Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2009 | 111 | 101–110 doi: 10.1111/j.1471-4159.2009.06300.x

Serine 209 resides within a putative p38(MAPK) consensus motif and regulates monoamine oxidase-A activity

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Abstract

The p38 mitogen-activated protein kinase (MAPK) cascade as well as the enzyme monoamine oxidase-A (MAO-A) have both been associated with oxidative stress. We observed that the specific inhibition of the p38(MAPK) protein [using either a chemical inhibitor or a dominant-negative p38(MAPK) clone] selectively induces MAO-A activity and MAO-A-sensitive toxicity in several neuronal cell lines, including primary cortical neurons. Over-expression of a constitutively active p38(MAPK) results in the phosphorylation of the MAO-A protein and inhibition of MAO-A activity. The MAO-A(Ser209Glu) phosphomimic – bearing a targeted substitution within a putative p38(MAPK) consensus motif – is neither active nor neurotoxic. In contrast, the MAO-A(Ser209Ala) variant (mimics dephosphorylation) does not associate with p38(MAPK), and is both very active and very toxic. Substitution of the homologous serine in the MAO-B isoform, i.e. Ser200, with either Glu or Ala does not affect the catalytic activity of the corresponding over-expressed proteins. These combined *in vitro* data strongly suggest a direct p38(MAPK)-dependent inhibition of MAO-A function. Based on published observations, this endogenous means of selectively regulating MAO-A function could provide for an adaptive response to oxidative stress associated with disorders as diverse as depression, reperfusion/ischemia, and the early stages of Alzheimer's disease.

Keywords: Alzheimer's disease, apoptosis, calcium, depression, mitochondrial permeability transition, phosphorylation, post-translation.

J. Neurochem. (2009) **111**, 101–110.

In the 1950s, the anti-tuberculosis drug, iproniazid, was found to exert mood-elevating effects by inhibiting monoamine oxidase [MAO; amine:oxygen oxidoreductase (deaminating) (flavin containing); EC 1.4.3.41]. The link between MAO and the synaptic depletion of biogenic neurotransmitters, particularly noradrenaline and serotonin, formed the neurobiological basis of depression (Coppen 1967). Both isoforms of MAO (i.e. MAO-A and MAO-B) produce NH_3 and H_2O_2 as reaction by-products (Bortolato *et al.* 2008) and these products are implicated in the oxidative stress associated with neurodegenerative processes including Alzheimer's disease (AD) (Crack *et al.* 2006) and Parkinson's disease (Jenner and Olanow 1996). Parkinson's disease centers on the H_2O_2/MAO-B-mediated disruption of dopaminergic function (Youdim *et al.* 2006; Czerniczyniec *et al.* 2007). In contrast, any contribution of MAO-A to neurodegeneration is often overshadowed by its established contribution to major depression.

There is a vast literature regarding the role of MAO-A in depression and it has recently been demonstrated that even a modest increase (~30%) in MAO-A protein in several brain regions is unequivocally associated with clinical depression (Meyer *et al.* 2006). Depression is clearly linked to cognitive impairment and to increased risk for AD (Rapp *et al.* 2006;

Received March 20, 2009; revised manuscript received July 2, 2009; accepted July 15, 2009.

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Abbreviations used: Δψm, mitochondrial membrane potential; 5-HT, 5-hydroxytryptamine; AD, Alzheimer's disease; CLG, clorgyline; HEK, human embryonic kidney; MAO, monoamine oxidase; MAPK, mitogen-activated protein kinase; p38(MAPK)-AF, active form of p38(MAPK); p38(MAPK)-DN, dominant-negative form of p38(MAPK); PEA, β-phenylethylamine; WT, wildtype.

Geerlings *et al.* 2008). It is not surprising, therefore, that MAO-A dysfunction is also a potential risk factor for AD (Emilsson *et al.* 2002; Takehashi *et al.* 2002; Nishimura *et al.* 2005) and that MAO-A inhibitors are efficacious in treating depression and cognitive disorders in the elderly (Malorni *et al.* 1998), or that they can protect against various cell stressors including AD-related β-amyloid peptides (Cao *et al.* 2007).

It is known that Ca^{2+} selectively enhances MAO-A activity in hippocampal HT-22 cells (Cao *et al.* 2007) and that the bacterial-derived toxin staurosporine can induce MAO-A-sensitive apoptosis in neuronal SH-Sy5y cells (Fitzgerald *et al.* 2007). Both of these effects occur independently of any change in *mao-A* mRNA expression and evidence provided by the latter authors suggests a possible post-translational modification of MAO-A by the p38 mitogen-activated protein kinase (MAPK) pathway (Fitzgerald *et al.* 2007). Interestingly, activation of the p38(MAPK) pathway, associated primarily with cellular stress, has been linked to induction of *mao-A* mRNA and an apoptotic phenotype in PC12 and SK-N-BE(2)-C cells (De Zutter and Davis 2001; Ou *et al.* 2006). While induction of *mao-A* mRNA can parallel an increase in MAO-A activity, for example in human brain (Mousseau *et al.* 1997), this is not always the case (Fowler *et al.* 2007). These combined data support cell type-dependent regulation of MAO-A function at both the mRNA and protein level, and implicate the p38(MAPK) pathway in these events. One must consider, however, that activation of the p38(MAPK) pathway using various external stressors (De Zutter and Davis 2001; Ou *et al.* 2006; Fitzgerald *et al.* 2007) would inevitably activate all of the kinases upstream of p38(MAPK), any one of which could initiate multiple signaling events. So as to better define the specific role of p38(MAPK) in the regulation of MAO-A function, we chose to focus on the p38(MAPK) protein itself using targeted pharmacological as well as molecular approaches. We compared and contrasted our results in several CNS-derived cell lines so as to exclude the possibility that our newly described mechanism is specific to a single clonal cell line. We now provide the first evidence for a potential selective, phosphorylation-dependent mechanism for the regulation of MAO-A function and clearly implicate the p38(MAPK) protein in the process.

