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Methionine sulfoxide reductase regulates brain catechol-*O*-methyl transferase activity

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Abstract

Catechol-*O*-methyl transferase (COMT) plays a key role in the degradation of brain dopamine (DA). Specifically, low COMT activity results in higher DA levels in the prefrontal cortex (PFC), thereby reducing the vulnerability for attentional and cognitive deficits in both psychotic and healthy individuals. COMT activity is markedly reduced by a non-synonymous SNP that generates a valine-to-methionine substitution on the residue 108/158, by means of as-yet incompletely understood posttranslational mechanisms. One posttranslational modification is methionine sulfoxide, which can be reduced by the methionine sulfoxide reductase (Msr) A and B enzymes. We used recombinant COMT proteins (Val/Met^{108}) and mice (wild-type (WT) and *MsrA* knockout) to determine the effect of methionine oxidation on COMT activity and COMT interaction with Msr, through a combination of enzymatic activity and Western blot assays. Recombinant COMT activity is positively regulated by MsrA, especially under oxidative conditions, while brains of *Msr*A knockout mice exhibited lower COMT activity (as compared with their WT counterparts). These results suggest that COMT activity may be reduced by methionine oxidation, and point to Msr as a key molecular determinant for the modulation of COMT activity in the brain. The role of Msr in modulating cognitive functions in healthy individuals and schizophrenia patients is yet to be determined.

Keywords

Methionine oxidation; Catechol-O-methyltransferase; prefrontal cortex; oxidative stress; postranslation modification

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Statement of Interest:
None.

Introduction

The enzyme catechol-*O*-methyltransferase (COMT) catalyzes the *O*-methylation of catecholamine neurotransmitters, such as dopamine (DA) and norepinephrine (Axelrod & Tomchick ,1958), using S-adenosylmethionine (SAM) as a methyl donor (Männistö & Kaakkola,1999). The COMT protein occurs as two distinct isoforms with identical kinetic mechanisms, which result from differential transcriptions of the corresponding gene: a soluble form (S-COMT), found in the cell cytoplasm; and a membrane-bound form (MB-COMT), which features 50 additional residues in the *N*-terminal portion of the protein (Männistö & Kaakkola,1999; Huh & Friedhoff 1979).

Several lines of evidence have shown that COMT serves a primary role in DA degradation in the prefrontal cortex (PFC) (Karoum *et al*. 1994; Matsumoto *et al*. 2003). Given the well-documented implication of DAergic neurotransmission in the modulation of these tasks (Miller & Cohen, 2001; Cohen *et al*. 2002; Nieoullon, 2002), the functional role of COMT in the PFC has garnered substantial interest. In particular, numerous investigations have focused on rs4680, one of the best-characterized single-nucleotide polymorphisms (SNPs) of the *COMT* gene, resulting in the substitution of a valine (*Val*) for a methionine (*Met*) residue at position 108 of S-COMT and 158 of MB-COMT ($Val^{108/158}Met$) (Lachman *et al*. 1996). The *Met* allele is associated with reduced COMT activity and lower DA metabolism; this functional characteristic has been shown to lead to a more efficient physiological response in the PFC across several functional domains, including cognitive flexibility, working memory, attentional control and emotional resilience (Malhotra *et al.* 2002; Goldberg *et al*. 2003; Blasi *et al*. 2005; Smolka *et al*. 2005).

The notion that low DAergic activity in the PFC may contribute to negative and cognitive symptoms of schizophrenia patients (Davis *et al*. 1991; Kahn & Davis, 1995), has also led several authors to investigate the potential influence of the rs4680 polymorphism of COMT on the severity of these deficits in psychotic disorders. Multiple studies have ascertained that the $Met^{108/158}$ variant is associated with a slightly lower schizophrenia risk, as well as less severity of attentional, cognitive and information-processing deficits (Bray *et al*. 2003; Egan *et al*. 2001; Bilder *et al*. 2002; Gallinat *et al*. 2003; Tunbridge *et al*. 2006; Ehlis *et al*. 2007).

