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ORIGINAL ARTICLE

Glutathione Transferase-M2-2 Secreted from Glioblastoma Cell Protects SH-SY5Y Cells from Aminochrome Neurotoxicity

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Abstract U373MG cells are able to take up aminochrome that induces glutathione transferase M2-2 (GSTM2) expression in a concentration-dependent manner where 100 μM aminochrome increases GSTM2 expression by 2.1-fold ($P < 0.001$) at 3 h. The uptake of ^{3}H-aminochrome into U373MG cells was significantly reduced in the presence of 2 μM nomifensine ($P < 0.001$) 100 μM imipramine ($P < 0.001$) and 50 mM dopamine ($P < 0.001$). Interestingly, U373MG cells excrete GSTM2 into the conditioned medium and the excretion was significantly increased (2.7-fold; $P < 0.001$) when the cells were pretreated with 50 μM aminochrome for 3 h. The U373MG-conditioned medium containing GSTM2 protects SH-SY5Y cells incubated with 10 μM aminochrome. The significant protection provided by U373MG-conditioned medium in SH-SY5Y cells incubated with aminochrome was dependent on GSTM2 internalization into SH-SY5Y cells as evidenced by (i) uptake of ^{14}C-GSTM2 released from U373MG cells into SH-SY5Y cells, a process inhibited by anti-GSTM2 antiserum; (ii) lack of protection of U373MG-conditioned medium in the presence of anti-GSTM2 antiserum on SH-SY5Y cells treated with aminochrome; and (iii) lack of protection of conditioned medium from U373MGsiGST6 that expresses an siRNA directed against GSTM2 on SH-SY5Y cells treated with aminochrome. In conclusion, our results demonstrated that U373MG cells protect SH-SY5Y cells against aminochrome neurotoxicity by releasing GSTM2 into the conditioned medium and subsequent internalization of GSTM2 into SH-SY5Y cells. These results suggest a new mechanism of protection of dopaminergic neurons mediated by astrocytes by releasing GSTM2 into the intersynaptic space and subsequent internalization into dopaminergic neuron in order to protect these cells against aminochrome neurotoxicity.

Keywords Glutathione transferase · Aminochrome · Glia cells · Dopaminergic neurons · Neuroprotection · Parkinson's disease

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Introduction

Astrocytes play a protective role in neighboring neurons by releasing energy substrates (Schousboe et al. 2007; Pellerin 2008; Pellerin et al. 2007; Nehlig and Coles 2007; McKenna 2007; Yang et al. 2008).The glutathion (GSH) synthesis in neurons is also dependent on neighboring astrocytes that supply all three constituent amino acids (L-glutamic acid, L-cysteine, and glycine) of GSH (Banerjee et al. 2008; Dringen and Hirrlinger 2003). GSH plays a key role in the protection against oxidative stress and toxicity from reactive chemical compounds formed by the metabolism of endogenous as well as exogenous sources. Loss of intracellular GSH will lead to apoptosis (Skindersoe et al. 2012).

Dopamine stored in monoaminergic vesicles in the presynaptic terminal of dopaminergic neurons is released to the synaptic cleft under neurotransmission. Synaptic dopamine is subsequently removed from the synaptic cleft via dopamine transporters localized on the plasma

membrane of dopaminergic neurons. Astrocytes surrounding dopaminergic neuron are also exposed to dopamine released from dopaminergic neurons under neurotransmission. Interestingly, dopamine reuptake is not restricted to dopaminergic neurons but also demonstrated by other cells such as astrocytes (Takeda et al. 2002).

A pertinent question is the fate of dopamine in astrocytes since at physiological pH, dopamine can undergo oxidization to aminochrome in the presence of oxygen (Linert et al. 1996). Aminochrome has been found to be involved in four of the five mechanisms that are involved in degeneration of the dopaminergic nigrostriatal system including mitochondrial dysfunction, protein degradation dysfunction, aggregation of alpha-synuclein to toxic protofibrils, and oxidative stress (Huenchuguala et al. 2014; Segura-Aguilar et al. 2014; Norris et al. 2005; Zafar et al. 2006; Muñoz et al. 2012a, b; Aguirre et al. 2012). It has been demonstrated that aminochrome can be conjugated by glutathione transferase M2-2 (GSTM2), and this reaction was proposed to be a protective reaction since the formed conjugate is stable and the conjugation prevents redox cycling promoted by one-electron reduction of aminochrome (Segura-Aguilar et al. 1997; Baez et al. 1997). In addition, GSTM2 intercepts the formation of aminochrome by conjugating the intermediate dopamine *o*-quinone to 5-glutathionyl-dopamine (Dagnino-Subiabre et al. 2000). The latter is the precursor of 5-cysteinyl dopamine which is a stable product (Shen et al. 1996).

We demonstrated that GSTM2 is released into the conditioned medium of the glioblastoma cell line U373MG. Therefore, the aim of this study was to investigate whether U373MG cells could protect SH-SY5Y cells against aminochrome neurotoxicity by releasing GST-M2-2 into the conditioned medium.

Materials and Methods

Chemicals

Dopamine and tyrosinase were purchased from Sigma-Aldrich (St Louis MO USA). A LIVE/DEAD Viability/Cytotoxicity kit was purchased from Molecular Probes (Eugene OR USA). ^{14}C-leucine was from PerkinElmer Inc. (Massachusetts USA). U373MG and SH-SY5Y cells were from ATCC.

Aminochrome Synthesis

Aminochrome was synthesized by oxidizing 0.1 M dopamine with 25 µl tyrosinase of a solution of 1 mg/ml during 15 min in an open Petri dish. Aminochrome was purified by adding 50 µl of oxidized dopamine to a column of 7.5 cm with a diameter of 0.5 cm containing 1 ml CM-Sepharose 50–100 column equilibrated with MES buffer pH 6.5. Aminochrome was separated from unreacted dopamine and tyrosinase, and the structure and purity were demonstrated using NMR (Paris et al. 2010). Aminochrome was quantified using the molar extinction coefficient of 3,058 M-1 cm-1 at 475 nm (Segura-Aguilar & Lind 1989).

Cell Lines

As a model cell line we used U373MG cells, a well-characterized permanent human astrocytoma cell line derived from a patient classified as having malignant anaplastic astrocytoma grade IV-glioblastoma. We also used U373MGsiGST6 cells expressing an siRNA for GSTM2 mRNA that only has 26 % of the normal GSTM2 expression as we described above (Huenchuguala et al. 2014). All cells were incubated in RPMI-1640 medium (HyClone Cat. # SH30011.04, GE Healthcare) containing 2 mM L-glutamine 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, and 1,500 mg/l sodium bicarbonate supplemented with 10 % fetal bovine serum (HyClone Fetalclone III SH30109.02, GE Healthcare), 10 U/ml sodium penicillin, 10 U/ml streptomycin sulfate, and amphotericin B at an atmosphere of 5 % CO_2 and at 37 °C. SH-SY5Y cells were grown in DMEM/F12 10 % fetal bovine serum, 2 mM L-glutamine, and 1 % of antibiotic and antimycotic mixture. SH-SY5Y cells were differentiated by incubating the cells with 10 µM retinoic acid during 8 days.

