouse types after nine months of caloric restriction (Fig. 1). This supports the idea that the reduced energy intake decreases the activity of reactive oxygen species, whereas normally fed *MsrA*$^{-/-}$ mice are hypersensitive to oxidative stress [16,25]. However, *MsrA*$^{-/-}$ mice were significantly more active than the WT mice before the end of the experiment (Fig. 1). This latter finding supports the concept that the food restriction had an effect by reducing methionine sulfoxide-mediated cellular changes while

increasing the function of already up-regulated genes in *MsrA*$^{-/-}$ brain [20]; alternatively, this could also be by activating specific compensatory mechanisms yet to be discovered. Regarding DA levels, it has been determined in this study (Fig. 2) that CR significantly alleviated the differences in DA levels observed between the *MsrA*$^{-/-}$ and WT mice when fed AL [23]. Thus, it may be that this is a result reflects the hypothesis above, which suggests the CR effects on cellular metabolism are linked to methionine oxidation via the absence of MsrA. It is important to note that in contrast to the elevated DA levels in 16-month-old WT mice fed on an AL diet, the WT mice fed on a CR diet at 17 months of age had a decreased concentration of DA in their brain tissue (Table 1). If indeed higher DA levels represent a response to elevated oxidative stress conditions, the latter decrease in DA levels in the WT brain may reflect lower oxidative stress conditions in older WT mice on food restriction.

The ability of CR diet in reducing the accumulations of MetO in specific brain proteins is evident from the data presented in Fig. 3, showing an example of one protein in which its MetO moiety can be reduced by the diet. Other tissues can be analyzed for the presence and reduction of methionine sulfoxide under AL diet versus CR diet using the anti-MetO antibodies; the positive effect of CR in lowering MetO content is probably not limited to brain. The data in Fig. 3 suggest that up-regulation of antioxidants during the course of the CR diet is a valid possible explanation for the prevention of brain abnormalities associated with oxidative stress in general, and methionine oxidation in particular.

Oxidative stress and methionine oxidation have been implicated in neurodegenerative movement disorders such as Parkinson's disease (PD) [2,5,17]. In PD, the DA-mediated movement pathways are disrupted. CR has been shown to improve the behavioral outcome and reduce neurodegeneration of the MPTP rodent model of this disease [6]. Furthermore, the addition of MsrA has been shown to prevent PD-like symptoms in *Drosophila* when alpha-synuclein is expressed [26]. Alpha-synuclein binds to DA, is associated with the pathogenesis of PD, and is a substrate for Msr [22,24]. However, the link between methionine oxidation, DA, and alpha-synuclein in movement disorders is still not clear. It is a plausible hypothesis that reactive oxygen species are causing modifications to methionine residues of proteins involved in DA signaling that may involve alpha-synuclein [23].

In summary, we suggest that CR reduces oxidative stress effects that are mediated by methionine oxidation. Furthermore, our studies support the concept that methionine oxidation alters protein function, which may contribute to neurological diseases associated with oxidative stress and aging.

Acknowledgments

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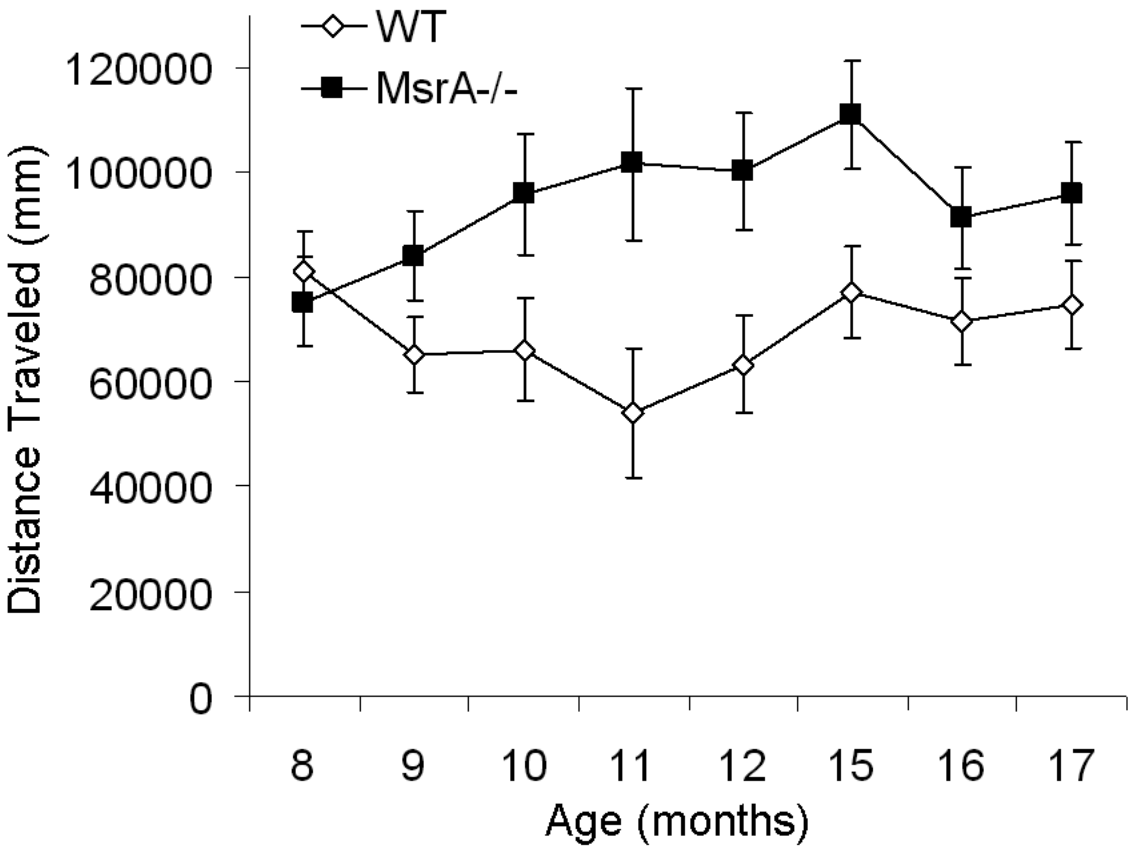