Previous research has shown that the $Val^{108/158}Met$ genotype affects protein expression, but not mRNA expression levels, indicating that this polymorphism may lead to post-translational changes in the protein (Matsumoto *et al*. 2003; Chen *et al*. 2004). Indeed, it has been shown that the methionine-to-valine substitution reduces the thermostability of the enzyme without affecting its structural and kinetic properties (Lachman *et al*. 1996; Lotta *et al*. 1995). Although the bases of this phenomenon are not fully elucidated, the lower catalytic activity of the $Met^{108/158}$ variant has been associated with a markedly higher susceptibility to oxidation (Cotton *et al*. 2004). The most oxidation-sensitive amino acids are the sulfur-containing methionine and cysteine. Indeed, Cotton and co-workers (Cotton *et al*. 2004) identified a key role of cysteine residues in the vulnerability of the $Met^{108/185}$ variant to oxidation; the specific role of methionine in this process, however, remains elusive. The oxidation of methionine residues leads to the formation of methionine sulfoxide (MetO); this well-known phenomenon is enhanced by oxidative stress and reversed by the methionine

sulfoxide reductase (Msr) system, consisting of two enzymes (termed A and B) that reduce either *S or R* enantiomers of MetO, respectively (Moskovitz, 2005; Moskovitz *et al*. 2000, 2002).

In this study, we hypothesized that the lower catalytic activity of the $Met^{108/158}$ variant may be also contributed by the oxidation of this and other methionine residues. To test for this possibility, we assessed whether COMT activity may be affected by Msr, using a number of complementary *in vitro* and *ex vivo* approaches.

Methods

Recombinant human COMT and MsrA proteins

Expression clones for recombinant His-tag soluble human COMT proteins (S-COMT, Val^{108} and Met^{108} forms) were kindly provided by Dr. Klinman (University of California, Berkeley). The expression and purification of the recombinant COMT proteins were performed as described by Zhang and Klinman (Zhang & Klinman, 2011). Recombinant His-tagged yeast MsrA protein was expressed and purified as previously described (Moskovitz *et al*. 1997). Oxidation of recombinant COMT proteins was performed by incubating the proteins with 200mM H_2O_2 for 24 h at room temperature. Then, the residual H_2O_2 was removed by dialysis against 25mM Tris-HCl (pH 7.4) at 4°C.

Animals

Wild type (WT) and *MsrA* knockout (KO) mice (n=10/group) were obtained as previously reported (Moskovitz *et al*. 2001). Animals were housed in group cages with *ad libitum* access to food and water. The room was maintained at 22°C, on a 12 h: 12 h light/dark cycle. Experimental procedures were in compliance with the accepted National Institute of Health guidelines (such as “Guiding principles in the care and use of animals” (DHEW Publications, NIH, 80-23) and approved by the Animal Use Committees of the University of Kansas.

Quantification of COMT levels in brains of WT and MsrA KO mice

Mice were euthanized at 6 and 12 months of age, by CO_2 asphyxiation followed by cervical dislocation (n=5 per age group). The brains were dissected within 2 minutes of euthanasia and frozen on dry ice. The tissues were stored in −80°C until use. Tissues were homogenized using a Teflon homogenizer in the presence of 25mM Tris-HCl (pH 7.4) and protease inhibitors cocktail (Roche Applied Science, Branford, CT) at 4°C. Following centrifugation at 10,000 x g for 20 minutes, the supernatants were collected and their protein concentrations were assessed using a Bio-Rad (Hercules, CA) assay kit. Equal protein amounts were subjected to SDS-gel electrophoresis followed by western blot analyses using anti-COMT mouse antibodies (BD Biosciences, San Jose, CA) as the primary antibodies. HRP-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology, Dallas, TX) were used as the secondary antibodies. Anti-ß-actin mouse antibodies (Abcam, Cambridge, MA) were also used on the same blot as primary antibodies (after stripping the blot) for the assessment of protein loading control. Following exposure of the blot to an X-ray film, the

resulting protein band images were quantified using the Image-J program (National Institute of Health).

COMT *mRNA* levels in brains of both mouse genotypes were determined by real-time RTPCR (using primers 1418701 and 144918, Affymetrix, Santa Clara, CA).

Statistical analyses

Normality and homoscedasticity of data distribution were verified by using Kolmogorov-Smirnov and Bartlett's tests. Non-parametric data were normalized by logarithmic transformation. Statistical analyses were performed using ANOVAs, followed by Newman-Keuls test for *post-hoc* comparisons. Significance threshold was set at 0.05. All statistical analyses were performed by STATISTICA 7 (Statsoft, Tulsa, OK).