Aminochrome Uptake

The synthesis of ^{3}H-aminochrome was performed in the presence of 8.5 µCi of 2,5,6-^{3}H-dopamine from Amersham Biosciences Limited, UK generating 2,5-^{3}H-aminochrome since the ^{3}H in the 6 position is removed when the amino chain of 2,5,6-^{3}H-dopamine *o*-quinone cyclizes to 2,5-^{3}H-aminochrome. U373MG cells were incubated for 5 to 120 min in 500 µl Hank's solution at 37 °C containing 10 mM HEPES, 130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM H_2PO_4, 10 mM glucose pH 7.4, and a final concentration of 50 µM ^{3}H-AM. At the end of the uptake period, the extracellular medium was removed and the cells were rapidly washed 5 times with 2 ml medium to remove residual 25-^{3}H-aminochrome tracers. Cell membranes were disrupted with 1 ml of 1 % Triton X-100 and after 15 min incubation, 900 µl of the cell/Triton X-100 extract was removed and analyzed for ^{3}H-aminochrome content by liquid scintillation counting. The remaining 100 µl was used for protein determination by bicinconinic acid method. The results were expressed in pmol/mg protein. Cells were also incubated with ^{3}H-aminochrome in the presence of 2 µM nomifensine (inhibitor of dopamine transporter), 100 µM imipramine (inhibitor of

serotonin transporter), and 2 mM dopamine. To measure 2,5-^{3}H-aminochrome efflux from U373MG cells, we washed the cells 5 times with Hank's solution and incubated with Hank's solution for 0, 20, and 40 min. After incubation, the cells were centrifuged at 3,000×*g* for 5 min and the supernatant was analyzed by scintillation counting.

GSTM2 Concentration in Conditioned Medium

The total volume of conditioned medium (8 ml) was passed through a 0.2-μm filter and was mixed with 1 ml of agarose-GSH gel (Pierce Chemical Company Rockford IL USA Cat. #16100) equilibrated with 50 mM Tris, 150 mM NaCl pH 8.0, and 1 ml of the same buffer (10 ml total). The tube was incubated for 2 h at room temperature and centrifuged for 2 min at 700×*g*. The GSTM2 was eluted by adding 1 ml of elution buffer containing 50 mM Tris, 150 mM NaCl pH 8.0, and 10 mM GSH.

Western Blot

The level of GSTM2 expression was determined as described before (Huenchuguala et al. 2014) by separating 100 μg U373MG cell homogenate on SDS-polyacrylamide gel electrophoresis (10 % w/v). The separated proteins were then transferred electrophoretically to a 0.2-μm nitrocellulose membrane. After blocking with 0.5 % skim milk containing 10 mM Tris–HCl of pH 7.6, 150 mM NaCl, and 0.1 % Tween 20 for at least 4 h, the membrane was incubated with polyclonal rabbit antiserum raised against GSTM2. Following overnight incubation at room temperature in the same buffer, the membrane was washed 3 × 15 min with a solution of 10 mM Tris–HCl pH 7.6, 150 mM NaCl, and 0.025 % Tween 20 and incubated 2 h with antibody linked to alkaline phosphatase antiserum. The incubation was followed by a final washing. The band of GSTM2 was detected using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium; Zymed laboratories Inc.). The polyclonal antibodies against GSTM2 were raised in rabbits using human GSTM2 obtained by heterologous expression in Escherichia coli and purified with affinity chromatography (Johansson et al. 1999).

Cell Death

Cell death was determined in culture medium in the absence of bovine serum and phenol red by incubating cells for 2 h in the presence of purified aminochrome (Paris et al. 2010). Cell death was measured by counting live and dead cells after staining with 0.5 μM Calcein AM and 5 μM ethidium homodimer-1 for 45 min at room temperature in the dark (LIVE/DEAD Viability/Cytotoxicity Kit Molecular Probes). Calcein AM is a marker for live cells, and ethidium homodimer-1 intercalates into the DNA of dead cells. Cells were counted with a phase contrast microscope equipped for fluorescence visualization using the following filters: Calcein AM 450–490 nm (excitation) and 515–565 nm (emission), and ethidium homodimer-1 510–560 nm (excitation) and LP-590 nm (emission).

GSTM2 Activity Assay

GSTM2 activity was measured in 0.1 M sodium phosphate pH 6.5, 1 mM GSH, and 100 μM aminochrome at 30 °C. The reaction, which is highly specific for GSTM2 (Segura-Aguilar et al. 1997), was started by the addition of enzyme and monitored by following the decrease in aminochrome absorption at 475 nm. The reaction rate was calculated using the molar extinction coefficient of aminochrome of 3,058 M^{-1} cm^{-1} (Segura-Aguilar and Lind 1989).

Protection of SH-SY5Y Cells by U373MG-Conditioned Medium

The protective effect of U373MG-conditioned medium on SH-SY5Y cells was studied by incubating SH-SY5Y cells with a mixture of conditioned medium from U373MG cells pretreated with 50 μM aminochrome for 3 h and an equal volume of normal SH-SY5Y medium. The effect was evaluated after cultivation of the SH-SY5Y cells for 24-h incubation with 10 μM aminochrome.

Uptake of ^{14}C-Labeled GSTM2 into SH-SY5Y Cells

U373MG cells were incubated with culture medium containing 2 μCi ^{14}C-labeled L-leucine for 1 h. The cells were washed (3×) with PBS and incubated with conditioned medium containing 50 μM aminochrome for 3 h. The cells were washed (3×) with PBS and were then incubated with normal conditioned medium for 24 h. Finally the 24-h culture medium was collected. SH-SY5Y cells were incubated with 1 ml of 24-h culture medium from the U373MG cells and 1 ml normal conditioned medium for 5 h. The cells were washed (3×) with PBS and centrifuged at 2,000 rpm. A part of the pellet was added to scintillation solution, and radioactivity was measured in a scintillation counter. An addition of 1:100 of anti-GSTM2 antiserum to the 24-h culture medium was used to inhibit ^{14}C-labeled GSTM2 uptake into SH-SY5Y cells.

Statistical Analysis

All data were expressed as mean ± SD values. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Student's *t* test.