Experimental procedures

Reagents, antibodies, and expression vectors

5-Hydroxytryptamine (5-HT), β-phenylethylamine (PEA), and the phospho-serine, phospho-MAPKAPK-2 (MAPK-activated protein kinase-2), and β-actin antibodies were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada). [^{14}C]-5-HT (NEC-225) and [^{14}C]-PEA (NEC-502) were purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). MAO-A and myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho- and total-p38α antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA), while IgG-horseradish peroxidase conjugates were from Cedarlane Laboratories Ltd. (Burlington, ON, Canada). pEBG-p38(MAPK)α was a gift from Dr B. Zanke (Cross Cancer Institute, Edmonton, AB, Canada). Substitution of serine 209 (Ser209 or S209) in human MAO-A and of the homologous Ser200 in MAO-B with alanine (Ala or A) or glutamic acid (Glu or E) was accomplished using targeted mutagenesis of the corresponding human cDNA (QuikChange® Mutagenesis system; Stratagene, La Jolla, CA, USA) and isoform-specific PCR primer pairs.

Clonal, immortalized, and primary cell cultures

The rat PC12 pheochromocytoma (CRL-1721), the mouse N2a neuroblastoma (CCL-131), and the human embryonic kidney (HEK293) (CRL-1573) clonal cell lines were maintained according to American Type Culture Collection (Manassas, VA, USA) specifications. The immortalized mouse hippocampal HT-22 cell line was kindly provided by Dr P. Maher (The Scripps Research Institute, La Jolla, CA, USA).

Primary cortical neuronal cultures were prepared as described previously (Cao *et al.* 2007) from embryonic day 20 (E20) tissue isolated from time-pregnant dams (Sprague–Dawley; Charles River, St Constant, QC, Canada). Dams were housed in accordance with the University Committee on Animal Care and Supply and the Canadian Council for Animal Care guidelines. Primary cultures were treated on day 7.

Monoamine oxidase activity

Monoamine oxidase-A and -B activities (nmol/h/mg protein) were estimated using 250 μM [^{14}C]-5-HT (for MAO-A activity) or 50 μM [^{14}C]-β-PEA (for MAO-B activity) (Holt and Baker 1996). Ca^{2+}-sensitive MAO-A activity was tested by adding 1 mM Ca^{2+} to the reaction mixture (Cao *et al.* 2007).

Determination of mRNA levels by semi-quantitative reverse transcriptase–PCR

Total RNA was isolated (TRIZOL™; GIBCO-BRL, Burlington, ON, Canada) and reverse-transcribed (SuperScript™III First-Strand Synthesis System; Invitrogen, Burlington, ON, Canada). Target genes were amplified using *Taq* polymerase and specific primer pairs (*mao-A*: forward, 5′-GAAGCTGAGCTCTCCTGTTAC-3′ and reverse, 5′-ACAAAGCAGAGAAGAGCCAC-3′; *mao-B*: forward, 5′-GCTGAAGAGTGGGACTACATGAC-3′ and reverse, 5′-GGAATGAACCTTGGGAGGTG-3′; *β-actin*: forward, 5′-TAGAAGCATTTGCGGTGCACG-3′ and reverse, 5′-TGCCCATCTATGAGGGTTACG-3′). Agarose gel-electrophoresed PCR products were visualized by ethidium bromide staining.

Transient transfections

Cells in log phase were transfected with plasmid DNA (i.e. 1–2 μg/well on a 24-well plate; seeded at 5×10^5 cells/well) using ExGen™500 (Fermentas, Burlington, ON, Canada) according to the manufacturer's directions. Expression of eGFP (enhanced Green Fluorescent Protein) revealed a transfection efficiency of ∼50%. Cells were routinely treated 24 h post-transfection.

Immunodetection and immunoprecipitation of target proteins

Standard protein extraction and sodium dodecyl sulfate–polyacrylamide gel electrophoresis immunoblot conditions were used to detect proteins in total cell lysates (pre-cleared; 5000 *g*, 10 min, 4°C; 20–30 μg/lane) or in immunoprecipitates (300–500 μg: pre-cleared using non-specific IgG) isolated with target-specific antibodies and precipitated with protein-A/G sepharose. Immunoblots are representative of two to three individual experiments.

Cell viability assays

Changes in the levels of cytoplasmic peroxy radicals were visualized using $DCFH_2$-DA (dichlorodihydrofluorescein diacetate) fluorescence (Molecular Probes, Eugene, OR, USA) (Cao *et al.* 2007). Mitochondrial membrane potential ($\Delta\psi_m$, which is reduced during apoptosis) was determined using the ratio of red (cytoplasmic) to green (mitochondrial) JC-1 fluorescence (Cell Technology Inc., Mountain View, CA, USA) and Hoechst 33258 staining in primary neuronal cultures ($n \geq 3$) was used to visualize chromatin condensation, another characteristic of apoptosis (Cao *et al.* 2007). Experimental means represent five or more individual experiments, each representing four to six replicates per test group.

Statistical analyses

Significance (set at $p < 0.05$) was assessed by unpaired *t*-test or by one-way ANOVA with *post hoc* analyses relying on Bonferroni's Multiple Comparison Test (GraphPad Prism v3.01; GraphPad Software Inc., San Diego, CA, USA). Data are represented as mean ± SEM.