Fig. 1. Total distance traveled by *MsrA*$^{-/-}$ mice on caloric restriction diet

Mouse spontaneous locomotor activity was measured starting before feeding the CR diet (8 months). These measurements were continued at the end of each month of the CR diet. Locomotor activity was measured as total distance traveled in a force-plate actometer for 30 minutes. Values represent average distance traveled of *MsrA*$^{-/-}$ mice (*filled squares*) and WT mice (*open diamonds*). Error bars represent standard deviation. The *MsrA*$^{-/-}$ mice traveled significantly more only in the intermediate months as judged by the polynomial trend order 2 post-hoc test (P=0.661, main effect; P=0.001, month*type interaction effect).

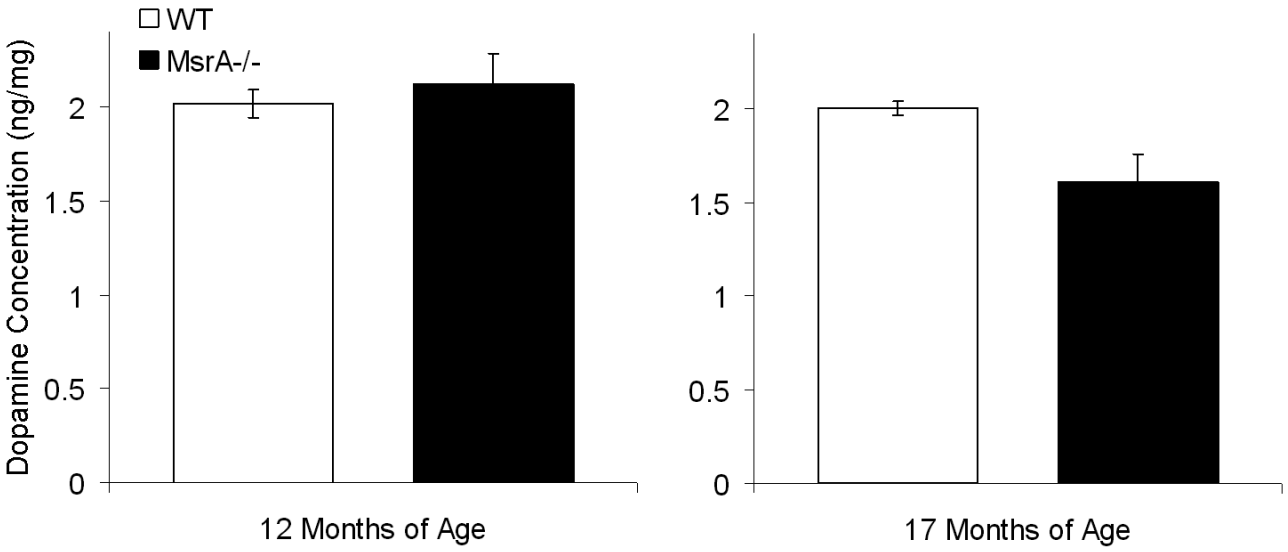
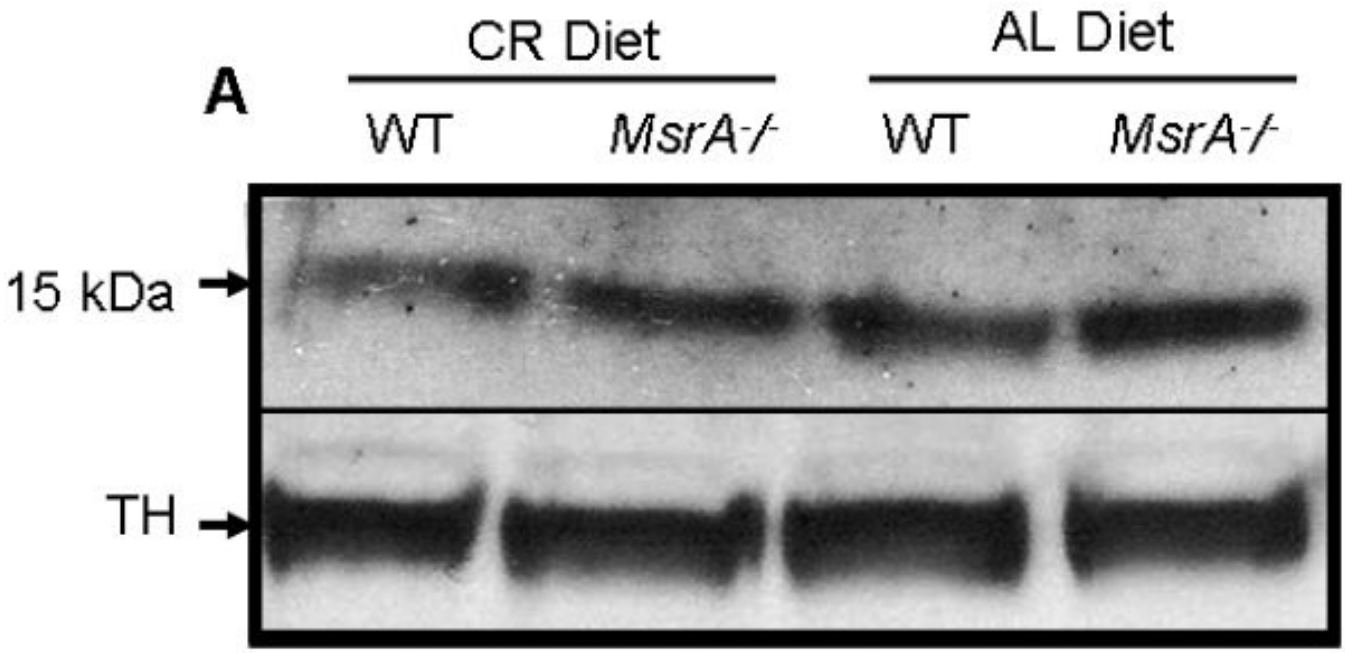


Fig. 2. Dopamine concentration in the brain tissue of *MsrA*$^{-/-}$ mice on caloric restriction diet

Cerebral DA levels were determined for *MsrA*$^{-/-}$ mice (*filled*) and WT mice (*open*) after reduced food intake as described in "Materials and Methods." Values represent the amount of DA per weight of cerebral brain tissue after four months (*left*) or after nine months (*right*) of caloric restriction diet. Error bars represent standard deviation of 6 *MsrA*$^{-/-}$ mice and 5 WT mice at four months and 5 *MsrA*$^{-/-}$ mice and 7 WT mice at nine months. P=0.597 and P=0.054, respectively, by the *student's t-test*.