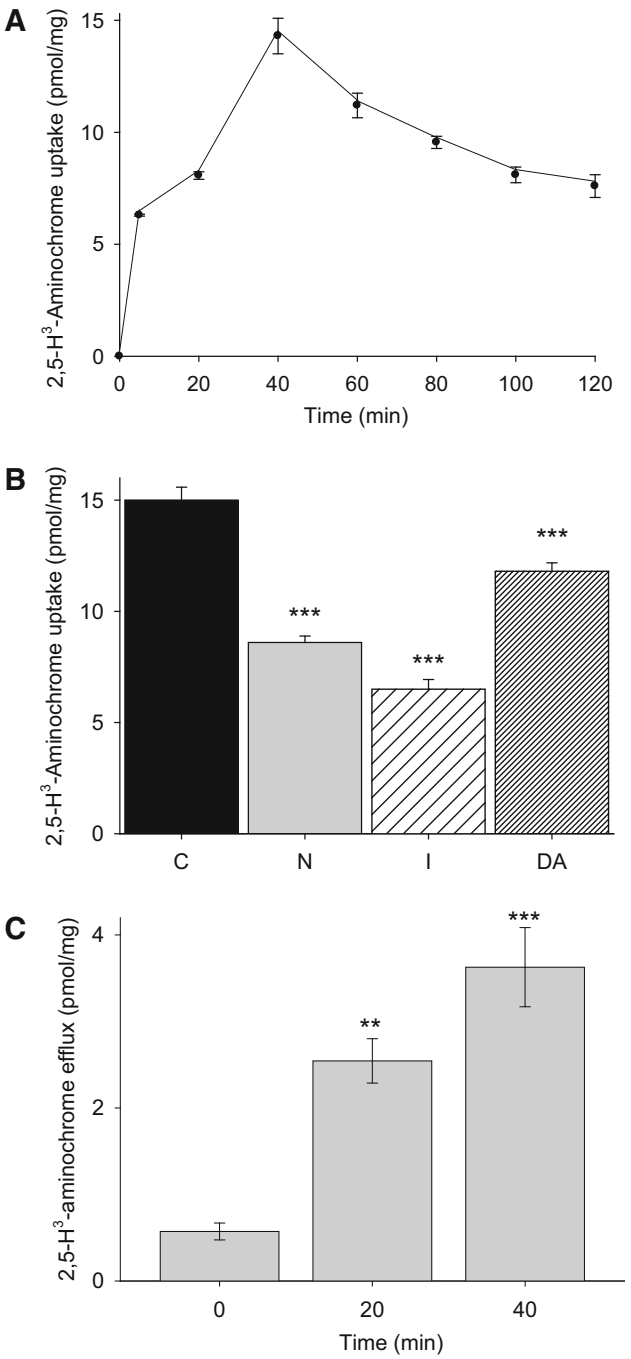

Fig. 1 Aminochrome uptake into U373MG cells. **a** The uptake of 25-^{3}H-aminochrome into U373MG cells increased during 40 min followed by a slow decline. **b** Aminochrome uptake in U373MG cells was inhibited by 2 μM nomifensine (N) and 100 μM imipramine (I) and 2 mM dopamine (DA) suggesting the presence of monoaminergic transporter in U373MG cells. In addition, U373MG cells have expression of organic cation transporter-1 and plasma membrane monoamine transporter that can also mediate 25-^{3}H-aminochrome transport (11). **c** The release of 25-^{3}H-aminochrome from U373MG cells increase with time [the values are the mean ± SD ($n = 3$)]

Results

Cellular Uptake and Disposition of 2,5-^{3}H-Aminochrome

The initial study was designed to investigate whether protection of dopaminergic neurons by astrocytes could be mediated via the secretion of GSTM2. The human astrocytoma cell line U373MG has constitutive expression of GSTM2 and dopamine uptake (Huenchuguala et al. 2014) and therefore we have used U373MG cells as a model for astrocytes. Aminochrome uptake into U373MG cells was determined by incubating the cells with 50 μM 2,5-^{3}H-aminochrome and determining the uptake after 5, 20, 40, 60, 80, 100, and 120 min. 2,5-^{3}H-Aminochrome uptake increased up to 40 min (14.3 ± 0.8 pmol/mg) and decreased after 60 min, although the uptake at 120 min (7.6 ± 0.5 pmol/mg) was still higher than at 5 min (6.3 ± 0.4 pmol/mg; Fig. 1a). 2,5-^{3}H-Aminochrome uptake at 40 min (14.0 ± 0.6 pmol/mg) was inhibited by 2 μM nomifensine (8.6 ± 0.3 pmol/mg; $P < 0.001$), also by 100 uM imipramine (6.5 ± 0.4 pmol/mg; $P < 0.001$) and 2 mM dopamine (11.8 ± 0.4 pmol/mg; $P < 0.001$; Fig. 1b). We expected that the uptake of 25-^{3}H-aminochrome into U373MG cells that have constitutive expression of GSTM2 (Huenchuguala et al. 2014) would generate 2,5-^{3}H-aminochrome-GSH conjugate that can be excreted from the cell. Therefore, we determined the efflux of radioactivity after 40-min treatment of U373MG cells with ^{3}H-aminochrome. After washing U373MG cells 5 times with Hank's solution at time zero, the conditioned medium contained 0.57 ± 0.09 pmol/mg protein 2,5-^{3}H-aminochrome equivalents that significantly increased after 20 min to 2.54 ± 0.3 pmol/mg ($P < 0.0$) and 40 min to 3.6 ± 0.5 pmol/mg ($P < 0.001$; Fig. 1c). The exact nature of the radioactive compound after entry into the cells was not determined.

Induction of GSTM2 in U373MG Cells by Aminochrome

The expression of GSTM2 in U373MG cells is constitutive (Huenchuguala et al. 2014), but the enzyme level can be increased by aminochrome in a concentration-dependent manner. Incubation of U373MG cells with increasing aminochrome concentrations (0, 30, 50, and 100 μM) demonstrated up to 2.1-fold increase of GSTM2 after treatment with 100 μM aminochrome for 3 h ($P < 0.001$; Fig. 2a, b) as determined from Western blot analysis. The time course