Results

MAO-A activity and levels of phospho-p38(MAPK) are inversely related

In our ongoing characterization of the contribution of post-translational modification of MAO-A to cell function, we screened several cell lines and observed that the different levels of MAO-A activity in HEK293A and HT-22 cell lines do not reflect the expression of endogenous MAO-A protein in the respective cell line (Fig. 1a and b). Given the reported role for p38(MAPK) in MAO-A function, we examined the activation (phosphorylation) of p38(MAPK) in corresponding lysates and observed that MAO-A activity is inversely related with the phosphorylation of p38(MAPK) in these cell lines (Fig. 1a and b).

For the remainder of this series of experiments, unless otherwise stated, we used the HT-22 cell line (high MAO-A activity: 107 nmol/h/mg protein) to study the effect of treatment on *endogenous* MAO-A activity and we used the HEK293A cell line (very low MAO-A activity: 8 nmol/h/mg protein) when characterizing an over-expressed MAO-A variant.

Treatment with the p38(MAPK) inhibitor SB203580 increased MAO-A activities in both the HEK293A (Fig. 1c) and HT-22 cell lines (Fig. 1d), and revealed Ca^{2+}-sensitive MAO-A activity in HT-22 cells. These data suggest that p38(MAPK) inhibits MAO-A activity.

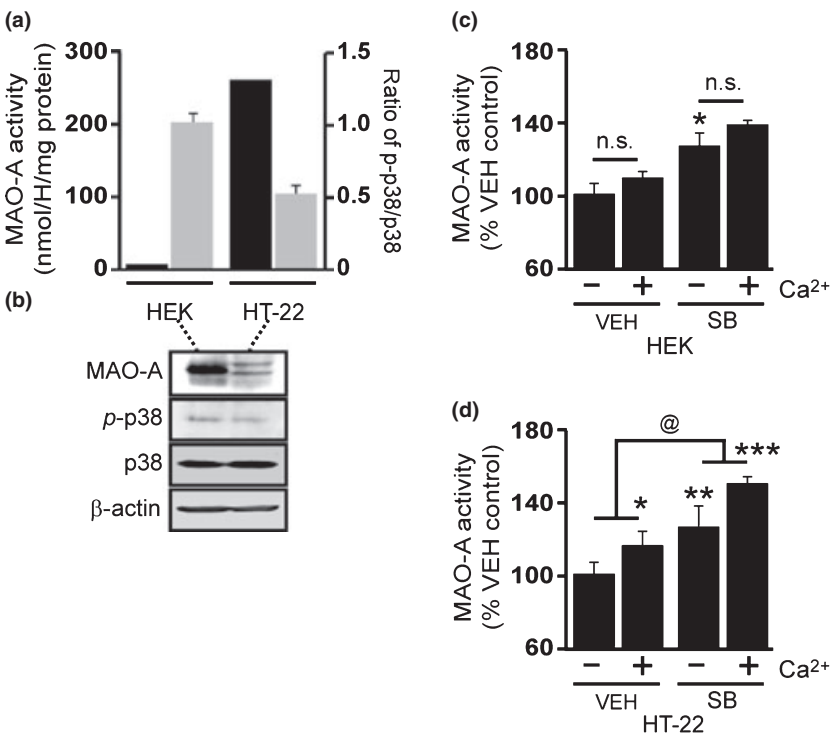

Fig. 1 MAO-A activity is inversely related with p38(MAPK) activation. (a) Comparison of MAO-A activities (black bars) and the ratio of phosphorylated (p-p38) to total p38(MAPK) (gray bars) in HEK293A cells and HT-22 cells ($n \geq 3$). (b) The expression of endogenous MAO-A and p38(MAPK) proteins in corresponding cell lysates was determined by western blot analysis. Levels of β-actin were used to demonstrate equal protein loading. Basal and Ca^{2+}-sensitive MAO-A activities (expressed as percentage of vehicle-treated control; VEH) were tested following treatment of (c) HEK293A cells [$F_{(3,12)} = 10.94$, $p = 0.0009$] and (d) HT-22 cells [$F_{(3,12)} = 29.67$, $p < 0.0001$] with the specific p38(MAPK) inhibitor SB203580 (SB; 10 μM, 1 h); $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus VEH control or between indicated groups; $^{@}p < 0.05$ between indicated groups; n.s., no significant difference.