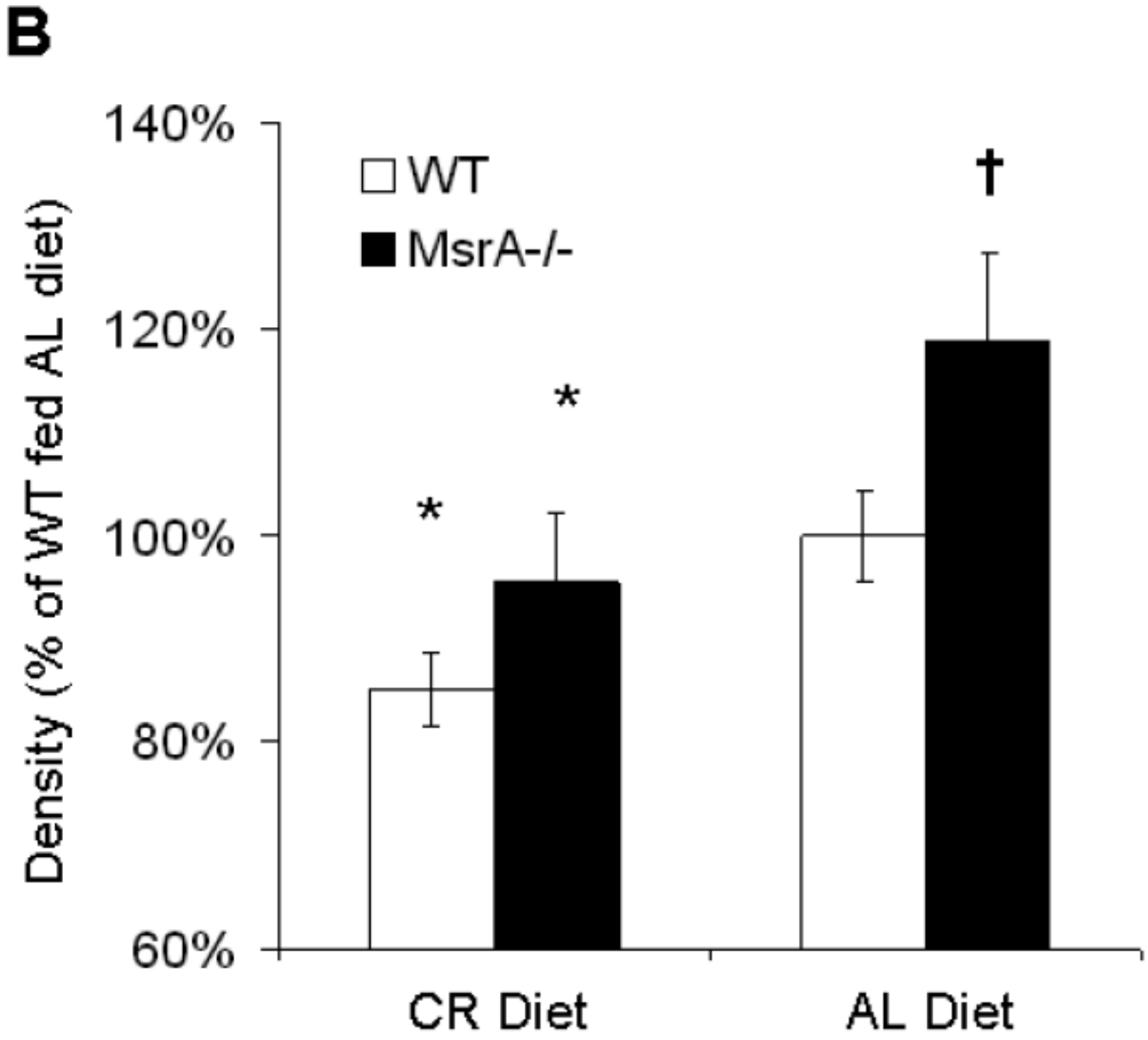


Fig. 3. Methionine sulfoxide detection in a 15 kDa protein from brain tissues of *MsrA*$^{-/-}$ and wild-type mice on caloric restriction and *ad libitum* diet

Soluble protein extracts were made from *MsrA*$^{-/-}$ and WT brain tissue of 12-month-old mice after four months of caloric restriction (CR diet) and from age-matched mice fed *ad-libitum* (AL diet). Equal protein amounts from each brain extract were separated by gel-electrophoresis followed by western blot analysis using the primary anti-methionine sulfoxide antibodies or anti-tyrosine hydroxylase (TH) antibodies, as previously described [21]. **(A)** Representative gel showing an unidentified protein with a mass of ~15kDa (mass was estimated by separated prestained molecular mass markers; data not shown). TH is shown as a loading control. **(B)** Average density of the 15 kDa protein. Density was determined by using the densitometry ImageJ (NIH). Error bars represent standard deviation of three brains per group. *P<0.05 for the effect from diet and $^{\dagger}P$<0.05 for the effect of genotype on the AL diet, *student's t-test*.

Table 1

Comparison of dopamine levels in brain tissue of *MsrA*$^{-/-}$ mice on caloric restriction diet and *ad libitum* diet

Cerebral DA levels are listed by the respective age after reduced food intake (CR diet) or on *ad-libitum* feeding (AL diet) from our previous study* [23]. Relative DA values and standard deviations are depicted as percentage of DA levels in WT control mice at 6 months of age fed on an AL diet. Variance (±) represents standard deviation of 5–7 mice per group.

Age (months)	**6**		**12**		**16–17**	
Genotype	**WT**	***MsrA*$^{-/-}$**	**WT**	***MsrA*$^{-/-}$**	**WT**	***MsrA*$^{-/-}$**
AL diet*	100±8	148±8	89±10	144±14	144±14	69±17
CR diet			90±7	94±16	89±4	72±15