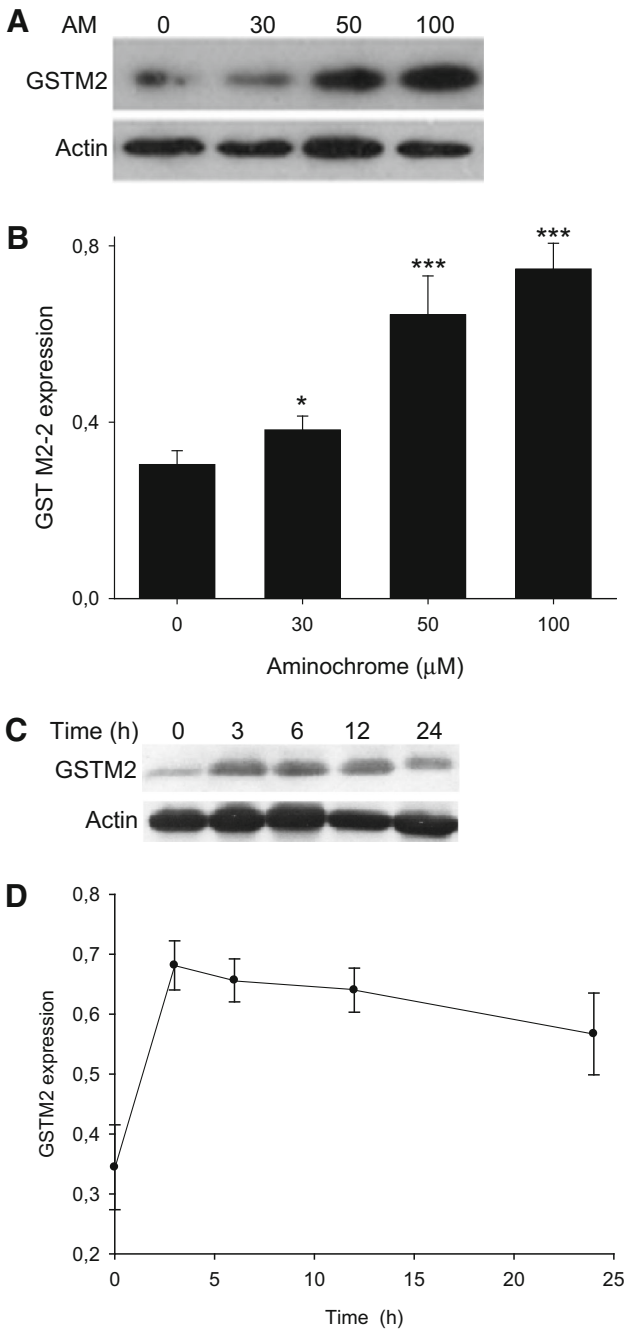

Fig. 2 GSTM2 expression in U373MG cells. **a** Aminochrome induces the expression of GSTM2 in U373MG cells in an aminochrome concentration-dependent manner. The expression of GSTM2 was determined using the Western blot technique and the quantified Western blot pixels were plotted in **b** and expressed in pixels of GSTM2/actin. The time course of GSTM2 induction was measured at 0, 5, 10, 20, and 25 h (**c**). The quantification of this Western blot was plotted in (**d**). The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons (*$P < 0.05$; ***$P < 0.001$)

of GSTM2 induction was measured at 0,3, 6, 12, and 24 h (Fig. 2c) showing that the induction was observed at 3 h and it was followed by a progressive decrease in GSTM2 expression (Fig. 2d).

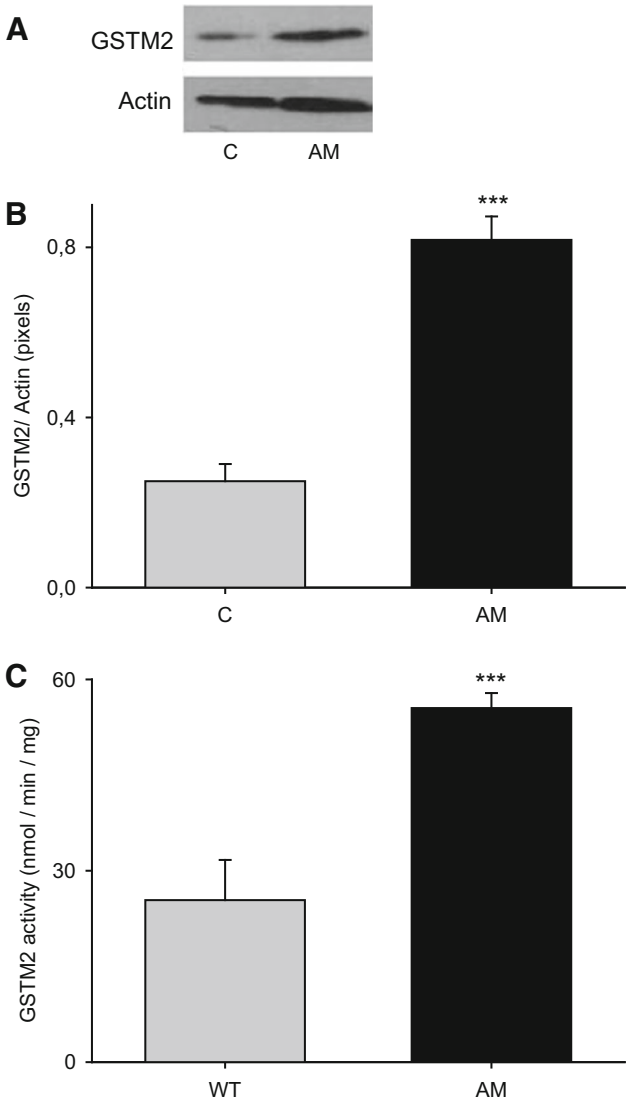

Fig. 3 GSTM2 release from U373MG cells into conditioned medium. **a** GSTM2 release from U373MG cells was measured in the absence (C) and presence of 50 μM aminochrome (AM) for 3 h. The quantification of the Western blot was plotted in **b** as pixels of GSTM2/actin. **c** A significant increase in GSTM2 activity was observed in U373MG cells treated with 50 μM aminochrome for 3 h (AM) in comparison with untreated U373MG cells (C), GSTMA2 activity was measured using 100 μM aminochrome as the substrate in 0.1 M sodium phosphate pH 6.5 and 1 mM GSH at 30 °C as is described under "Materials and Methods" section. The values are the mean ± SD ($n = 3$) and the statistical significance was determined by Student's T test (***$P < 0.001$)

Release of GSTM2 from U373MG Cells

It has been reported that astrocytes release several proteins including superoxide dismutase and thioredoxin peroxidase into the conditioned medium (Lafon-Cazal et al. 2003) and the question was whether U373MG cells in a similar manner release GSTM2 into the culture medium. We evaluated the secretion of GSTM2 isoform into the

conditioned medium by collecting the spent medium after 24-h incubation for GST affinity adsorption on agarose-GSH gel. Any adsorbed GST was eluted with GSH and analyzed with a Western blot stained with antiserum specific for GSTM2. Western blot analysis revealed the presence of GSTM2 in the conditioned medium of U373MG cells that significantly increased when U373MG cells were pretreated with 50 μM aminochrome for 3 h (Fig. 3a). We measured the enzyme activity of GSTM2 in the conditioned medium using aminochrome as substrate. The activity of GSTM2 with aminochrome exceeds by several orders of magnitude those of all other human GSTs tested with this substrate (Segura-Aguilar et al. 1997; Baez et al. 1997). The untreated conditioned medium had an enzymatic activity of 25 ± 4 nmol/(min mg) protein after incubation for 24 h that significantly increased to 56 ± 2 nmol/(min mg) protein ($P < 0.001$) in medium from U373MG cells pretreated for 3 h with 50 μM aminochrome (Fig. 3c).