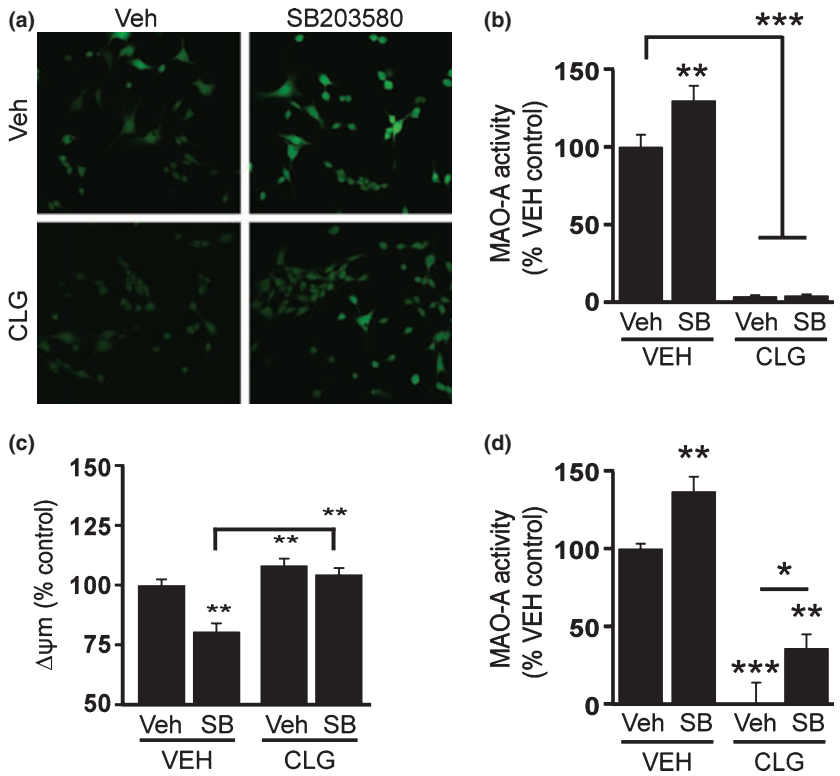

Fig. 2 Pharmacological inhibition of p38(MAPK) is toxic to neuronal cells. Neuronal cell lines were treated with the specific p38(MAPK) inhibitor SB203580 (SB; 10 μM, 1 h) and/or the selective MAO-A inhibitor clorgyline (CLG; 1 μM, 1 h). Neurotoxicity was determined by (a) changes in peroxy radical/H_2O_2-sensitive DCFH2-DA fluorescence in HT-22 cells, and (c) changes in mitochondrial membrane potential ($\Delta\psi_m$; quantitated using the JC-1 stain) in N2a cells [$F_{(3,8)} = 17.03$, $p = 0.0008$]. MAO-A activities were determined in corresponding (b) HT-22 [$F_{(3,21)} = 707$, $p < 0.0001$] and (d) N2a [$F_{(3,19)} = 40.03$, $p < 0.0001$] homogenates; $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus vehicle control or between indicated groups.

Chemical inhibition of p38(MAPK) induces MAO-A-sensitive toxicity in immortalized and clonal neuronal cultures

Treatment with SB203580 increased the generation of peroxy radicals in HT-22 cells (Fig. 2a) and induced, as expected (see Fig. 1d), MAO-A activity (Fig. 2b). Pre-treatment with the selective MAO-A inhibitor, clorgyline (CLG; 1 μM, 60 min), reversed the SB203580-induced generation of peroxy radicals (Fig. 2a) and inhibited MAO-A catalytic activity in corresponding cell homogenates (Fig. 2b). Similarly, SB203580 treatment resulted in the reduction of $\Delta\psi_m$ in N2a cells (such a reduction is an early indicator of apoptosis) (Fig. 2c) and induced MAO-A activity (Fig. 2d). These effects of SB203580 in N2a cells were also sensitive to pre-treatment with CLG (Fig. 2c and d). Finally, the SB203580-induced increase in the number of apoptotic nuclei (determined using chromatin condensation as a measure) in primary neuronal cultures was also CLG-sensitive (Fig. 3). These combined data indicate that inhibition of p38(MAPK) promotes MAO-A-dependent neurotoxicity and that this is neither specific to a particular neuronal cell preparation nor is it an artifact of any individual means of determining cell viability.

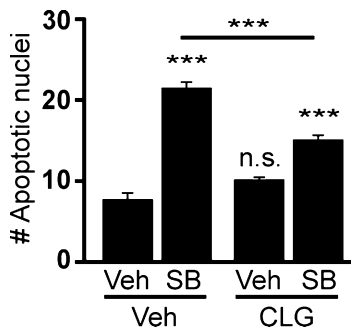

Fig. 3 SB203580 induces CLG-sensitive toxicity in primary neuronal cultures. Chromatin condensation (based on Hoechst 33258 staining) was examined on day 7 of culture of E20 primary cortical neurons treated with SB203580 (SB; 10 μM, 1 h) and/or CLG (1 μM, 1 h) [$F_{(3,17)} = 75.34$, $p < 0.0001$]; $^{***}p < 0.001$ versus vehicle control or between indicated groups; n.s., no significant difference.

Over-expression of a constitutively active p38(MAPK)α exerts cell line-dependent effects on mao-A mRNA expression and MAO-A activity

As the specificity of any chemical inhibitor is not absolute, we chose to confirm the role of p38(MAPK)α in the regulation of MAO-A function using a constitutively active form of p38(MAPK) [i.e. p38(MAPK)-AF; bearing a T180E/Y182D substitution within the TGY dual phosphorylation motif] as well as a catalytically inactive, dominant-negative form [i.e. p38(MAPK)-DN: bearing a T180A/Y182F substitution].

The activity of over-expressed p38(MAPK)-AF in PC12 cells was confirmed by the phosphorylation of the downstream target, MAPKAPK-2 (Fig. 4a). Over-expression of p38(MAPK)-AF resulted in a significant (and selective)

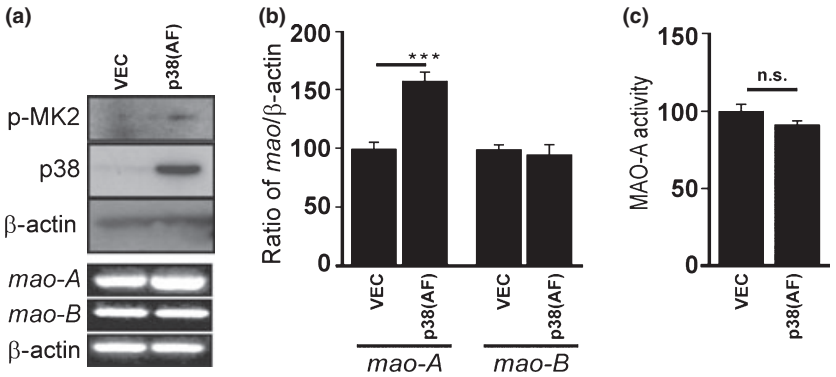

Fig. 4 Over-expression of a constitutively active p38(MAPK) increases *mao-A* mRNA expression, but not MAO-A catalytic activity, in PC12 cells. (a; upper panels) PC12 cell protein expression of the transduced activated form of p38(MAPK) [p38(AF)] and the phosphorylation of its downstream target, MAPKAPK-2 (MK2), were examined by western blot analysis. (a; lower panels) The effect of over-expression of p38(AF) on *mao-A* and *mao-B* mRNA expression in PC12 cultures were examined by RT-PCR and (b) expressed relative to the housekeeping gene *β-actin* (*mao-A*: $p = 0.0001$, $t = 15.33$, df = 4). (c) MAO-A activity was determined in corresponding PC12 cultures ($p = 0.1577$, $t = 1.735$, df = 4); $^{***}p < 0.001$ versus vector control; n.s., no significant difference.