Results

Effect of MetO reduction on recombinant COMT activity

To determine the combined effect of the *rs4680* polymorphism and methionine oxidation on S-COMT activity, we monitored the activity of purified His-tagged human recombinant COMT of Val^{108} (native) and Met^{108} forms. First, we analyzed the activity of the recombinant enzymes in the presence of 2mM DTT, a reducing agent that maintains cysteine residues in their reduced form (Fig. 1*a*). Under these conditions, the mean activity (± SEM) of the Met^{108} COMT variant was 69 ± 7.72% of the native form ($P<0.001$) (Fig. 1*a*). Increasing the DTT concentration to 20mM caused a significant increase in the activity of both COMT forms ($P<0.001$) (Fig. 1*a*), without affecting the activity gap between them (Fig.1*a*). The addition of recombinant MsrA in the presence of 20mM DTT further increased the activities of both forms of S-COMT ($P<0.05$), and ablated their difference in catalytic activity (Fig. 1*a*), suggesting that COMT activity is negatively affected by methionine oxidation *in vitro*, especially in the Met^{108} form. To further elucidate the possible negative effect of oxidizing COMT methionine residues (including Met^{108}) on enzyme activity, both versions of the recombinant S-COMT were exposed to high concentrations of H_2O_2 prior to monitoring COMT activity. As shown in Fig. 1*b*, COMT activity was reduced in the Met^{108} variant in comparison to the Val^{108} variant, similar to the levels observed under non-oxidizing conditions, in the presence of DTT only. However, although the addition of recombinant MsrA caused significant increases in the activities of both COMT variants ($P<0.001$) (Fig. 1*b*), there was a significant gap between the two COMT variants. These data suggest that, under severe oxidative conditions *in vitro*, the difference in activity between Val^{108} and Met^{108} forms is not fully rescued.

Genetic ablation of MsrA leads to significant reduction of COMT activity in the mouse brain

To test the relevance *in vivo* of the relation between MsrA and COMT, we measured the activity of the latter enzyme in the brains of *MsrA* KO mice, as compared with WT counterparts. The primary sequences of murine and human COMT share sequence homology for 5 methionine residues. The mouse protein contains additional methionine residues at positions 93 and 244 (Table 1), and features a leucine residue (a hydrophobic

amino acid like valine and methionine) in the position 151, homologue to 108/158 in human COMT (Table 2).

The brains of MsrA KO mice exhibited a marked reduction in COMT activity [Main genotype effect: $F(1,24)=175.05$, $P<0.001$] (Fig. 2*a*). The increase of DTT concentration to 20mM produced a significant enhancement in COMT activity in both WT and KO mice (173% and 132% in comparison to WT and *MsrA* KO treated with 2 mM DTT, respectively; Main treatment effect: $F(1,24)=105.46$, $P<0.001$ in comparison with 2 mM DTT). Finally, the addition of recombinant MsrA protein to the assay reaction (in the presence of 20mM DTT) further significantly increased COMT activity across both genotypes (*Ps*<0.001 compared with both 2 mM and 20 mM DTT (Fig. 2*a*). Notably, ANOVA failed to identify a significant genotype x treatment interaction, indicating that the treatment with DTT and MsrA did not abrogate the difference between genotypes.

We then analyzed whether the differences in COMT activity between WT and *MsrA* KO brains may be influenced by aging (Fig. 2*b*). While ANOVA found significant main effects for genotype [$F(1,16)=47.40$, $P<0.001$] and age [$F(1,16)=32.64$, $P<0.001$], no significant interaction between these two effects was found, indicating that the age-dependent decline in COMT activity was not dependent on *MsrA* genotype.

To confirm that the observed difference in COMT activity between the two mouse genotypes is not due to a difference in COMT mRNA/ protein expression levels, COMT protein and *mRNA* levels were determined in brains of both WT and *MsrA* KO mice. Accordingly, western blot analysis and real-time PCR determination of COMT's *mRNA* levels were performed. As shown in Fig. 3, the protein levels of COMT were equivalent across both mouse genotypes. Complementary to these data, there was no significant difference in the *mRNA* levels between the two mouse genotypes (data not shown).

Discussion

The findings of this study converge in support of a regulatory role of Msr on COMT activity in the brain, and, more specifically, the PFC. We documented that MsrA countered the reduction of COMT activity induced by oxidation; this effect was not only limited to the Met^{108} variant, but targeted also other methionine residues, as suggested by the ability of MsrA to enhance the catalytic activity of the Val^{108} variant (Fig. 1). In addition, we found that in the mouse brain, COMT activity was reduced by genetic MsrA deficiency and increased by addition of recombinant MsrA; nevertheless, the latter intervention did not fully rescue the deficits of COMT activity in *MsrA* KO mice. This phenomenon suggests that in these mutants, COMT may feature posttranslational modifications that may interfere with the reduction of *R*-MetO residues (Fig. 2). Supportive evidence for the involvement of posttranslational modifications to the observed reduced COMT activity in *MsrA* KO mice arises from the similar expression levels of COMT's mRNA (data not shown) and protein (Fig. 3) in both mouse strains.