Effect of U373MG-Conditioned Medium on SH-SY5Y Cells

To study the possible protective role of U373MG-conditioned medium on SH-SY5Y cells, we first determined the toxic effect of aminochrome on SH-SY5Y cells by incubating the cells with 0, 10, 30, and 50 μM aminochrome. The cell death increased by raising the aminochrome concentration from 10 μM (26 ± 3, $P < 0.001$) via 30 μM (35 ± 0.6 %, $P < 0.001$) to 50 μM (47 ± 3 %, $P < 0.001$) (Fig. 4a). We incubated SH-SY5Y cells with the conditioned medium of U373MG cells in order to determine the protective effect of GSTM2 secreted into U373MG-conditioned medium against aminochrome neurotoxicity. In order to increase the level of secreted GSTM2 in the U373MG-conditioned medium, we pre-incubated U373MG cells with 50 μM aminochrome for 3 h before changing the culture medium and incubating cells for another 24 h. SH-SY5Y cells incubated with 10 μM aminochrome for 24 h showed a 31.6 ± 2.1 % cell death rate (Fig. 4b). Remarkably, the addition of U373MG-conditioned medium to SH-SY5Y cells incubated with 10 μM aminochrome significantly decreased cell death to 14.5 ± 1 % ($P < 0.001$). This result raised the question whether the protection observed with the U373MG-conditioned medium was dependent on GSTM2 or another component in the medium. Therefore, we incubated SH-SY5Y cells with 10 μM aminochrome and U373MG-conditioned medium in the presence of antiserum against GSTM2 which was expected to abrogate the effect of GSTM2. In the presence of the antiserum, the protective effect of U373MG-conditioned medium on the SH-SY5Y cells disappeared. A 30.3 ± 2.4 % cell death was observed similar to the 31.6 % obtained in the absence of U373MG-conditioned medium. As control we incubated SH-SY5Y cells with 10 μM aminochrome in the presence of antiserum against GSTM2; 1 mM GSH; 1 mM GSH and antiserum against GSTM2; 1 mM GSH and U373MG-conditioned medium; or 1 mM GSH, antiserum against GSTM2 and U373MG-conditioned medium. Under all these conditions, we observed protection similar to the conditioned medium (Fig. 4b).

Effect of siRNA Directed to GSTM2 RNA

The above results support the notion that GSTM2 secreted from U373MG cells into conditioned medium protects SH-SY5Y cells from aminochrome toxicity. For additional confirmation, we wanted to test whether the conditioned medium from U373MGsiGST6 cells which express siRNA against GSTM2 mRNA (Huenchuguala et al. 2014) also provide protection in SH-SY5Y cells. The incubation of SH-SY5Y cells with 10 μM aminochrome induced a 31 ± 2 % cell death, while the SH-SY5Y cells incubated with U373MG cell-conditioned medium pre-incubated with 50 mM aminochrome during 3 h induced a significant decrease to 14.3 ± 1 % cell death ($P < 0.001$). By contrast, the incubation of SH-SY5Y cells with U373MGsiGST6-conditioned medium pre-incubated with 50 μM aminochrome during 3 h did not significantly protect SH-SY5Y cell from aminochrome neurotoxicity (29 ± 2 % cell death; Fig. 5a) presumably because the U373MGsiGST6 cells have only 26 % expression of GSTM2 as compared to U373MG wild-type cells (Huenchuguala et al. 2014).

Effect of Purified Recombinant GSTM2-2

The additional result presented above supports the interpretation that GSTM2 released into U373MG cell medium protects SH-SY5Y from aminochrome toxicity. Therefore, we could expect that incubation of SH-SY5Y cells with pure GSTM2 would prevent the toxic effects of aminochrome. The incubation of SH-SY5Y cells with 200 ng pure GSTM2 protected these cells against aminochrome toxicity (10 μM) since the cell death decreased from 26 ± 1.5 % cell death to 10 ± 1 % cell death ($P < 0.001$). A partial reversal of this protective effect was demonstrated by the inclusion of antiserum against GSTM2 in the incubation of SH-SY5Y cells with 200 ng pure GSTM2 and 10 μM aminochrome. A significant increase of cell death (14.4 ± 1.5 %) from the value in the absence of antiserum (10 ± 1 %; $P < 0.05$; Fig. 5b) was observed.

Uptake of ^{14}C-Labeled GSTM2 by SH-SY5Y Cells

For induction of ^{14}C-labeled-GSTM2 synthesis, U373MG cells were treated with 50 μM aminochrome for 3 h in the presence

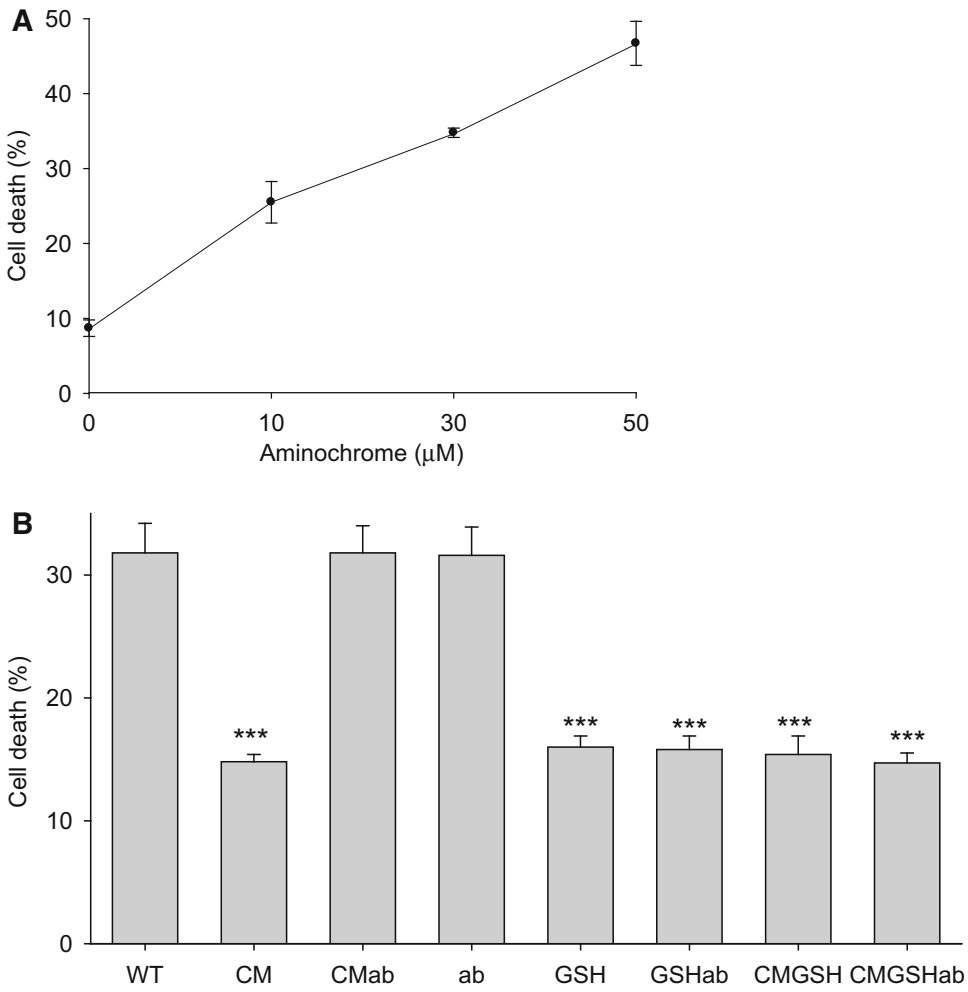

Fig. 4 The effect of U373MG-conditioned medium on aminochrome-induced cell death of SH-SY5Y cells. **a** Aminochrome induces a concentration-dependent cell death in SH-SY5Y cell line, where 50 μM induces approximately 50 % cell death. **b** A significant decrease in cell death was observed in SH-SY5Y cells treated with 10 μM aminochrome when the cells were incubated in the presence of conditioned medium of U373MG cells (CM) pretreated with 50 μM aminochrome for 3 h. Interestingly, this effect disappeared in the presence of antiserum against GSTM2 (ab). The possible role of 1 mM GSH (GSH) was determined in the presence of anti-GSTM2 antiserum (GSHab); GSH in the presence of conditioned medium (CMGSH) and GSH conditioned medium and antiserum against GSTM2 (CMGSHab). The values are the mean ± SD ($n = 3$) and the statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons, (***$P < 0{,}001$)