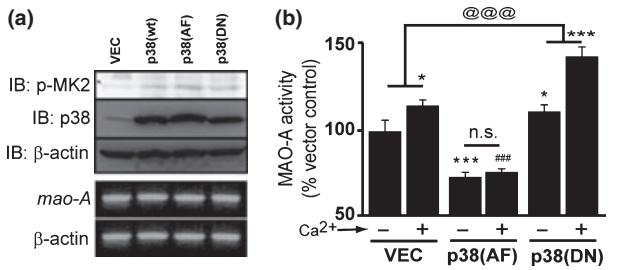

Fig. 5 Over-expression of a constitutively active p38(MAPK) affects MAO-A activity, but not *mao-A* mRNA expression, in HT-22 cells. (a; upper panels) Transfection with the vector (VEC) or over-expression of the p38(MAPK) wildtype (wt), activated form (AF), or dominant-negative (DN) proteins in HT-22 cultures was confirmed by western blot analysis. Corresponding HT-22 cultures were used to examine (a; lower panels) the expression of *mao-A* gene and *β-actin* gene expression, and (b) basal and Ca^{2+}-sensitive MAO-A activities [$F_{(5,24)} = 78.48$, $p < 0.0001$]; $^{*}p < 0.05$ and $^{***}p < 0.001$ versus vector control or between indicated groups; $^{\#\#\#}p < 0.001$ versus Ca^{2+} (+)/vector control; $^{@@@}p < 0.001$ between Ca^{2+} (–) and (+) in vector- and p38(DN)-transfected cells; n.s., no significant difference.

increase in *mao-A* mRNA expression in these cells (Fig. 4b), thus corroborating a previous report (De Zutter and Davis 2001), but, rather surprisingly, p38(MAPK)-AF had no effect on MAO-A activity in corresponding cultures (Fig. 4c). In contrast, over-expression of p38(MAPK)-AF had no effect on *mao-A* mRNA expression in HT-22 cells, but it did significantly reduce basal as well as Ca^{2+}-sensitive MAO-A activities (Fig. 5). Furthermore, over-expression of p38(MAPK)-DN enhanced basal as well as Ca^{2+}-sensitive MAO-A activities in this same cell line (Fig. 5b), thus corroborating our data obtained with the chemical p38(MAPK) inhibitor SB203580 (*see* Fig. 1d). These data support a cell line-dependent role for the p38(MAPK) protein in the post-translational regulation of MAO-A activity.

The activity and neurotoxic potential of MAO-A responds to disruption of Ser209, a putative target for p38(MAPK)

Protein–protein interactions rely on specific motifs. If p38(MAPK) is influencing MAO-A directly, then MAO-A should contain a p38(MAPK) consensus motif and targeted disruption of this motif should appropriately affect MAO-A function.

Examination of the deduced amino acid sequence for MAO-A [NCBI: NP_000231] reveals that the flanking sequence for S209, i.e. RXXS, is a motif that can be targeted by several kinases including p38(MAPK) (Bulavin *et al.* 2001). Human *MAO-A* cDNA (Miller and Edmondson 1999) was mutagenized so as to generate a phosphorylation mimic (substituting the serine for a glutamic acid, e.g. S209E) as well as a protein that cannot be phosphorylated at this position (substituting the serine for an alanine, e.g. S209A). The activities of the over-expressed MAO-A (S209) variants were examined in the MAO-A-deficient HEK293A cell line. The wildtype MAO-A [MAO-A(WT)] protein and the MAO-A(S209A) dephosphorylation mimic both have similar inherent MAO-A activities, but Ca^{2+}-sensitive activity was only evident with the MAO-A(S209A) protein (Fig. 6a). In contrast, the MAO-A(S209E) phosphomimic was significantly less active and clearly did not respond to Ca^{2+}. We then questioned whether these differing activities could be reflecting any interference by the individual substitutions on the ability of the over-expressed MAO-A protein to dimerize, which is a conformation invariably associated with catalytic activity in both purified rat and human MAO-A (Ma *et al.* 2004; Upadhyay *et al.* 2008). We treated corresponding cell cultures with the cell-permeable cross-linking reagent, disuccinimidyl suberate. While protein cross-linking did not reveal a band on western blot corresponding to the molecular weight of a dimeric MAO-A species, it did reveal that the activities of the over-expressed proteins correlated