These results suggest that COMT activity may be reduced by the oxidation of its methionine residues, and complements previous evidence documenting the implication of cysteine (the

other sulfur-containing amino acid) in the higher susceptibility of the $Met^{108/158}$ variant to oxidation (Cotton *et al*. 2004). Previous data have shown that methionine oxidation causes conformational changes (Berlett *et al*. 1996) and increases the hydrophobicity of proteins (Chao *et al*. 1997). Interestingly, the increase in hydrophobicity due to MetO formation has also been shown to enhance the proteolytic susceptibility of proteins (Levine *et al*. 1996), suggesting that the $Met^{108/158}$ variant may be more vulnerable to protease-mediated degradation. Accordingly, the $Met^{108/158}$ variant exhibits 20% of its activity at physiological temperature; this phenomenon may be due to the observed reduction in the protein expression level (possibly because of its enhanced degradation rate), but not mRNA expression level in brain tissue (Matsumoto *et al*. 2003; Chen *et al*. 2004; Lotta *et al*. 1995). The robust association between Msr and COMT is particularly noteworthy, in view of the primary role of COMT in the regulation of DA homeostasis in the PFC (Matsumoto *et al*. 2003; Gogos *et al*.1998). High COMT activity, such as that associated with the $Val^{108/158}$ variant, leads to increased DA turnover, which may result in a higher predisposition for a number of information-processing, attentional, executive and cognitive deficits in patients with psychosis, as well as healthy individuals (Kahn & Davis, 1995; Weinberger *et al*. 2001, 2002; Barnett *et al*. 2007; Ira *et al*. 2013). Furthermore, it should be noted that alterations in oxidative stress in the PFC have been associated with multiple psychiatric disorders, including schizophrenia, bipolar disorder and major depression (Michel *et al*. 2007; Gawryluk *et al*. 2011; Andreazza *et al*. 2013), as well as cognitive and motivational deficits in mouse models (Johnson *et al*. 2013). These premises highlight the potential importance of Msr as a moderator of COMT function with respect to these functional domains regulated by the PFC. Future studies are needed to test this interesting hypothesis.

Previous studies have shown that *MsrA* KO mice exhibit high levels of DA in the brain (and, particularly, in the striatum), raising the possibility that the reduction in COMT activity may be partially responsible for such changes. Nevertheless, the contribution of COMT is unlikely to fully account for the enhancement of DA levels in *MsrA* KO mice; indeed, *COMT* KO mice only featured increases in DA in the PFC, but not in the striatum (Gogos *et al*.1998).

One of the main limitations of this study is that our observations were based on *S*-COMT, which is posited to play a relatively less important role in DA degradation in comparison with MB-COMT. Even with this limitation, the identification of a link between Msr and COMT activity points to the potential importance of methionine oxidation in the modulation of prefrontal activity and the pathophysiology of cognitive impairments of schizophrenia. Future clinical studies are warranted to test the implication of Msr in the function of COMT, particularly with respect to the regulation of dopamine neurotransmission and pathophysiology of schizophrenia.

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Abbreviations

COMT	Catechol-O-methyltransferase
DA	dopamine
WT	wild-type
KO	knockout
Msr	Methionine sulfoxide reductase
PFC	prefrontal cortex
SAM	S-adenosylmethionine
MetO	Methionine sulfoxide
DTT	dithiothreitol