of 2 μCi ^{14}C-leucine. The expression of GSTM2 was continued for 24 h in the absence of aminochrome but still with ^{14}C-labeled leucine to obtain ^{14}C-labeled GSTM2 in the conditioned medium. SH-SY5Y cells were incubated with an equal mix of U373MG-conditioned medium containing ^{14}C-labeled GSTM2 and normal conditioned medium in order to determine whether GSTM2 was internalized into SH-SY5Y cells. A significant increase in radioactivity was observed in SH-SY5Y cells (54.1 ± 3.6 cpm; $P < 0.001$) incubated with U373MG-conditioned medium that had been treated with ^{14}C-leucine and 50 μM aminochrome, compared to SH-SY5Y cells incubated with conditioned medium of U373MG cells (26.7 ± 5 cpm) or conditioned medium incubated with only ^{14}C-leucine (22.6 ± 1.5 cpm). To determine whether the observed radioactivity taken up in SH-SY5Y cells derived from GSTM2, we incubated SH-SY5Y cells with U373MG cell medium containing ^{14}C-labeled GSTM2 and anti-GSTM2 antiserum. A significant decrease in the measured radioactivity (31.4 ± 1.5 cpm; $P < 0.001$) was observed in SH-SY5Y cells incubated in the presence of anti-GSTM2 antiserum (Fig. 6).

Discussion

GSTM2 Protective Role

We have previously proposed that GSTM2 serves a protective role based on the fact that 4-*S*-glutathionyl-5,6-dihydroxyindoline conjugate is stable in the presence of biologically oxidizing agents such as dioxygen superoxide radicals and hydrogen peroxide (Segura-Aguilar et al. 1997). Furthermore, 5-glutathionyl-dopamine (Dagnino-Subiabre et al. 2000) is the precursor of 5-cysteinyl dopamine (Shen et al. 1996) which has been detected in cerebrospinal fluid of Parkinson's disease patients in dopamine-rich brain regions such as caudate nucleus, putamen, globus pallidus, and substantia nigra and in neuromelanin (Cheng et al. 1996; Carstam et al. 1991; Rosengren et al. 1985). Aminochrome is one of the best substrates for GSTM2 and this isoform is by far the most active isoenzyme in aminochrome conjugation among the human glutathione transferases. For example, GSTM2 is 4,774-fold

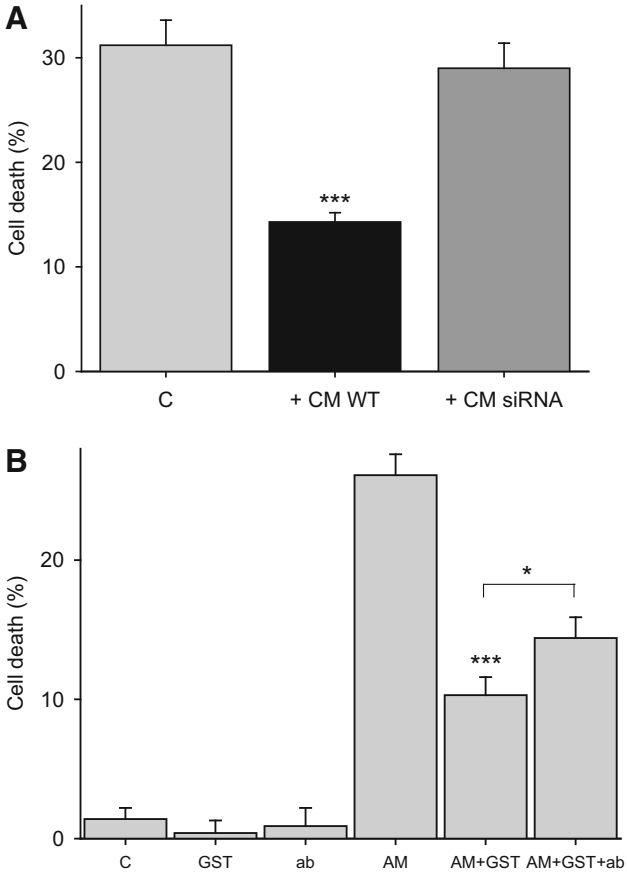

Fig. 5 GSTM2 protection against cell death in SH-SY5Y cells caused by aminochrome. **a** A significant decrease in cell death was observed in SH-SY5Y cells incubated with 10 μM aminochrome when U373MG-conditioned medium (+CM WT) was added to the culture. However, the culture medium from U373MGsiGST6 cells in which GSTM2 expression is nearly completely silenced by an siRNA provided no protection. **b** The incubation of SH-SY5Y cells with pure recombinant GSTM2 significantly decreased aminochrome-induced cell death and antiserum against GSTM2 decreased the protective effect of recombinant GSTM2, The values are the mean ± SD ($n = 3$) and the statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons (***$P < 0{,}001$; *$P < 0.05$)

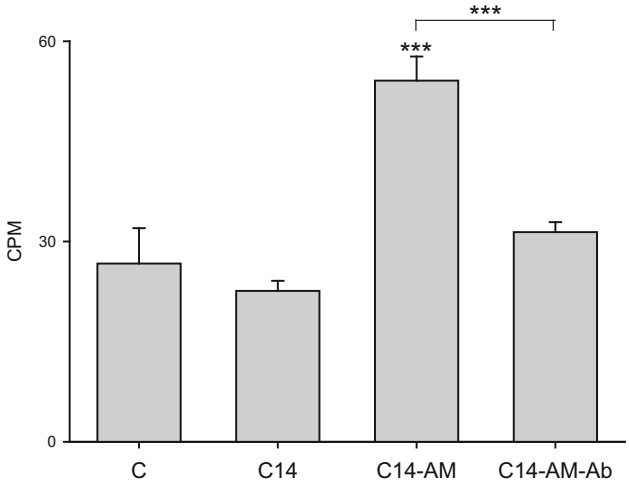

Fig. 6 Uptake of ^{14}C-labeled GSTM2 into SH-SY5Y cells. The uptake of ^{14}C-labeled GSTM2 into SH-SY5Y cells was determined by incubating the cells with (C) the cell medium from U373MG cells incubated with conditioned medium; (C14) U373MG cells incubated with 2 μCi ^{14}C-leucine L; (C14-AM) U373MG cells incubated with 2 μCi C14-leucine L and 50 μM aminochrome; and (C14-Am-Ab) U373MG cells incubated with 2 μCi **^{14}C** -leucine L, 50 μM aminochrome, and GSTM2 antiserum. The values are the mean ± SD ($n = 3$) and the statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons, (***$P < 0{,}001$)

more active than GST A2-2 and 194-fold more active than GST M1-1 (Segura-Aguilar et al. 1997). A protective role has been proposed for GSTM2 in astrocytes based on the fact that GSTM2 prevents aminochrome-induced toxicity mediated by autophagy and lysosomal dysfunction in U373MG cells (Huenchuguala et al. 2014).