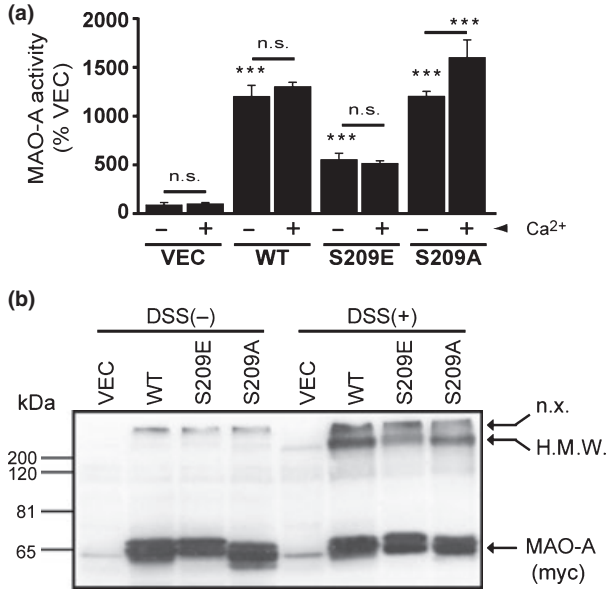

Fig. 6 Serine 209 regulates MAO-A activity. (a) Basal and Ca^{2+}-sensitive MAO-A activities were examined in HEK293A cells over-expressing the myc-tagged MAO-A wildtype (wt) protein, the S209E phosphorylation mimic or the S209A unphosphorylatable protein [$F_{(7,16)} = 184.2$, $p < 0.0001$]. (b) HEK293A cells expressing the MAO-A proteins were cultured in the absence (left) or presence (right) of the crosslinking reagent, disuccinimidyl suberate (DSS), and examined for MAO-A (anti-myc) expression by western blot analysis. n.x., non-cross-linked band, i.e. it is also present in control, vehicle-treated lysates; H.M.W., high molecular weight; kDa, kiloDalton); ***$p < 0.001$ versus vector control or between indicated groups; n.s., no significant difference.

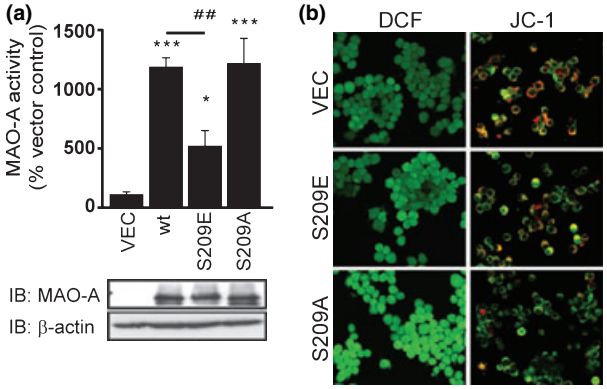

Fig. 7 Serine 209 contributes to MAO-A-mediated neurotoxicity. (a; upper panel) MAO-A activities were examined in N2a cells expressing the myc-tagged MAO-A wildtype (wt) protein, the S209E phosphomimic or the S209A non-phosphorylatable protein [$F_{(3,15)} = 70.2$, $p < 0.0001$]. (a, lower panel) The expression of the MAO-A variants was confirmed by western blot analysis and β-actin was included as a loading control. (b) Neurotoxic relevance of the over-expressed MAO-A proteins was demonstrated by H_2O_2-sensitive DCFH2-DA fluorescence imaging (left panels) and JC-1-sensitive changes in $\Delta\psi_m$ (right panels: *note*, the reduced red signal indicates a loss of $\Delta\psi_m$) in N2a cells; *$p < 0.05$ and ***$p < 0.001$ versus vector control; ##$p < 0.01$ between indicated groups.

with their appearance in high molecular weight complexes (Fig. 6b).

We also examined the inherent activities of the over-expressed MAO-A variants in N2a cells, as this is a *neuronal* cell line with very little endogenous MAO-A activity (e.g. 2 nmol/h/mg protein). The activity of the MAO-A(S209A) variant was similar to that of the WT protein (Fig. 7a) [thus corroborating what we had already observed in HEK293A cells (see Fig. 6a)] and corresponded with a significant increase in DCFH2-DA-sensitive peroxy radical production as well as a loss of $\Delta\psi_m$ (Fig. 7b). As expected, the MAO-A(S209E) phosphomimic was barely active, and did not influence either DCFH2-DA-sensitive peroxy radical production or $\Delta\psi_m$. Therefore, mimicking the phosphorylation of MAO-A(S209) is an inhibitory event (in several cell lines) that diminishes the activity (both basal and Ca^{2+}-sensitive) and neurotoxic potential of the expressed MAO-A protein.

Activated p38(MAPK) interacts with and phosphorylates over-expressed MAO-A

If S209 in MAO-A is targeted by p38(MAPK), then substitution of this residue should impede the ability of the

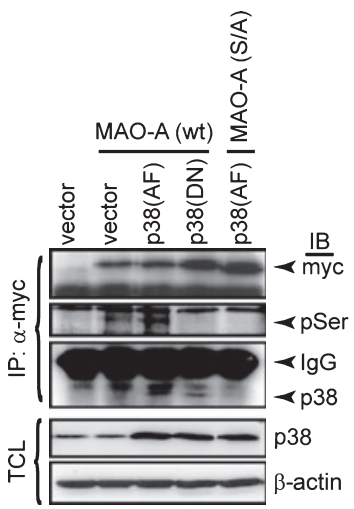

Fig. 8 MAO-A Ser209 is a putative target for p38(MAPK). HEK293A cultures over-expressing myc-tagged MAO-A wildtype (wt) or S209A (S/A) protein in combination with p38(MAPK)-activated form (AF) or dominant-negative (DN) protein were immunoprecipitated (IP) with anti-myc and then examined for myc (i.e. MAO-A expression), phospho-serine (pSer) status and the presence of p38(MAPK) (p38) using western (immuno)blot (IB) analysis. The over-expression of p38(MAPK) in corresponding total cell lysates (TCL) was confirmed by immunoblot [*note*, endogenous p38(MAPK)α is clearly detected in the vector-transfected controls, but at significantly lower levels than those found in the cultures over-expressing the p38(MAPK) AF and DN forms]. The expression of β-actin was used to demonstrate equal protein loading.

MAO-A protein to be phosphorylated by p38(MAPK). In HEK293 cells, MAO-A(WT)-myc co-immunoprecipitated with both p38(MAPK)-AF and p38(MAPK)-DN, but serine phosphorylation of MAO-A(WT)-myc was only observed in cultures expressing the constitutively active p38(MAPK)-AF (Fig. 8). We were unable to detect any association between the MAO-A(S209A) variant and p38(MAPK)-AF, nor could we detect a seryl-phosphorylated MAO-A species in these extracts (Fig. 8).