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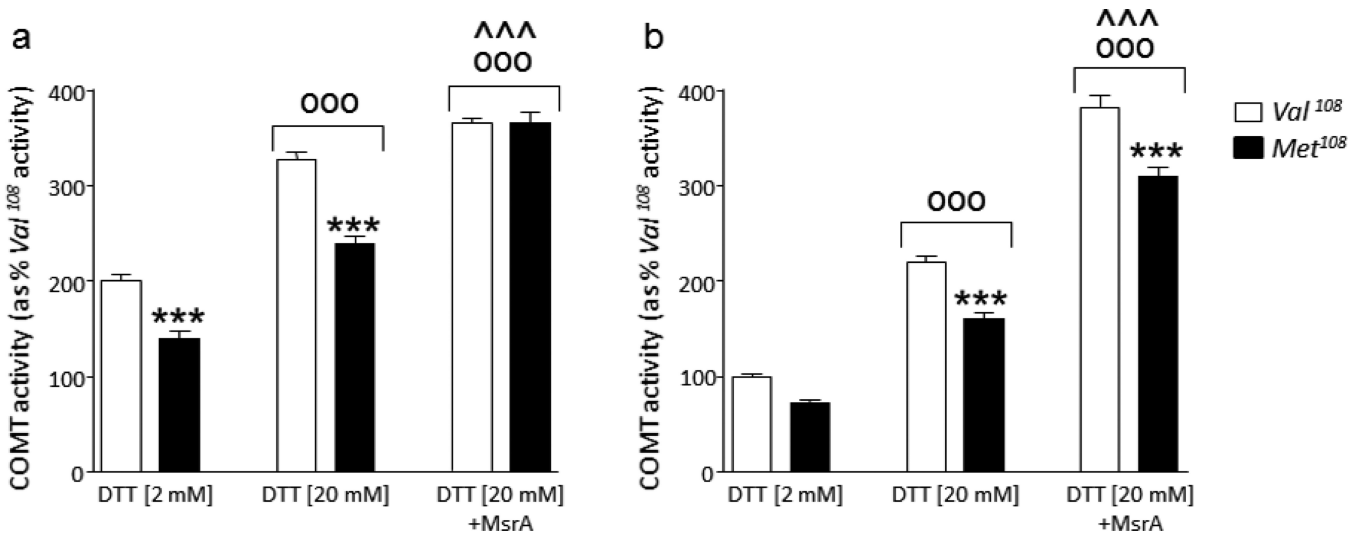

Figure 1.

Effects of dithiothreitol (DTT) and MsrA on the activities of COMT Val^{108} and Met^{108} variants in vitro under (*a*) standard conditions and (*b*) high H_2O_2 concentrations. Values are displayed as means ± SEM. All analyses were run by 2-way ANOVAs, followed by Newman-Keuls test for post-hoc comparisons. ***, $P<0.001$ for comparisons vs corresponding Val^{108} (genotype x treatment interaction); °°°, $P<0.001$ for comparison vs 2mM DTT (Main treatment effect); ^^^, $P<0.01$ for comparison vs 20mM DTT (Main treatment effect). Main genotype effects are not shown.

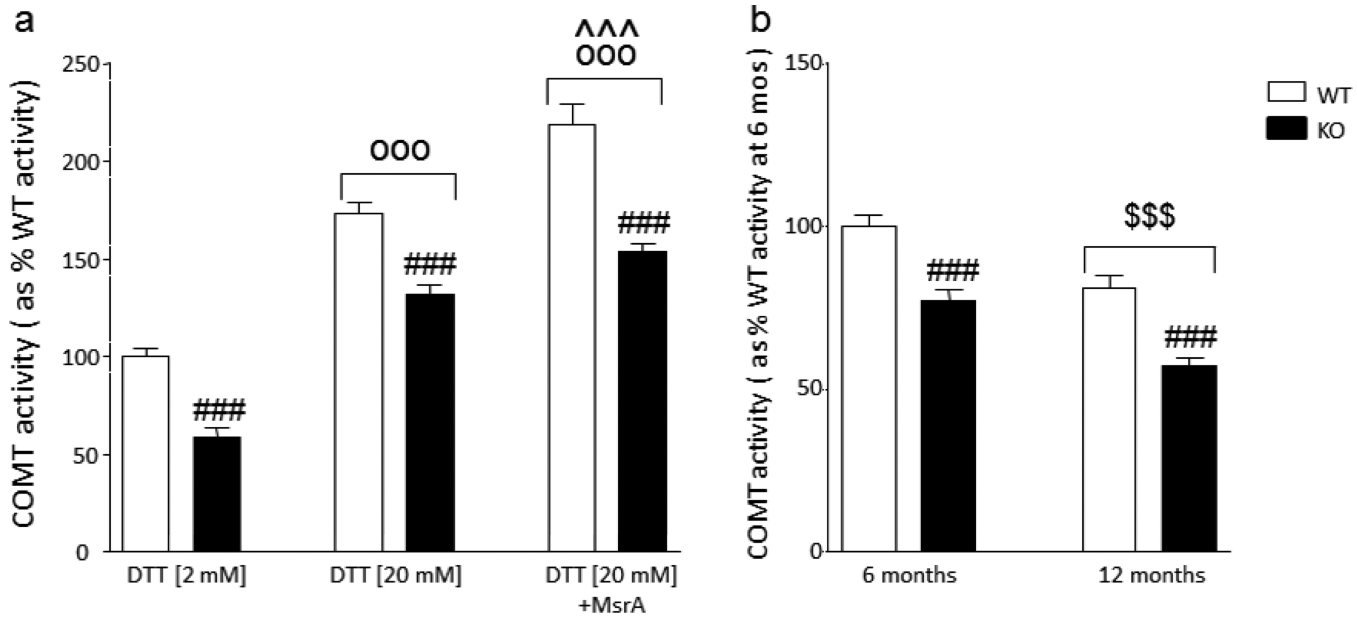

Figure 2.