Astrocytes Protect Dopaminergic Neurons

We used U373MG cells as model cell line for astrocytes that are derived from a patient classified as having malignant anaplastic astrocytoma grade IV-glioblastoma since (i) primary cultures of astrocytes from animals were not possible to use due to the fact that GSTM2 is expressed only in human and primary cell culture from humans, and these were not available; (ii) the cells have constitutive expression of GSTM2 (Huenchuguala et al. 2014); (iii) the cells have dopamine transporters (Huenchuguala et al. 2014); and (iv) constitutive expression of glial fibrillary acidic protein (GFAP) (Huenchuguala et al. 2014) that it is a marker of glia cells (Koh et al. 2009). Finally, (iv) we have U373MGsiGST6 cells expressing an siRNA against GSTM2 that have only 26 % expression of GSTM2 in comparison with U373MG wild-type cells (Huenchuguala et al. 2014).

Astrocytes are able to protect dopaminergic neurons as demonstrated by several lines of evidence: Astrocytes protect tyrosine hydroxylase-positive neurons when astrocytes over-expressing glial cell line-derived neurotrophic factor (GDNF) are transplanted prior to intracerebral 6-hydroxydopamine (6-OHDA) lesioning (Ericson et al. 2005); other experiments also demonstrated that astrocytes protect neurons against 6-OHDA toxicity (Safi et al. 2012; Gardaneh et al. 2011); astrocyte activation protects dopaminergic neurons against MPTP neurotoxicity in mice (Ericson et al. 2005); astrocytes release the precursors for GSH synthesis in neurons (Banerjee et al. 2008; Dringen and Hirrlinger 2003); proteomic analysis revealed that mouse astrocytes release more than 30 proteins including superoxide dismutase, thioredoxin peroxidase-2, metalloproteinase inhibitor-1 and -2, and GSTM 1-1 (Lafon-Cazal); our results with U373MG suggest the protective role of astrocytes on dopaminergic neurons since U373MG

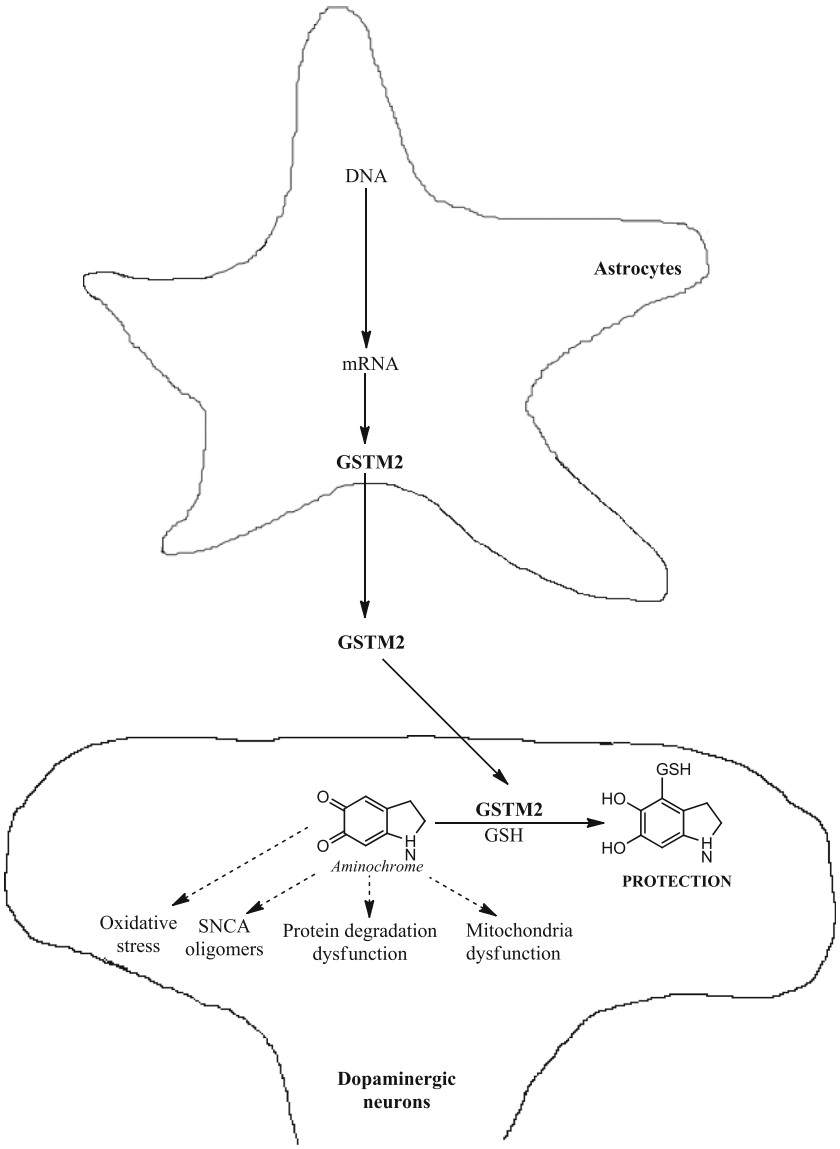

Fig. 7 Protection of dopaminergic neurons by astrocytes. Astrocytes have constitutive as well as inducible expression of GSTM2 that provides protection against aminochrome toxicity by glutathione conjugation. The effect in astrocytes is shared with dopaminergic neurons by secretion of GSTM2 that can be taken up by neurons to counteract aminochrome neurotoxicity

cells release GSTM2 into the conditioned medium which protects the surrounding neurons against aminochrome toxicity. Interestingly, the protection provided by GSTM2 excreted by U373MG cells on SH-SY5Y cells against aminochrome was dependent on GSTM2 uptake into SH-SY5Y cells (Fig. 7). Cellular uptake of GSTs has been reported previously. It has been reported that *Schistosoma japonicum* GST 26 is internalized into several cell lines using an energy-dependent event involving endocytosis based on the finding that GST 26 co-localized with transferrin within the cell. Other proteins containing a GST-fold structure were also internalized by several cell lines (Morris et al. 2009). The possibility that the observed protective effect of U373MG-conditioned media on SH-SY5Y cells was dependent on GSH release from U373MG cells to the conditioned media was refuted since (i) extracellular GSH alone provides protection in SH-SY5Y cells treated with aminochrome, but the supplementation of U373MG conditioned media with fresh extracellular GSH did not increase the level of protection as expected, and (ii) the presence of antiserum against GSTM2 completely abolished the protective effect of U373MG-conditioned media on SH-SY5Y cells treated with aminochrome. The protective role of GSTM2, released from U373MG cells to the conditioned media, on SH-SY5Y treated with aminochrome is also supported by the fact that conditioned media from U373MGsiGST6 cell line expressing an siRNA against GSTM2 did not provide significant protection on SH-SY5Y cells. Furthermore, ^{14}C-labeled GSTM2 synthesized in U373MG cells and secreted into the conditioned medium was internalized by SH-SY5Y cells supporting the notion that U373MG cells protect SH-