Catalytic regulation via the RXXS motif is specific to MAO-A

It has previously been demonstrated that substitution of the phenylalanine residue immediately upstream of Ser209, i.e. Phe208, is sufficient to affect the substrate specificity for MAO-A (Tsugeno and Ito 1997). However, the substitution of Ser209 for Ala or Glu did not affect MAO-A substrate preference (i.e. over-expression of MAO-A variants does not alter the cell's capacity to metabolize the MAO-B substrate, [^{14}C]-PEA) (Fig. 9a). Furthermore, homologous mutations in the *RXXS* motif in MAO-B (e.g. on Ser200) did not affect the activity of the individual over-expressed MAO-B proteins (Fig. 9b and c).

These combined data strongly suggest that S209 in MAO-A is a target for p38(MAPK) and indicate that phosphorylation of S209 not only inhibits MAO-A activity, but also diminishes its cytotoxic potential. Furthermore, p38(MAPK)α-mediated phosphoregulation *via* the *RXXS* motif appears to be specific for MAO-A function.

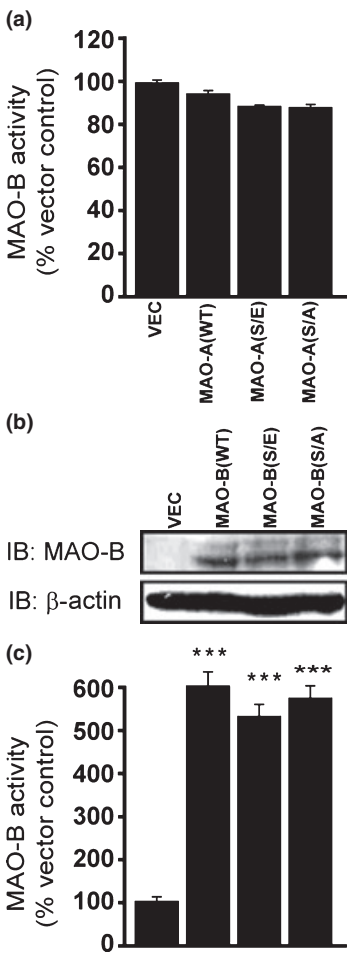

Fig. 9 Targeting the *RXXS* motif affects MAO-A catalytic activity specifically. (a) MAO-A wildtype and the Ser209 variants were over-expressed in HEK293A cells and corresponding cultures were assayed for MAO-B activity [$F_{(3,19)} = 412.1$, $p < 0.0001$]. (b) MAO-B proteins bearing substitution of Ser200 (homologous to Ser209 in MAO-A) were expressed and (c) tested for their inherent activities in HEK293A cells; ***$p < 0.001$ versus vector control.

Discussion

The p38(MAPK) pathway exerts distinct cell line-dependent effects on *mao-A* mRNA expression and/or activity (De Zutter and Davis 2001; Ou *et al.* 2006; Fitzgerald *et al.* 2007). While these reports suggest that the p38(MAPK) *pathway* might augment MAO-A function, our data indicate that specific activation of the p38(MAPK)α *protein* itself, rather than the entire p38(MAPK) *pathway*, actually inhibits MAO-A in several, but not all (e.g. PC12), clonal cell lines, and that the Ser209 residue appears to be pivotal for this effect. Preliminary investigations reveal that over-expression of an activated form of MEK3 (MAPK/ERK kinase 3), the immediate upstream kinase for p38(MAPK), induces MAO-A activity without any concurrent change in *mao-A* mRNA expression in the SH-Sy5y human neuroblastoma cell line (Xia Cao and Darrell D. Mousseau, unpublished data). Not only does this support the proposal by the Billett Laboratory of a transcription-independent regulation of MAO-A function following activation of the p38(MAPK) pathway in this same cell line (Fitzgerald *et al.* 2007), but it also implicates another pivotal cell signaling kinase in the process, and suggests the potential for interplay between these two kinases, i.e. MEK3 (MAPK/ERK kinase 3) and p38(MAPK), and MAO-A in different cellular contexts.

We found that MAO-A catalytic activity tends to correlate with its detection within a (cross-linked) high molecular weight complex of ~250 kDa. MAO-A is most often viewed as a homodimeric species anchored to the outer mitochondrial membrane through its C-terminal transmembrane helix tail (Ma *et al.* 2004; Upadhyay *et al.* 2008). While its stoichiometry within the observed high molecular weight complex presently remains unclear, we must assume that, as reported (Ma *et al.* 2004; Upadhyay *et al.* 2008), only its C-terminus is membrane-bound, thus leaving most of the protein exposed to the cytoplasm for potential modification. The 3D structure of human MAO-A (PDB code: 2BXR) reveals Ser209 is exposed on the protein surface adjacent to the 'cavity shaping loop.' The hydroxyl group of Ser209 is situated ~4 Å from an anionic carboxyl group of Glu216;