(*a*) Effects of DTT and MsrA on the brain activity of COMT in WT and *MsrA* KO mice, and (*b*) Effects of aging on COMT activity in WT and MsrA KO mice. Values are displayed as means ± SEM. All analyses were run by 2-way ANOVAs, followed by Newman-Keuls test for post-hoc comparisons. $^{###}$, $P<0.001$ for comparisons vs corresponding WT group (Main genotype effect); °°°, $P<0.001$ for comparison vs 2mM DTT (Main treatment effect); ^^^, $P<0.01$ for comparison vs 20mM DTT (Main treatment effect); $^{\$\$\$}$, $P<0.001$ for comparison vs 6 months (Main age effect).

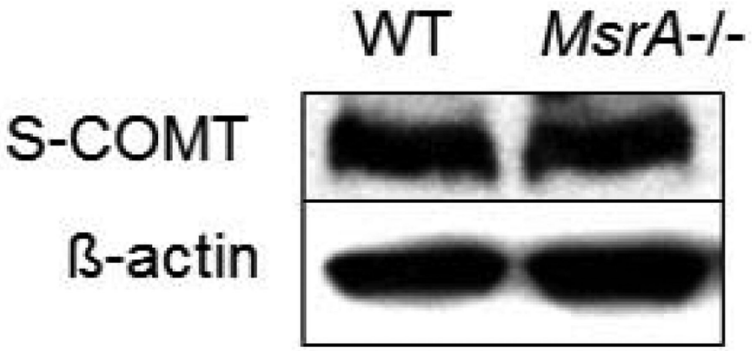

Figure 3.

Representative blots from Western blot analyses (*n*=5) showing COMT expression in comparison with *β*-actin expression in brain samples from WT and *MsrA* KO mice.

Table 1

Protein sequence homology alignment between mouse and human catechol-O-methyl transferase (COMT). *Met* (M) residues are in bold and ^{158} *Val* of the human COMT is both in bold and underlined

Mouse	20	RHLGWGLVAIGWFEFVQQPVHNLLMGGTKEQRILRHVQQHAKPGDPQSVLEAIDTYCSEK RH GWGL IGW EF+QP+HNLLMG TKEQRIL HV QHA+PG+QSVLEAIDTYC+K	79
Human	27	RHWGWGLCLIGWNEFILQPIHNLLMGDTKEQRILNHVLQHAEPGNAQSVLEAIDTYCEQK	86
Mouse	80	EWAMNVGDAKGQIMDAVIREYRPSLVLELGAYCGYSAVRMARLLPPGARLLTMEINPDYA EWAMNVGD KG+I+DAVI+E++PS++LELGAYCGYSAVRMARLL PGARL+T+EINPD A	139
Human	87	EWAMNVGDKKGKIVDAVIQEHQPSVLLELGAYCGYSAVRMARLLSPGARLITIEINPDCA	146
Mouse	140	AITQQMLDFAGLQDKVSILIGASQDLIPQLKKKYDVDTLDMVFLDHWKDRYLPDTLLLEE AITQ+M+DFAG++DKV++++GASQD+IPQLKKKYDVDTLDMVFLDHWKDRYLPDTLLLEE	199
Human	147	AITQRMVDFAG VKDKVTLVVGASQDIIPQLKKKYDVDTLDMVFLDHWKDRYLPDTLLLEE	206
Mouse	200	CGLLRKGTVLLADNVIVPGTPDFLAYVRGSSSFECTHYSSYLEYMKVVDGLEKAVYQGPG CGLLRKGTVLLADNVI PG PDFLA+VRGSS FECTHY S+LEY+VVDGLEKA+Y+GPG	259
Human	207	CGLLRKGTVLLADNVICPGAPDFLAHVRGSSCFECTHYQSFLEYREVVDGLEKAIYKGPG	266
Mouse	260	SS S	261
Human	267	SE	268