SY5Y cells from aminochrome toxicity by releasing GSTM2 into conditioned media and subsequently internalized by SH-SY5Y cells. The possibility that the protection of U373MG-conditioned medium on SH-SY5Y cells treated with aminochrome was depending on extracellular inactivation of aminochrome by GSTM2 released from U373MG cells was likewise refuted because glutathione conjugation of aminochrome requires GSH, and significant protection was observed when SH-SY5Y cells treated with aminochrome were incubated with recombinant GSTM2 in the absence of GSH.

The degenerative process in Parkinson's disease is initiated long before (Braak et al. 2004) the massive loss of dopaminergic neurons containing neuromelanin induces the motor symptoms. It seems to be plausible that astrocyte loss is an event preceding the degeneration of dopaminergic neurons since astrocytes protect neighboring neurons by releasing different molecules—including glutamine and glutamate—that are substrates of neurotransmitter precursors for GSH synthesis and energy substrates (Schousboe et al. 2007; McKenna 2007; Yang et al. 2008; Banerjee et al. 2008; Dringen and Hirrlinger 2003). The release of GSTM2 from U373MG cells to the intersynaptic space and its uptake by SH-SY5Y suggest that GSTM2 can also be released from astrocytes and protect dopaminergic neurons against aminochrome toxicity. This mechanism seems to be important to protect both the astrocytes and dopaminergic neurons from aminochrome toxicity, preventing the loss of dopaminergic neurons in Parkinsońs disease.

Dopamine Oxidation

Dopamine released from dopaminergic neurons under neurotransmission into postsynaptic space is removed by dopamine reuptake mediated by dopamine transporters expressed in dopaminergic neurons. However, other cells that are able to take up dopamine also participate in dopamine clearance from the postsynaptic space. This means that the dopamine uptake into astrocytes may result in the formation of aminochrome. Astrocytes have expression of monoamino oxidase-B (MAO-B; Westlund et al. 1988) that may play a role in dopamine degradation by catalyzing dopamine oxidative deamination in order to remove free dopamine from the cytosol. However, MAO-A has been reported as the isoform that has more affinity to dopamine and other monoaminergic neurotransmitters, while MAO-B has more affinity to tyramine and phenylethylamine (Strolin-Benedetti et al. 1992). Dopamine oxidation and formation of aminochrome in astrocytes can induce toxicity. Aminochrome has been found to be toxic in dopaminergic neurons (Arriagada et al. 2004; Lozano et al. 2010; Paris et al. 2011; 2010; Muñoz et al. 2012b) as well as in astrocytes (Huenchuguala et al. 2014). Aminochrome is toxic due to (i) aminochrome inducing and stabilizing the formation of neurotoxic protofibrils of alpha-synuclein by forming adducts in alpha-synuclein motif 125YEMPS129 (Norris et al. 2005; Conway et al. 2001; Dibenedetto et al. 2013); (ii) inducing protein degradation dysfunction by impairing both the proteasome system (Zafar et al. 2006; Zhou and Lim 2009); and the autophagy/lysosomal system (Huenchuguala et al. 2014; Muñoz et al. 2012a, b); (iii) aminochrome impairs mitochondria electron transport by inhibiting complex I (Aguirre et al. 2012); (iv) aminochrome induces oxidative stress during its one-electron reduction to leukoaminochrome *o*-semiquinone radical that it is extremely reactive with oxygen generating a redox cycling between aminochrome and leukoaminochrome when flavoenzymes that transfer one electron catalyze this reaction. This redox cycling depletes NADH required for ATP production in mitochondria, depletes NADPH required for biosynthesis reactions, and depletes oxygen required for ATP production with concomitant formation of superoxide radical, hydrogen, and hydroxyl radicals (Segura-Aguilar et al. 1998; Segura-Aguilar and Lind 1989; Arriagada et al. 2004). Aminochrome also affects anterograde axonal transport of mitochondria since it forms adducts with α- and β-tubulin, generating abnormal aggregates that prevent microtubules formation required for anterograde transport in the axon (Paris et al. 2010). Dopamine oxidation produces three *o*-quinones in a sequential manner since dopamine oxidizes to dopamine *o*-quinone that at physiological pH undergoes immediately intramolecular cyclization to form aminochrome with a constant rate of 0.15 s^{-1} (Tse et al. 1976). Aminochrome rearranges to 5,6-indole quinone with a rate constant of 0.06 min^{-1} (Bisaglia et al. 2007) suggesting that aminochrome is the most stable *o*-quinone. Several reports reveal that dopamine *o*-quinone is able to form adducts with several proteins (Van Laar et al. 2009) such as with (1) complexes I, III, and V of the mitochondrial electron transport chain, (2) processes in oxidative phosphorylation, (3) DJ-1, a protein that protects mitochondria from oxidative stress, and (4) UCHL-1, involved in the proteasome system, by hydrolyzing peptide–ubiquitin bonds and recycling ubiquitin monomers inactivating the proteasome system (Van Laar et al. 2009; Canet-Avilés et al. 2004; Blackinton et al. 2009; Trempe and Fon 2013). The question is whether the adducts were formed by dopamine *o*-quinone or aminochrome due to the short life of dopamine *o*-quinone. Dopamine oxidation inactivates parkin, a component of ubiquitin ligase 3 of the proteasome system (LaVoie et al. 2005). In dopaminergic neurons, aminochrome toxicity can be prevented by the two-electron reduction of aminochrome to leukoaminochrome (Segura-Aguilar and Lind 1989) that prevents aminochrome neurotoxic reactions (Arriagada et al. 2004; Lozano et al. 2010; Paris et al. 2010 2011; Huenchuguala et al. 2014). However, in astrocytes, aminochrome and its precursor dopamine *o*-quinone can be conjugated with GSH by GSTM2 to 4-*S*-glutathionyl-56-dihydroxyindoline and 5-glutathionyl-dopamine, preventing the toxic effects of the *o*-quinones (Segura-Aguilar et al. 1997; Baez et al. 1997).

In conclusion, our results demonstrated that GSTM2 expressed in U373MG cells provides protection to SH-SY5Y cells against aminochrome toxicity by releasing GSTM2 to the conditioned media and subsequent internalization into SH-SY5Y cells (Fig. 7). These results suggest a new mechanism for protection of dopaminergic neurons by astrocytes by releasing GSTM2 to the intersynaptic space where dopaminergic neurons can internalize GSTM2 in order to prevent aminochrome neurotoxicity.

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