therefore, incorporation of an anionic phosphate moiety on the Ser209 side chain would necessarily result in electrostatic repulsion with the Glu216 carboxyl group. The ensuing change in the conformation of the loop could explain the observed reduction in catalytic activity. Our ongoing characterization of the potential for post-translational regulation of catalytic activity of MAO-A reveals that the S209E substitution significantly alters the activity and stability of the expressed MAO-A protein [expressed and purified using the *Pichia pastoris* (yeast) expression system] as well as its ability to recognize substrates and inhibitors (Wang *et al.* 2009). We also found that this behavior was not as prominent in its membrane-bound form, but this may simply be reflecting different membrane-protein interactions in the yeast expression system when compared with mammalian cell cultures (used in the present series of experiments). In support of this, recent comparisons of pargyline spin-labeled inhibition of MAO-A in intact mitochondria from the yeast expression system with mitochondria from human placenta demonstrated differing orientations (Upadhyay and Edmondson 2009). Furthermore, our ongoing investigations have also revealed that N2a cell toxicity induced by SB203580 and C6 cell toxicity induced by pervanadate (a pan-inhibitor of tyrosine phosphatases and other enzymes) are both associated with the appearance of a pool of MAO-A that is no longer strictly bound to the mitochondrial membrane, but that can also be detected within the nucleus (Zhongjian Jiang and Darrell D. Mousseau, unpublished data). Extra-mitochondrial MAO is not uncommon (Arnold *et al.* 1990), but to our knowledge, the only report of pathology-associated nuclear MAO-A focused on pre-eclampsic–eclampsic placenta (the tissue expressing the highest levels of MAO-A protein) and described how the severity of eclampsia was directly correlated with the redistribution of MAO-A protein from the mitochondrial to the nuclear fraction (Gujrati *et al.* 1996).

Comparison of the human MAO-A and MAO-B deduced amino acid sequences reveals that MAO-B has a homologous Ser200 residue. However, the 'cavity shaping loop' of MAO-B is in a more compact conformation than that of MAO-A and there are no anionic groups adjacent to the hydroxyl of the Ser200 side chain to elicit a conformational change should the Ser200 residue be phosphorylated. These structural considerations might explain why phosphorylation within the RXXS motif regulates MAO-A activity, but not that of MAO-B. We continue to examine how the manipulation of this motif affects modification, regulation, localization, and overall function of the MAO-A protein.

Given that MAO-A activity is associated with oxidative stress/cell death (Maher and Davis 1996; Malorni *et al.* 1998; De Zutter and Davis 2001; Maragos *et al.* 2004; Ou *et al.* 2006), the observed inhibition of MAO-A activity (and associated neurotoxicity) by p38(MAPK) would surely imply a pro-survival role for p38(MAPK). While this might initially be counterintuitive, high basal activity of p38(MAPK) in healthy adult mouse brain certainly argues for a role for this enzyme in normal brain cell physiology and survival (Jiang *et al.* 1996; Lee *et al.* 2000). p38(MAPK) has been associated with major depressive illness (Miller and Raison 2006) and has been presumed to play a precipitating/exacerbating role. This conclusion would be reasonable if p38(MAPK) were strictly a cell stressor (Lee *et al.* 1994; Wysk *et al.* 1999; Porras *et al.* 2004; Jayakumar *et al.* 2006). However, it is now known that H_2O_2, the by-product of MAO reactions, can actually activate p38(MAPK) (Blanc *et al.* 2003) and inhibit MAO-A activity (Konradi *et al.* 1986), apparently *via* a Ca^{2+}-dependent mechanism (Blanc *et al.* 2004). This suggests, in principle, that a p38(MAPK)-mediated feedback regulation of MAO-A could present an antidepressant-like effect.

Both p38(MAPK) and MAO-A have been linked with the neurodegeneration associated with AD (Emilsson *et al.* 2002; Takehashi *et al.* 2002; Sun *et al.* 2003; Zhu *et al.* 2003; Nishimura *et al.* 2005; Franciosi *et al.* 2006; Cao *et al.* 2007). In the later stages of AD, and as a result of sustained activation, the proinflammatory function of p38(MAPK) clearly prevails (Franciosi *et al.* 2006). However, the activation of p38(MAPK) in the early stages of AD (Sun *et al.* 2003; Zhu *et al.* 2003), when markers of apoptosis are uncharacteristically absent, supports the potential for an oxidative stress-induced adaptive (*see* pro-survival) response (Zhu *et al.* 2003). Our current observations clearly indicate that activation of p38(MAPK) results in the inhibition of both basal as well as Ca^{2+}-sensitive MAO-A activity. p38(MAPK)-mediated-negative feedback of an H_2O_2-generating mitochondrial enzyme such as MAO-A would have obvious survival benefits, particularly in situations where intracellular Ca^{2+} availability would be increased, as it is in AD (Thibault *et al.* 1998; Palop *et al.* 2003). A preliminary examination reveals induction of the p38(MAPK) and MAO-A proteins, and their co-localization, in numerous cells in layers III/IV of the cortex of T*g*CRND8 mice (a mouse model of AD-related amyloidosis) as young as 3 months (Jennifer Chlan-Fourney and Darrell D. Mousseau, unpublished data). This is about 2 months before the reported appearance of some of the hallmarks of AD, such as dense-core plaques and neuritic pathology (Chishti *et al.* 2001). We continue to examine the role of the interaction between p38(MAPK) and MAO-A in T*g*CRND8 mouse neuropathology.

Finally, p38(MAPK)-mediated regulation of MAO-A function could have implications beyond neuropsychiatric disorders. Indeed, pre-ischemic treatment of myocytes with low doses of H_2O_2 (i.e. pre-conditioning) not only activates p38(MAPK) (Blunt *et al.* 2007; Wu *et al.* 2007), but also mitigates post-ischemic myocardial damage induced by a Ca^{2+} overload (Blunt *et al.* 2007). Post-ischemic myocardial damage is known to rely on MAO-A-mediated oxidative stress (Bianchi *et al.* 2005).

Given that the literature suggests a potential association between p38(MAPK) and MAO-A in normal physiology as well as in pathologies as diverse as depression, AD and heart disease, further examination of this unique *endogenous* means of regulating MAO-A function is certainly warranted.

Acknowledgments

This work was funded, in part, by Departmental Aruna and Kripa Thakur Research Awards (to XC) and, in part, by a Canadian Institutes of Health Research-Saskatchewan Health Research Foundation Operating Grant (to DDM). The TgCRND8 mice used in some of our preliminary investigations were a generous gift from Dr D. Westaway (University of Alberta, Edmonton, AB, Canada).

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